

Joanna Jankowicz-Cieslak · Thomas H. Tai
Jochen Kumlehn · Bradley J. Till *Editors*

Biotechnologies for Plant Mutation Breeding

Protocols



IAEA

Joint FAO/IAEA Division
of Nuclear Techniques in Food and Agriculture



Springer Open

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Joanna Jankowicz-Cieslak
Plant Breeding and Genetics Laboratory
Joint FAO/IAEA Division of Nuclear
Techniques in Food and Agriculture
Vienna, Austria

Thomas H. Tai
Crops Pathology and Genetics Research Unit
USDA-ARS
Davis
California, USA

Jochen Kumlehn
Plant Reproductive Biology
Leibniz Institute of Plant Genetics and
Crop Plant Research (IPK)
Gatersleben, Germany

Bradley J. Till
Plant Breeding and Genetics Laboratory
Joint FAO/IAEA Division of Nuclear
Techniques in Food and Agriculture
Vienna, Austria

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*In memory of Dr Pierre J.L. Lagoda
(1961–2016) for his tireless efforts and
endless enthusiasm for plant mutation
breeding.*

Preface

Sustainable global food security remains a serious challenge. The compounding factors of a growing population, a changing climate and finite and dwindling natural resources mean that food production needs to increase dramatically in the coming decades. Genetic improvement of crops has historically led to vast gains in yields and subsequently has reduced famine. Variation is the source of all breeding, and inducing mutations is an important and successful approach for generating novel variation and developing new crop varieties that are climate smart and nutritious and increase farmer's incomes.

The use of induced mutations dates back to the 1920s, and today there are over 3200 mutant crop varieties registered in a database curated by the FAO/IAEA. The process can be improved through the development, adaptation and transfer of technologies for optimizing the density of induced mutations and increasing the efficiency of phenotypic and genotypic screening of large mutant plant populations. Towards this end, the Joint FAO/IAEA Programme of Nuclear Techniques for Food and Agriculture initiated a collaborative Coordinated Research Project (CRP) titled "Enhancing the Efficiency of Induced Mutagenesis through an Integrated Biotechnology Pipeline". The project brought together researchers from developing and developed countries with the aim to develop protocols and guidelines to improve the efficiency of the different steps of the plant mutation breeding process. This book provides protocols resulting from this CRP. A range of methods are provided suitable for those new to the field

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as well as to those seeking more advanced phenotypic and genotypic screening techniques. To guarantee high scientific quality, all chapters of this book have been peer reviewed. We are thankful to the participants of the CRP, authors of the chapters and also to all the reviewers (for list, see overleaf) who donated their time to improve the chapters.

Vienna, Austria

Bradley J. Till

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Contents

Part I Introduction

- 1 Mutagenesis for Crop Breeding and Functional Genomics** 3
Joanna Jankowicz-Cieslak, Chikelu Mba, and Bradley J. Till

Part II Mutation Induction and Chimera Dissociation

- 2 Chemical and Physical Mutagenesis in *Jatropha curcas*** 21
Fatemeh Maghuly, Souleymane Bado, Joanna Jankowicz-Cieslak,
and Margit Laimer
- 3 Chemical Mutagenesis and Chimera Dissolution in Vegetatively
Propagated Banana** 39
Joanna Jankowicz-Cieslak and Bradley J. Till
- 4 Mutation Induction Using Gamma Irradiation and Embryogenic
Cell Suspensions in Plantain (*Musa spp.*)** 55
Jorge López, Aymé Rayas, Arletys Santos, Víctor Medero,
Yoel Beovides, and Milagros Basail
- 5 Optimisation of Somatic Embryogenesis in Cassava** 73
Kenneth E. Danso and Wilfred Elegba
- 6 Creation of a TILLING Population in Barley After Chemical
Mutagenesis with Sodium Azide and MNU** 91
Iwona Szarejko, Miriam Szurman-Zubrzycka, Malgorzata Nawrot,
Marek Marzec, Damian Gruszka, Marzena Kurowska,
Beata Chmielewska, Justyna Zbieszczyk, Janusz Jelonek,
and Mirosław Maluszynski
- 7 Site-Directed Mutagenesis in Barley by Expression of TALE
Nuclease in Embryogenic Pollen** 113
Maia Gurushidze, Stefan Hiekel, Ingrid Otto, Götz Hensel,
and Jochen Kumlehn

- 8 Doubled Haploidy as a Tool for Chimaera Dissolution of TALEN-Induced Mutations in Barley 129**
Maia Gurushidze, Hannes Trautwein, Petra Hoffmeister, Ingrid Otto, Andrea Müller, and Jochen Kumlehn

Part III Phenotypic Screening

- 9 Field Evaluation of Mutagenized Rice Material 145**
Sydney D. Johnson, Dennis R. Taylor, Thomas H. Tai, Joanna Jankowicz-Cieslak, Bradley J. Till, and Alpha B. Jalloh
- 10 Root Phenotyping Pipeline for Cereal Plants 157**
Michal Slota, Mirosław Maluszynski, and Iwona Szarejko
- 11 Breeding New Aromatic Rice with High Iron Using Gamma Radiation and Hybridization 173**
Phuong Tan Tran and Cua Quang Ho
- 12 Utilising NIRS for Qualitative and Non-destructive Identification of Seed Mutants in Large Populations 193**
Johann Vollmann and Joanna Jankowicz-Cieslak
- 13 Proteome Analyses of *Jatropha curcas* 203**
Fatemeh Maghuly, Gorji Marzban, Ebrahim Razzazi-Fazeli, and Margit Laimer

Part IV Genotypic Screening

- 14 Low-Cost Methods for DNA Extraction and Quantification 227**
Owen A. Huynh, Joanna Jankowicz-Cieslak, Banumaty Saraye, Bernhard Hofinger, and Bradley J. Till
- 15 A Protocol for Benchtop Extraction of Single-Strand-Specific Nucleases for Mutation Discovery 241**
Bernhard J. Hofinger, Owen A. Huynh, Joanna Jankowicz-Cieslak, and Bradley J. Till
- 16 A Protocol for Validation of Doubled Haploid Plants by Enzymatic Mismatch Cleavage 253**
Bradley J. Till, Bernhard J. Hofinger, Ayşe Şen, Owen A. Huynh, Joanna Jankowicz-Cieslak, Likyelesh Gugsa, and Jochen Kumlehn
- 17 Bioinformatics-Based Assessment of the Relevance of Candidate Genes for Mutation Discovery 263**
Michal Slota, Mirosław Maluszynski, and Iwona Szarejko
- 18 Mutation Detection by Analysis of DNA Heteroduplexes in TILLING Populations of Diploid Species 281**
Miriam Szurman-Zubrzycka, Beata Chmielewska, Patrycja Gajewska, and Iwona Szarejko

19 Determining Mutation Density Using Restriction Enzyme Sequence Comparative Analysis (RESCAN) 305
Diana Burkart-Waco, Isabelle M. Henry, Kathie Ngo, Luca Comai, and Thomas H. Tai

20 Next-Generation Sequencing for Targeted Discovery of Rare Mutations in Rice 323
Diana Burkart-Waco, Helen Tsai, Kathie Ngo, Isabelle M. Henry, Luca Comai, and Thomas H. Tai

Chapter Reviewers

Yosvanis Acanda University of Florida, Lake Alfred, FL, USA

Carlos Alonso-Blanco Dpt Genetica Molecular de Plantas, Centro Nacional de Biotecnologia, Madrid, Spain

Saleha Bakht John Innes Centre, Norwich, UK

Babita Dussoruth Food Agricultural Research and Extension Institute, Reduit, Mauritius

Anne Edwards John Innes Centre, Norwich, UK

Brian P. Forster Biohybrids International Ltd, Earley Reading, UK

Ricardo F.H. Giehl Molecular Plant Nutrition, Leibniz Institute of Plant Genetics & Crop Plant Research, Seeland/OT, Gatersleben, Germany

Tilo Guse Gregor Mendel Institute of Molecular Plant Biology, Vienna, Austria

Thomas Halbach Strube Research GmbH & Co. KG, Söllingen, Germany

Isabelle M. Henry Davis Genome Center and Department of Plant Biology, University of California, Davis, CA, USA

John S. (Pat) Heslop-Harrison Department of Genetics, University of Leicester, Leicester, UK

Inger Holme Department of Molecular Biology and Genetics, Slagelse, Denmark

Ivan Ingelbrecht Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, Vienna, Austria

Daniela Isola Laboratorio di Botanica Sistemática e Micologia, Università delle Tuscia—DEB, Viterbo, Italy

Shri Mohan Jain Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland

Yulin Jia U.S. Department of Agriculture, Agricultural Research Service, Dale Bumpers National Rice Research Center, Stuttgart, AR, USA

Kamila Kozak-Stankiewicz Kutnowska Hodowla Buraka Cukrowego Sp. z o.o., Kłodawa, Poland

Jens Leon Institute of Crop Science and Resource Conservation (INRES), Rheinische Friedrich-Wilhelms-University of Bonn, Bonn, Germany

Fatemeh Maghuly Plant Biotechnology Unit (PBU), Department of Biotechnology, BOKU-VIBT, University of Natural Resources and Life Sciences, Vienna, Austria

Martina Marchetti-Deschmann Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria

Chikelu Mba Seeds and Plant Genetic Resources Team, Plant Production and Protection Division, Food and Agriculture Organization of the United Nations, Rome, Italy

Heinrich Orsini-Rosenberg Bruker Austria GmbH, Wien, Austria

Peggy Ozias-Akins Department of Horticulture, University of Georgia, Tifton, GA, USA

Andy Phillips Plant Biology and Crop Science Department, Rothamsted Research, Harpenden, United Kingdom

J. Neil Rutger (retired) U.S. Department of Agriculture, Agricultural Research Service, Dale Bumpers National Rice Research Center, Stuttgart, AR, USA

Qing-Yao Shu Institute of Crop Science and Centre for Bio-breeding, Zhejiang University, Hangzhou, China

Nils Stein Leibniz Institute of Plant Genetics and Crop Plant Research, Seeland/OT, Gatersleben, Germany

Michael J. Thomson Texas A&M AgriLife Research, Department of Soil and Crop Sciences, Texas A&M University, College Station, TX, USA

Trevor Wang John Innes Centre, Norwich, UK

Hao Wu University of Florida, Lake Alfred, FL, USA

Janice Zale University of Florida, Lake Alfred, FL, USA

Contributors

Souleymane Bado Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, Vienna, Austria

Milagros Basail Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba

Yoel Beovides Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba

Diana Burkart-Waco Crops Pathology and Genetics Research Unit, USDA-ARS, Davis, CA, USA

Department of Plant Sciences, University of California, Davis, CA, USA

Beata Chmielowska Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Luca Comai Genome Center, University of California, Davis, CA, USA

Department of Plant Biology, University of California, Davis, CA, USA

Kenneth. Ellis Danso Ghana Atomic Energy Commission, Biotechnology and Nuclear Agriculture Research Institute, Legon-Accra, Ghana

Wilfred Elegba Ghana Atomic Energy Commission, Biotechnology and Nuclear Agriculture Research Institute, Legon-Accra, Ghana

Patrycja Gajewska Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Damian Gruszka Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Likyelesh Gugsu Ethiopian Institute of Agricultural Research, Holetta, Ethiopia

Maia Gurushidze Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Isabelle M. Henry Genome Center, University of California, Davis, CA, USA
Department of Plant Biology, University of California, Davis, CA, USA

Götz Hensel Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Stefan Hiekel Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Cua Quang Ho SocTrang Department of Agriculture and Rural Development, SocTrang, VietNam

Petra Hoffmeister Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Bernhard Hofinger Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, Vienna, Austria

Owen A. Huynh Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, Vienna, Austria

Alpha B. Jalloh Rokupr Agricultural Research Centre (RARC), Sierra Leone Agricultural Research Institute (SLARI), Freetown, Sierra Leone

Joanna Jankowicz-Cieslak Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, Vienna, Austria

Janusz Jelonek Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Sydney D. Johnson Rokupr Agricultural Research Centre (RARC), Sierra Leone Agricultural Research Institute (SLARI), Freetown, Sierra Leone

Jochen Kumlehn Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Marzena Kurowska Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Margit Laimer Plant Biotechnology Unit (PBU), Department of Biotechnology, BOKU-VIBT, University of Natural Resources and Life Sciences, Vienna, Austria

Jorge López Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba

Fatemeh Maghuly Plant Biotechnology Unit (PBU), Department of Biotechnology, BOKU–VIBT, University of Natural Resources and Life Sciences, Vienna, Austria

Mirosław Maluszynski Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Gorji Marzban Plant Biotechnology Unit (PBU), Department of Biotechnology (DBT), University of Natural Resources and Life Sciences (BOKU), Vienna, Austria

Marek Marzec Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Chikelu Mba Seeds and Plant Genetic Resources Team, Plant Production and Protection Division, Food and Agriculture Organization of the United Nations, Rome, Italy

Víctor Medero Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba

Andrea Müller Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Malgorzata Nawrot Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Kathie Ngo Genome Center, University of California, Davis, CA, USA
Department of Plant Biology, University of California, Davis, CA, USA

Ingrid Otto Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Seeland/OT, Gatersleben, Germany

Ebrahim Razzazi-Fazeli VetCore Facility, University of Veterinary Medicine Vienna, Vienna, Austria

Aymé Rayas Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba

Arlety Santos Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba

Banumaty Saraye Food Agricultural Research and Extension Institute, Reduit, Mauritius

Ayşe Şen Faculty of Science, Department of Biology, Istanbul University, Vezneciler, Istanbul, Turkey

Michał Słota Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Iwona Szarejko Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Miriam Szurman-Zubrzycka Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Thomas H. Tai Crops Pathology and Genetics Research Unit, USDA-ARS, Davis, CA, USA

Phuong Tan Tran Department of Agriculture and Rural Development, SocTrang, VietNam

Dennis R. Taylor Rokupr Agricultural Research Centre (RARC), Sierra Leone Agricultural Research Institute (SLARI), Freetown, Sierra Leone

Bradley J. Till Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, Vienna, Austria

Hannes Trautwein Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Plant Reproductive Biology, Seeland/OT, Gatersleben, Germany

Helen Tsai Genome Center, University of California, Davis, CA, USA

Department of Plant Biology, University of California, Davis, CA, USA

Johann Vollmann Plant Breeding Division, Department of Crop Science, University of Natural Resources and Life Sciences Vienna (BOKU), Tulln an der Donau, Austria

Justyna Zbieszczyk Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

Part I
Introduction

Chapter 1

Mutagenesis for Crop Breeding and Functional Genomics

Joanna Jankowicz-Cieslak, Chikelu Mba, and Bradley J. Till

Abstract Genetic variation is a source of phenotypic diversity and is a major driver of evolutionary diversification. Heritable variation was observed and used thousands of years ago in the domestication of plants and animals. The mechanisms that govern the inheritance of traits were later described by Mendel. In the early decades of the twentieth century, scientists showed that the relatively slow rate of natural mutation could be increased by several orders of magnitude by treating *Drosophila* and cereals with X-rays. What is striking about these achievements is that they came in advance of experimental evidence that DNA is the heritable material. This highlights one major advantage of induced mutations for crop breeding: prior knowledge of genes or gene function is not required to successfully create plants with improved traits and to release new varieties. Indeed, mutation induction has been an important tool for crop breeding since the release of the first mutant variety of tobacco in the 1930s. In addition to plant mutation breeding, induced mutations have been used extensively for functional genomics in model organisms and crops. Novel reverse-genetic strategies, such as Targeting Induced Local Lesions IN Genomes (TILLING), are being used for the production of stable genetic stocks of mutant plant populations such as *Arabidopsis*, barley, soybean, tomato and wheat. These can be kept for many years and screened repeatedly for different traits. Robust and efficient methods are required for the seamless integration of induced mutations in breeding and functional genomics studies. This chapter provides an overview of the principles and methodologies that underpin the set of protocols and guidelines for the use of induced mutations to improve crops.

Keywords Mutation breeding • Reverse-genetics • Forward-genetics • Phenotyping • Genotyping • Technology packages

J. Jankowicz-Cieslak • B.J. Till (✉)

Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria
e-mail: b.till@iaea.org

C. Mba

Seeds and Plant Genetic Resources Team, Plant Production and Protection Division, Food and Agriculture Organization of the United Nations, Rome, Italy

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1.1 Inducing Genetic Variation

The genetic improvement of crops is a crucial component of the efforts to address pressures on global food security and nutrition (Ronald 2011). It is estimated that food production should be at least doubled by the year 2050 in order to meet the needs of a continually growing population (Ray et al. 2013; Tester and Langridge 2010; FAO 2009). The availability of heritable variation is a prerequisite for genetic improvement of crops. Where sufficient variation does not exist naturally, it can be created through either random or targeted processes (Fig. 1.1). Aside from recombination, the treatment of plant materials with chemical or physical mutagens is the most commonly reported approach for generating novel variation. While various mutagens have different effects on plant genomes, and some positional biases have been reported, irradiation and chemical mutagenesis are generally considered random mutagenesis as the location of DNA lesions cannot be effectively predicted in advance (Greene et al. 2003). The effect of different mutagens on the DNA sequence also varies with mutagen type and dosage. Once sufficient genetic variation is induced, the next step is to select materials that have the desired altered traits (see Fig. 1.1 and Sects. 1.2 and 1.3).

1.1.1 Practical Considerations in Induced Crop Mutagenesis

Mutation breeding is a three-step process consisting of (a) inducing mutations, (b) screening for putative mutant candidates and (c) mutant testing and official release (Fig. 1.2). The last step tends to be standardised in specific countries and is not an area where research and development can (easily) improve efficiencies. While not trivial, mutation induction has been widely used and highly successful in most species. Screening of mutants and selection of desired variants remain the most intensive step. Incredible advances have been made in the field of phenomics over the past 5 years, however, phenotyping remains more specialised and labour intensive than genotypic selection (Fiorani and Schurr 2013; Cobb et al. 2013).

The choice of which type of mutagen to use for mutation breeding is often based on past successes reported for the species and other considerations such as the availability of mutagens, costs and infrastructure (Bado et al. 2015; Mba 2013; MVD 2016). Mutant varieties produced with ionising radiation, specifically gamma rays, predominate in the database of registered mutant varieties (MVD 2016). This may be due primarily to the active promotion of the use of gamma irradiation by the Food and Agriculture Organisation of the United Nations and the International Atomic Energy Agency (FAO/IAEA) Joint Programme, but also may be biologically significant as physical mutagens tend to induce larger genomic aberrations than some chemical mutagens, and more dominant or more easily observable traits could be created at a higher frequency (Jankowicz-Cieslak and Till 2015). Standardised protocols and general considerations for induced mutations in seed

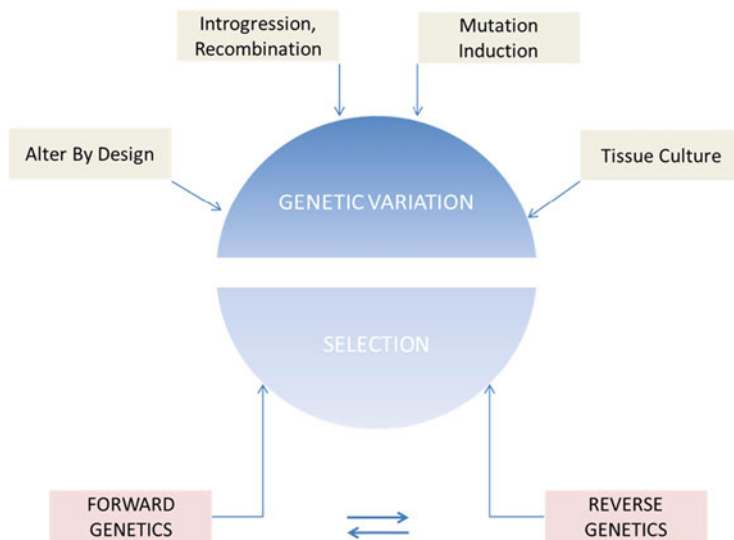


Fig. 1.1 Crop improvement strategies based on the generation and harnessing of genetic variation. There are many methods to introduce novel genetic variation into a specific line. The most common is through outcrossing, whereby introgression and recombination generate new combinations of alleles. This may include wide intraspecific and interspecific crosses. Passaging of cells through tissue culture has also been used to generate what is known as somaclonal variation. ‘Alter by design’ refers to any method whereby genetic variation is induced through thoughtful modifications. These include methods such as transgenics or genome editing (see Chap. 7). Mutagenesis provides a low-cost means to rapidly generate novel variation. The next step is to select plants that have the desired mutation or phenotype. Here, the researcher can choose between forward and reverse-genetic approaches depending on prior knowledge of genes and hypotheses of gene function. In addition to direct traditional phenotyping, the emerging fields of genomics and phenomics offer opportunities for more precise breeding and large gains in efficiencies while reducing the time for recovery of desired variants (see Sects. 1.2 and 1.3). Figure adapted from Novak and Brunner (Novak and Brunner 1992)

and vegetatively propagated plants using the physical mutagen (gamma rays) and the chemical mutagen (ethyl methanesulfonate, EMS) have been previously discussed (Lee et al. 2014; Bado et al. 2015; Till et al. 2006; Mba et al. 2010). Chapters 2, 3, 4 and 6 of this book describe chemical and/or physical mutagenesis protocols for obligate vegetatively propagated banana (*Musa acuminata*), facultative vegetatively propagated *Jatropha* (*Jatropha curcas*) and seed-propagated barley (*Hordeum vulgare*).

A major bottleneck in plant mutation breeding is the imperative of generating and evaluating large mutant populations in order to increase the chance of identifying a desirable variant. Efforts are devoted to the dissociation of chimeras, also known as mosaics or sectoral differences, whereby cells of different genotypes exist side by side in the tissues of the same mutant plant. This is straightforward in sexually produced crops owing to the fact that single cells in the form of gametes are the basis for the next generation, thus resolving any chimeras. For vegetatively

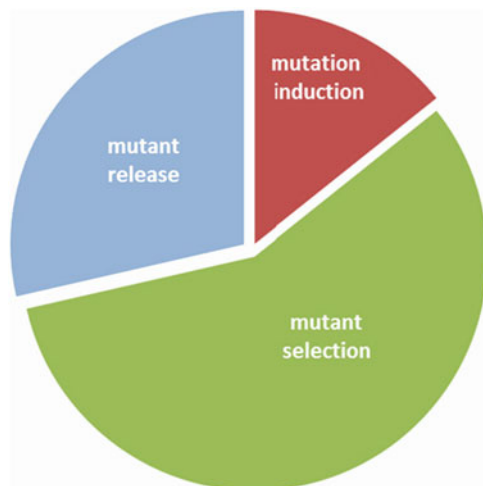


Fig. 1.2 A three-step mutation breeding scheme for direct release of improved crops. Each part is drawn proportional to the estimated time needed for development of a seed-propagated cereal (7–10 years). The first step is mutation induction which may take up to a year. The most time consuming and complicated step is mutant selection. Several years are typically needed to identify useful traits that are stable through propagation cycles. The third step, mutant varietal release, follows standardised procedures of the country where the material is grown. This often requires multilocational trials with farmer involvement. While the timing of this may vary, it is usually a shorter duration than the selection and testing phase. The procedure becomes longer and more complicated if the selected mutants are used as pre-breeding material in hybridisations (see Chap. 11)

propagated crops, several cycles of regeneration may be required to produce solid homohistonts or genotypically homogeneous material (van Harten 1998; Mba et al. 2009). One way to avoid chimerism in vegetatively propagated species is to mutagenise individual cells that can regenerate into plants, either using cell suspensions or (embryogenic) callus (van Harten 1998). Protocols for these strategies are provided in Chaps. 4 and 5. These approaches have been less often used than those involving multicellular organs and tissues, and so there is less information available on the possibility of chimerism at the DNA sequence. It is interesting to speculate on the fate of induced DNA modifications in single cells. For example, EMS mutagenesis results in alkylation, whereby the original base is not physically altered, but the mutation is only fixed due to an error in replication of the affected base. Here, two daughter cells could be produced with distinct genotypes.

1.1.2 Developing Crop Varieties Using Induced Mutations

Once a mutant population has been developed, the next steps of the mutation breeding process mirror traditional breeding procedures (Fig. 1.3). One issue to

consider is in which generation the selection for desirable putative mutants could begin. Depending on the density of mutations, selection of stable phenotypes in the M_2 may be difficult. This is due to the potential confounding factors of combinations of deleterious lesions (which affect the function of different proteins) and epistasis. One consequence of selecting phenotypes too early is that the observed trait may be lost in segregation in subsequent generations as non-linked alleles assort independently. The researcher may choose to accept this risk and select everything of interest in the first non-chimeric generation (M_2 for seed) for further

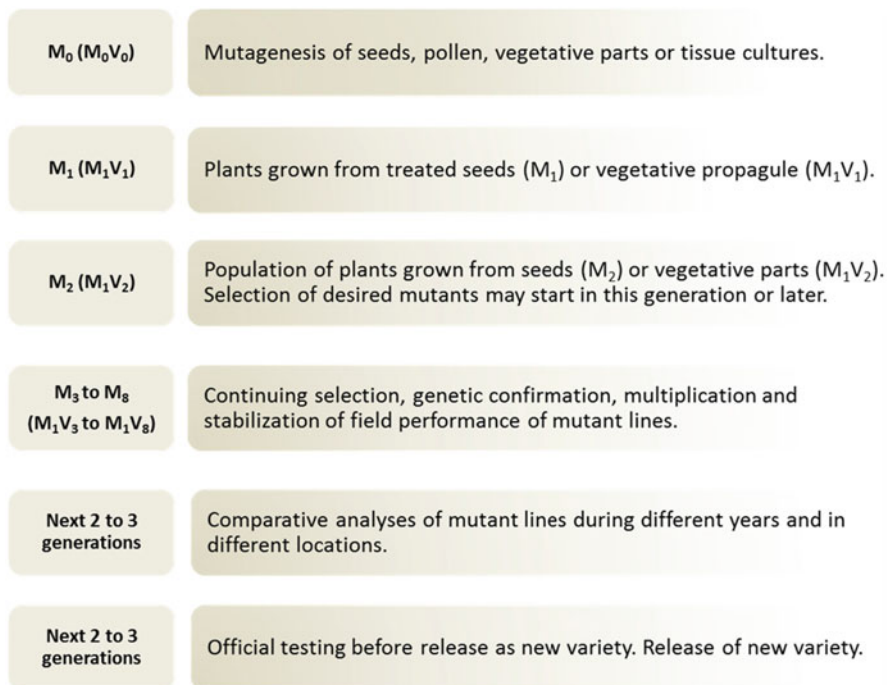


Fig. 1.3 Traditional mutation breeding scheme. Each row describes the steps for a specific generation. The generation nomenclature starts with M_0 for seed or pollen mutagenesis and M_0V_0 for vegetative organs, where M stands for the meiotic and V for the vegetative generation. All materials are labelled with a '0' prior to mutagenesis and with a '1' after mutagenesis is performed. The first generation is not suitable for evaluation when multicellular material is mutagenised because resulting plants will be genotypically heterogeneous (chimeric). The first non-chimeric (homohistont) generation in a seed-mutagenised and seed-propagated material is the M_2 . It may take several cycles to make a vegetatively propagated material genotypically homogeneous and to stabilise the inheritance of mutant alleles. Screening and selections can begin as early as the first non-chimeric generation. Subsequent generations typically involve selection and evaluation of mutant phenotypes to ensure that the traits are reproducible. Once this is complete, the materials can enter trials for varietal release. Alternatively, materials can be used as parents in breeding programmes. Officially released mutant crop varieties which are reported to the Joint FAO/IAEA Programme are recorded in the searchable Mutant Variety Database (MVD 2016). According to the MVD, approximately 62 % of all mutant varieties are directly released. Figure adapted from Novak and Brunner (Novak and Brunner 1992)

characterisation (see Chap. 9). On the other hand, when considering reverse-genetic strategies, it is often preferable to employ molecular screens on the first non-chimeric generation in order to maximise the discovery of unique mutations (Jankowicz-Cieslak and Till 2015). Also of critical importance is the method employed to select desirable phenotypes. While phenomic strategies have been rapidly developing in recent years (Cobb et al. 2013), the diversity of physiological parameters, disease responses and morphological variations from crop to crop complicates the task of developing standardised species-independent protocols, as can be done with most genomic screening tools (see Sect. 1.2).

1.1.3 Elite Crop Varieties Developed Through Induced Mutations

Shortly after scientists discovered that mutations could be induced through work on *Drosophila* (Muller 1927) and cereals (Stadler 1929, 1928a, b), plant breeders started using this as a tool to develop novel varieties. The first example was a mutant of tobacco called ‘Chlorina’ that was developed through the X-ray irradiation of floral buds in the 1930s (Tollenaar 1934, 1938; Konzak 1957; Coolhaas 1952). The Mutant Varieties Database maintained by the Joint Programme of the Food and Agriculture Organisation of the United Nations and the International Atomic Energy Agency (Joint FAO/IAEA) in Vienna, Austria, has searchable data for over 3220 crop varieties that have been developed using induced mutations and are being grown in different countries of the world (Fig. 1.4 and (MVD 2016)). Nearly 80 % of these crop varieties are seed propagated, almost half of which (48 %) are cereals.

Ahloowalia et al. (2004) and Kharkwal and Shu (2009) provided overviews of the contributions of these mutant crop varieties to food security and nutrition and economic wellbeing. Widely cultivated rice varieties in Australia, China, India, Pakistan and Thailand; sunflower and peppermint in the USA; barley in many countries of Europe; sorghum in Mali; and several ornamental plants in India, the Netherlands and Germany are a few such examples. The high-yielding and dwarf mutant cultivars of barley, ‘Diamant’ and ‘Golden Promise’, for instance, and their progenies are credited with additional billions of dollars in revenues for the brewing and malting industries in Europe (Ahloowalia et al. 2004). Other examples include most of the varieties of durum wheat grown in Italy for pasta and marketed worldwide; the Rio Star grapefruit in the USA; the Japanese pear variety, ‘Gold Nijesseiki’; and the cotton variety, NIAB78, in Pakistan.

It is difficult to estimate the precise extent to which novel alleles created through mutagenesis have been used in developing superior crop varieties worldwide. From a practical standpoint in most parts of the world, they are treated like any allele that the breeder may knowingly or unknowingly incorporate into a new improved cultivar.

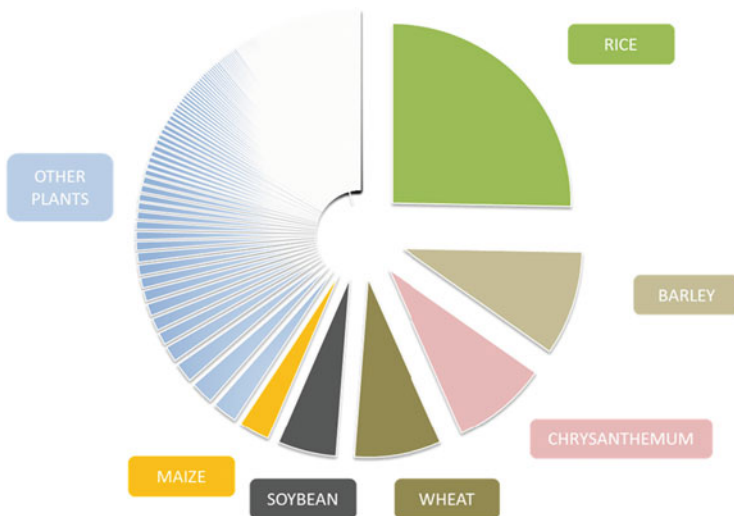


Fig. 1.4 Pie chart representing officially registered mutant crop varieties. The Mutant Variety Database contains 3222 entries out of which 2456 are seed propagated and 367 vegetatively propagated plants. The above grouping is based on the common name of the entry. The top six are rice, barley, chrysanthemum, wheat, soybean and maize. Data comes from (MVD 2016) accessed on May 4th, 2016

1.2 Phenotypic Screening

The origin of agriculture can be traced back to more than 10,000 years ago when the first crops were domesticated in the Fertile Crescent (Brown et al. 2009). The identification (phenotyping) and deliberate selection of off-type plants (i.e. spontaneous mutants) by the then hunters and gatherers were the initial efforts at plant breeding. The first plants to undergo domestication are thought to be the cereals wheat, barley, millet and emmer (Sang 2009). Early phenotypers selected plants with increased grain size and loss of seed shattering. They created and improved crops without any knowledge of why the selected differences occurred or were heritable. The first written reports of the earliest description of the selection of (presumably) spontaneous mutants appear to date back to around 300 BC when selection of plants with abnormal but improved traits were described in an ancient Chinese book, ‘Lulan’ (Shu et al. 2012). Among the improved characters were ‘days to maturity’ and other easily visible traits in cereal crops. The term ‘mutation’ was coined much later by Hugo de Vries to describe sudden genetic change in higher plants which was stably inherited through many years (de Vries 1901). Spontaneous mutants remain valuable sources of diversity and variation, but many years of intensive breeding has resulted in the narrowing of the genetic base of many crop species necessitating the need to create new variation through means such as induced mutations.

Accurate plant phenotyping remains as critically important today as it has ever been and is arguably the major bottleneck in plant breeding (Fiorani and Schurr 2013). This is especially true in plant mutation breeding where a novel trait may appear only once in a population of several thousand mutant lines. Therefore, high-throughput and cost-efficient screening methods are required for the rapid identification and characterisation of putative mutants.

1.2.1 Phenotypic Traits Developed Through Plant Mutation Breeding

Plant phenotyping can broadly be described as the evaluation of plant traits defined by the researcher (breeder) and may include yield, quality and resistance to biotic/abiotic stresses. The list can be further extended depending on the need and question asked. The Mutant Variety Database contains released and registered mutant plants with improved traits (characters) in five main categories: ‘agronomic and botanic traits’, ‘quality and nutrition traits’, ‘yield and contributors’, ‘resistance to biotic stresses’ and ‘tolerance to abiotic stresses’ (Fig. 1.5 and Table 1.1). For the 3222 officially registered mutants, 5569 improved characters are listed, implying that many mutants show several improved traits. For instance, higher yield is observed when a plant has improved resistance to abiotic or biotic stresses. Such a mutant, therefore, will have more than one improved character. It remains difficult to even speculate on the number of mutated alleles and genes that are causing the induced variation. Genomic technologies will allow the elucidation of mutant alleles causing altered traits (see Sect. 1.3).

Interestingly, the majority (48 %) of released mutant varieties that are registered in the Mutant Variety Database are characterised by improved agronomic and botanic traits. This could be due to the fact that botanic and agronomic traits are easily observable, and for most of them, no specialised equipment is needed for screening. The least represented mutants are in the biotic and abiotic stresses category. It is notable that these characters, though complex and difficult to screen for, are important breeding objectives. Examples of officially released mutant varieties in the five trait categories are listed in Table 1.1.

There remains a clear need to develop methods and protocols to enhance the efficiency of the mutation breeding process. The protocols in this book aim to do this, but it remains an incredibly challenging task. Every step of the procedure can differ depending on the parental genotype, propagation mode (seed versus vegetatively), the trait of interest which needs to be improved and available facilities. For example, Near-Infrared Reflectance Spectroscopy (NIRS) is a method that can be used to screen for seed composition. Traditional methods used a destructive approach which is suitable for characterising an advanced mutant line where many seeds are available. Screening is rapid, and non-destructive methods that measure whole seed allow NIRS to be used as a fast prescreen of large mutant

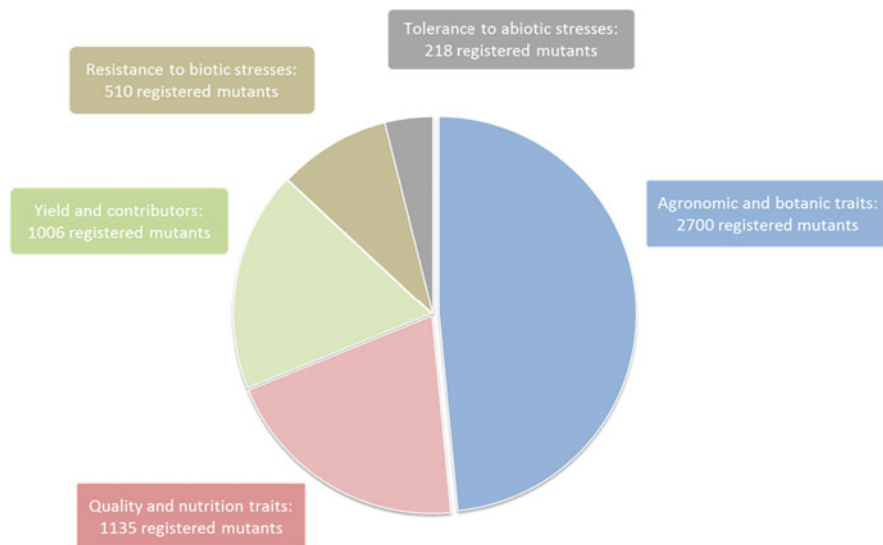


Fig. 1.5 Mutants registered in the MVD classified according to improved characters (traits). In total, improved characters are described 5569 times for 3222 varieties. These are classified in five general categories: ‘agronomic and botanic traits’ (48 %), ‘quality and nutrition traits’ (20 %), ‘yield and contributors’ (18 %), ‘resistance to biotic stresses’ (9 %) and ‘tolerance to abiotic stresses’ (4 %). Agronomic and botanic traits include maturity, flowering time and plant structure. Data comes from (MVD 2016) accessed on May 4, 2016

populations. Protocols for both destructive and non-destructive screening of rice are provided in Chap. 12. Calibration standards can be applied to NIRS spectra to evaluate seed components such as protein content. Once interesting mutants are identified in a rapid prescreen, detailed characterisation can be undertaken. Proteomic analysis allows a detailed cataloguing of the effect of genetic variation on the collection of expressed proteins in grains or tissues. Chapter 13 of this book provides detailed methods for protein analysis in seed and leaves of *Jatropha curcas*. Digital imaging is another non-destructive method that can be adapted for phenotypic evaluation of morphological variations induced by treatment with mutagens. Root architecture, for example, is an important component in abiotic stress responses such as drought. A low-cost approach for digital analysis of root traits is provided in Chap. 10.

Table 1.1 Examples of released improved varieties registered in the Mutant Variety Database under five main trait categories

Trait category	Mutant's name (<i>species</i>)	Description ^a	Development type ^b	References
Agronomic and botanic traits	'Above' (<i>Triticum aestivum</i> L.)	Awned, white glumed, early maturing and semidwarf	Treatment of seed with chemical mutagen, sodium azide (NaN ₃)	Newhouse et al. (1992)
Quality and nutrition traits	'Aldamla' (<i>Prunus avium</i> L.)	Compact growth habit (70–80 %), long petioles and improved fruit quality	Irradiation of dormant buds with gamma rays	Kunter et al. (2012)
Resistance to biotic stresses	'Akita Berry' (<i>Fragaria x ananassa</i>)	Improved resistance to black leaf spot disease (<i>Alternaria alternata</i>)	Somaclonal mutation by meristem culture	MVD (2016)
Tolerance to abiotic stresses	'Maybel' (<i>Lycopersicon esculentum</i> M.)	Very high performance under drought conditions	Treatment of seed with gamma rays	MVD (2016)
Yield and contributors	'Early Blenheim' (<i>Prunus armeniaca</i> L.)	Early maturity, higher yield, large fruits and self-compatible pollen	Treatment of dormant scions with thermal neutrons (thN)	Sigurbjoernsson and Micke (1974)

^aSome mutants listed have more than one character type

^bNo molecular characterisation reported concerning novel variation causing phenotype

1.3 Genotypic Screening of Mutant Plants

1.3.1 Genotypic Methods

Plant genotyping can be broadly considered as any experimental assay that aims to evaluate differences in the nucleotide sequence within or between species. This is an especially powerful approach because nucleotide variation is the major contributor to heritable phenotypic variation. Methods to uncover nucleotide variation also provide important information on plant evolution and enable efficient selections that avoid the confounding effects of genotype by environment (GxE) interactions (Annicchiario 2002). Protocols for genomic DNA acquisition and evaluation have been improving to the point where resequencing of hundreds to thousands of plant genomes is now a reality (Weigel and Mott 2009).

1.3.1.1 Lower-Cost Mutation Discovery and Genotyping Methods

The risk of new technologies, however, is that they tend to be expensive and require a high level of technical expertise. New tools, therefore, are not available to all

laboratories. Yet many powerful methods can be developed that are lower cost and suitable for laboratories with varying infrastructure. One example is the starting point of all genotyping experiments: the extraction of DNA. While long-term storage of plant tissues prior to DNA extraction often involves the use of liquid nitrogen and -80°C freezers, these can be avoided by desiccating and storing leaf material in silica gel at room temperature (Till et al. 2015). Extraction of high-quality genomic DNA from leaf material is typically performed using expensive kits or with more manual methods that require toxic organic chemicals such as the CTAB method. These can be avoided by using the protocol described in Chap. 14 of this book. DNA is extracted by binding to silica in the presence of chaotropic salts. This mirrors the chemistry used in expensive kits but at about only 10 % of the price. Importantly, therefore, high-quality genomic DNA can be extracted without specialised equipment for tissue grinding and without the use of any toxic organic compounds that require specialised waste disposal.

Low-cost methods do not end with the extraction of genomic DNA. The process of altering the expression or activity of a gene in order to evaluate its function *in vivo* is known as reverse-genetics. This term was coined because it is essentially the reverse of the process of forward-genetics which starts with a phenotype and ends with a gene sequence. While endogenous transposons have been used for gene disruptions in some crops such as maize and rice, a major development came in the late 1990s with a reverse genetic approach known as TILLING that uses induced mutations (Meeley and Briggs 1995; McCallum et al. 2000; Hirochika 2001; Conrad et al. 2008; Hunter et al. 2014). TILLING, short for Targeting Induced Local Lesions IN Genomes, typically utilises mutagens that induce a high density of induced mutations randomly throughout the genome (Kurowska et al. 2011; Greene et al. 2003; Jankowicz-Cieslak et al. 2011). A population of between 3000 and 6000 mutant lines can be developed that contains multiple mutations in every gene in the genome. A library of DNA and seed can be prepared and used as a resource for many years. In traditional TILLING, the DNA library is screened by PCR and enzymatic mismatch cleavage to identify mutations in target genes of choice. The entire TILLING process can be made low cost. In addition to low-cost methods for DNA extraction found in Chap. 14, Chap. 15 describes a rapid method for the extraction of single-strand-specific nucleases for TILLING and other applications that costs less than 1 cent per assay. Standard agarose gels can be used as a readout platform for mutation discovery.

1.3.1.2 Higher-Throughput Genotyping and Mutation Discovery Methods

Where budgets permit, next-generation sequencing technologies offer significant gains in screening throughput over low-cost methods. For example, the TILLING by sequencing protocol described in Chap. 20 provides a three-dimensional pooling strategy for 768 individuals and simultaneous mutation discovery in many gene targets (Tsai et al. 2011). The use of advanced tools is not limited to reverse-genetics. The majority of officially released mutant crop varieties are produced

from forward-genetic screens from plant materials treated with ionising radiation (MVD 2016). Genomic techniques promise to greatly enhance the efficiency of traditional forward mutation breeding that has been a mainstay for over 70 years. The challenge remains to determine if a population truly harbours a high density of desirable mutations. While visual evaluation of M_1 plants as described above is advantageous in that it is rapid and low-cost, it is known that variations observed in the M_1 do not represent heritable DNA mutations (Preuss and Britt 2003). Therefore, mutation density need not correlate with phenotypic variations observable in the M_1 . Next-generation sequencing technologies now provide rapid methods for the evaluation of mutation density and spectra in the M_2 generation. Many plant genomes are prohibitively large to consider whole-genome sequencing of the requisite number of plants for all but the very highly funded laboratories. Reduced representation genome sequencing offers a solution. Chapter 19 provides a protocol for Restriction Enzyme Sequence Comparative Analysis (RESCAN) where a fraction of a plant genome can be sequenced for discovery of induced point mutations. Here, tens of millions of base pairs can be sequenced from each mutant plant to recover sufficient mutations for a suitable estimation of mutation density.

1.3.1.3 Cloning Mutant Alleles Causative for Improved Traits

The same protocols described above can be adapted for another major challenge of forward mutation breeding: the identification and cloning of mutations causing the improved trait. With smaller genome plants, it is possible to sequence whole genomes and clone genes by associating co-segregation of genotype to phenotype (Schneeberger et al. 2009; Cuperus et al. 2010). An approach known as MutMap has been described for cloning EMS-induced alleles in rice using a bulked segregant strategy, and the method further adapted so that alleles can be cloned without outcrossing (Abe et al. 2012; Fekih et al. 2013). This is much more challenging in larger genome crops due to throughput and cost limitations of whole-genome sequencing. Targeted capture-resequencing methods offer a way for reduced representation genome sequencing of specific regions designed by the researcher. Coding sequences are an excellent choice when mutations affecting gene function are sought. Henry and colleagues describe exome capture methods to recover EMS-induced mutations in rice and wheat (Henry et al. 2014). With large genomes like wheat, this approach allows massive enrichment of functional regions of the genome and makes applications such as MutMap feasible, so long as causative mutations lie within regions that the researcher has selected for sequencing. To date, the majority of efforts have focused on recovery of point mutations such as those induced by treatment with the chemical mutagen EMS. Less is known about the effects of mutagens such as gamma irradiation, but recent experiments suggest that mutagen causes primarily large genomic deletions. For example, deletions of 1.2 million base pairs and 232,000 base pairs were recovered in *Zea mays* treated with gamma irradiation (Yuan et al. 2014). In sorghum, deletions ranging between 100,000 kb and 700,000 kb were recovered in materials treated with 75 and 300 Gy

(B.J Till, I.M. Henry and L. Comai, unpublished). In contrast to what is emerging from gamma irradiation, whole-genome sequencing studies of fast neutron-irradiated rice suggest a broader spectrum of mutations (Li et al. 2016).

The presence of large genomic deletions may make the task of cloning much easier. For example, a diploid such as sorghum with a ~ 730 Mbp genome that is treated with EMS may harbour 3000 induced mutations making the job of finding the one mutation causing the trait difficult. The same genome treated with gamma irradiation may only be able to accumulate a small number of large genomic indels. Thus, identifying the mutation causing the phenotype is severalfold easier. Discovery of large genomic indels via sequencing may also prove more efficient as lower depth of coverage is needed for accurate variant calling compared to SNPs, and therefore more samples can be screened per run. This approach has been used to catalogue gamma-induced mutations created through irradiation of pollen from poplar (Henry et al. 2015). Continued improvement in sequencing technologies suggests that cloning both SNP and large indel mutations will become more common in the near future. This will make valuable mutant alleles available to breeders for marker-assisted introgression into elite germplasm.

1.4 Conclusion

There is little controversy that growing pressures on agricultural productivity such as increasing population, reduction of arable land and new and geographically shifting biotic and abiotic stresses demand serious attention and innovative approaches. Genetic improvement of crops is fundamental to long-term success, and a combination of novel developments and translational science is required. We predict that induced mutagenesis will remain an important tool for the breeder as it is a rapid and relatively low-cost approach to generate novel alleles and phenotypes. Further, new technologies will enable determination of mutant alleles used to create successful mutant varieties and will shed light on gene function and crop productivity.

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Part II
Mutation Induction and Chimera
Dissociation

Chapter 2

Chemical and Physical Mutagenesis in *Jatropha curcas*

Fatemeh Maghuly, Souleymane Bado, Joanna Jankowicz-Cieslak,
and Margit Laimer

Abstract Mutagenized populations are important resources to generate and identify desirable genetic variation of useful traits for crop improvement. When the lack of genetic variability hampers the breeding progress, mutagenesis can introduce genetic variation, reveal gene function, and aid in the characterization of candidate genes involved in biological functions. Mutagenized populations are useful for screening for altered phenotypes and physiological responses, and as a genomics tool. *Jatropha curcas* is a semi-wild, economically important shrub useful as a source of biofuel or in soil reclamation, but it requires genetic improvement in order to select the best genotypes for these purposes. Therefore, this chapter describes the general methods for mutation induction (chemical and physical mutagenesis) using ethyl methanesulfonate (EMS) treatment, gamma irradiation, X-rays, and the procedures that can be used to generate large numbers of induced mutants in different tissues of *J. curcas* under in vitro and in vivo conditions.

Keywords Chemical mutagenesis • Physical mutagenesis • Gamma • X-ray • Mutation induction

2.1 Introduction

Jatropha curcas is one of the most valuable crops for its ability to produce seeds, which contain 60–63 % of protein and 30–45 % of toxic oil that renders the seedcake and oil unsuitable for animal or human consumption (Maghuly and Laimer 2013). The narrow genetic base in *J. curcas* hinders efficient genetic

F. Maghuly • M. Laimer (✉)

Plant Biotechnology Unit (PBU), Department of Biotechnology, BOKU–VIBT, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria
e-mail: fatemeh.maghuly@boku.ac.at

S. Bado • J. Jankowicz-Cieslak

Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria

improvement (Maghuly and Laimer 2013). In fact, the ultimate breeding objectives of the *J. curcas* accessions are to reduce toxicity and improve productivity under adverse climatic conditions. To increase genetic diversity, mutagenesis can be applied for plant improvement (Maghuly and Laimer 2013; Carels 2013).

One of the most effective and commonly used chemical mutagens is ethyl methanesulfonate (EMS; $\text{CH}_3\text{SO}_2\text{OC}_2\text{H}_5$), a monofunctional alkylating agent with a formula weight of 124 able to induce chemical modification of nucleotides, resulting in base changes, breakage of the DNA backbone, and mispairing (Kodym and Afza 2003; Kim et al. 2006). The most frequent alkylating event of nitrogen occurs with guanine (G) at the 6'-O position, forming O-6-ethylguanine, which can pair with thymine (T) instead with cytosine (C), resulting in base pair errors. In this way, during DNA repair, the original G/C site is replaced by A/T in the majority of nucleotide changes (99 %) obtained with EMS (Greene et al. 2003). In addition, depurination of alkylated G will form gaps in the DNA and, therefore, after replication will result in deletions and in frameshift mutations. The gap can fill with any of four bases, obtaining a normal copy, a G/C to A/T transition, or a G/C to C/G or G/C to T/A transversion. Further, alkylation of nitrogen can occur at a lower frequency with G at N-7, forming 7-ethylguanine, representing G/C to C/G or T/A transversions, or A at N-3, forming 3-ethyladenine, representing A/T to G/C transitions, or C at N-1, forming 1-ethylcytosine (Kodym and Afza 2003; Krieg 1963; McCallum et al. 2000).

While chemical mutagenesis primarily causes base substitution, ionizing radiation causes base modification and single-/double-stranded breaks by the production of reactive oxidative species, which interact with DNA, causing oxidative damages (Morita et al. 2009). In physical mutagenesis, physical events such as ionization and extension lead to modifications of the DNA, cell membranes, lipids, enzymes, and other cellular constituents (Kodym and Afza 2003).

Physical mutagens based on linear energy transfer (LET), physical properties, and mutagenesis activity are divided into two main classes. Alpha particles, fast neutrons and heavy ion beams have high LET using subatomic particles such as electrons, protons, neutrons, deuterons, and alpha and beta particles, while gamma rays, X-rays, cosmic rays, and electron beams have a low LET and produce energy in the form of electromagnetic waves, which are the most commonly used for mutation breeding (Mba et al. 2012). Ionizing radiation causes biological injuries in higher plants through two main interactions with genetic material, and light can cause photochemical damages and is effective in producing purine or pyrimidine dimers, resulting in point mutations in the DNA (Esnault et al. 2010; Pathirana 2011; Lagoda 2012). This may result in small to large deletions, point mutations, single- and double-stranded breaks, and even chromosome deletions.

Physical and chemical mutagens have been applied to all types of plant material. Thus, soft materials such as in vivo and in vitro cuttings as well as embryogenic callus require lower doses in comparison to seeds. In fact, the water content, storage time, applied mutagen dose, and temperature represent important factors influencing mutagens in all types of plant material (Mba et al. 2010). The most important and difficult step is to screen the entire mutant population, which can be overcome

by forward and reverse genetic technologies (Maghuly and Laimer 2016). These approaches provide excellent tools for developing efficient strategies for the identification of useful alleles in a breeding program and for functional genomic analyses.

To induce variability in *Jatropha curcas*, chemical and physical techniques for crop improvement have been adapted for the treatment of seeds, as well as for in vivo and in vitro cuttings and somatic embryos (Kodym and Afza 2003; Bado et al. 2013, 2015). This chapter describes commonly used techniques for physical and chemical mutation induction on different *J. curcas* tissues. The mutated populations (M_1) are generated, and to reduce chimerism M_2 or higher populations are produced. Entire mutant populations are screened by either phenotypic evaluation for selection of phenotype of interest (forward genetics) or by genotypic evaluation for detection of novel allele in gene of interest as well as study of gene function (reverse genetics) (Fig. 2.1.).

2.2 Materials

2.2.1 *In Vivo Material*

1. High quality, disease-free seeds, clean and uniform in size (*see Note 1*).
2. In vivo stem cuttings (*see Note 1*).
3. Sterilized soil mixture.
4. Glasshouse facility.

2.2.2 *In Vitro Material*

1. High-quality embryogenic callus cultures and cuttings of in vitro grown cultures in defined genotypes (*see Note 1*).
2. Laminar flow cabinet for in vitro work (in vitro tissue culture facility).
3. Tissue culture media.
4. 70 % ethanol.
5. Parafilm.
6. Whatman filter paper circles (90 mm diameter).
7. Petri dishes (90 mm diameter).
8. Regeneration media.

2.2.3 *Mutagenesis by Chemical Agents (See Note 2)*

1. Ethyl methanesulfonate (EMS) AR grade (*see Note 3*).
2. Dimethyl sulfoxide (DMSO).

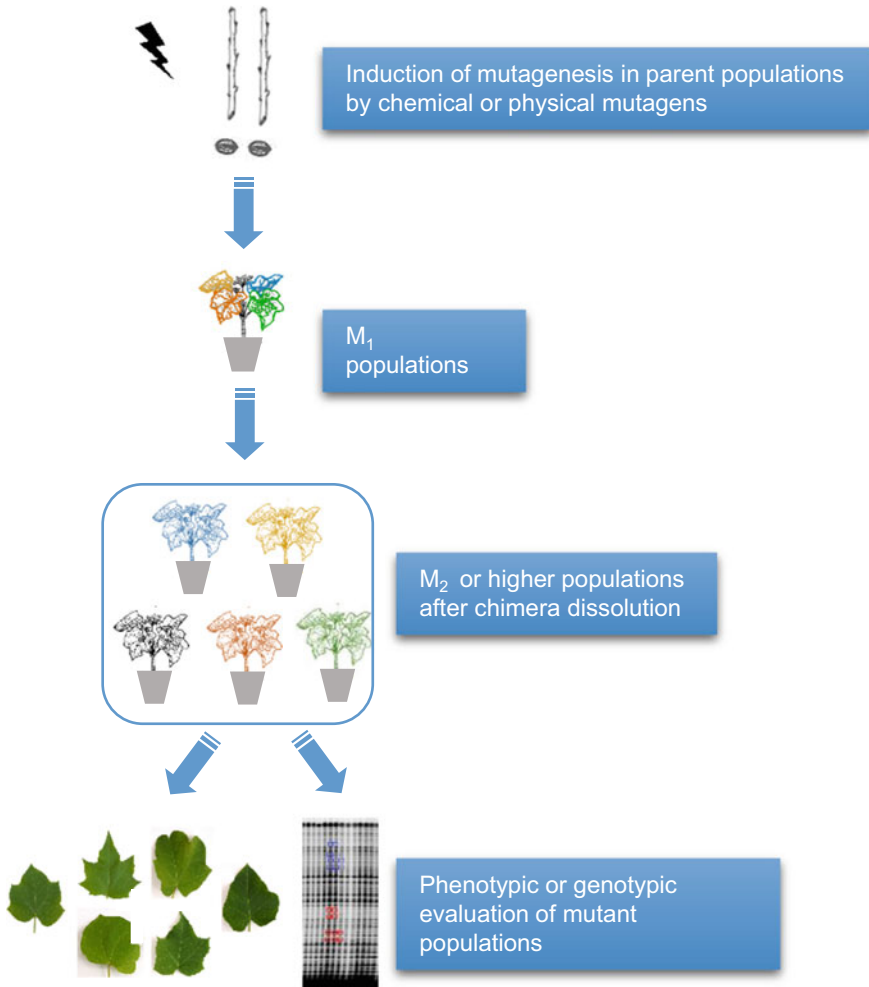


Fig. 2.1. Schematic diagram of the basic steps in physical and chemical mutagenesis of *Jatropha curcas* and characterization of mutant plants

3. 10 % (w/v) sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) for decontamination of EMS solution and tools (*see Note 4*).
4. Sterile deionized water (*see Note 5*).
5. Beakers (500 ml and 1000 ml).
6. Wash bottles.
7. Sterile sieves (metal, 70 mm diameter, 70–100 μm pore size).
8. Forceps.
9. Flat containers for mutagenesis of in vivo cuttings.

10. Sterile membrane filters for filtering the EMS solution (25 mm diameter, 0.2 μm pore size).
11. Syringe.
12. Laboratory fume hood for solution preparation.
13. Polyethylene mesh bags (ca. 11 \times 7 cm in dimension).
14. Orbital shaker.
15. Personal protective equipment (dedicated laboratory coat, protective eyewear, shoe protection, nitrile gloves).
16. Hazardous liquid waste receptacle (collection vessels for EMS waste solution).
17. Box for dry hazardous material disposal.

2.2.4 Mutagenesis by Physical Agents

1. Gamma radiation source.
2. X-ray irradiator RS-2400.
3. Paper envelopes (air and water permeable).
4. Vacuum desiccator.
5. Petri dishes (90 mm diameter).
6. Whatman filter papers for 90 mm Petri dishes.
7. 60 % glycerol (v/v).
8. Sterile and non-sterile deionized water.
9. Parafilm.

2.3 Methods

2.3.1 In Vivo Material

1. Select clean, homogeneous, and disease-free seeds (*see Note 1*).
2. Check the viability and homogeneity by performing a germination test (*see Note 6*).
3. Select seeds with good viability (i.e. >90 %) that allow for the assessment of the mutagen effects.
4. Separate scions of *Jatropha* according to the stage of maturity (from bottom to top: hardwood, semihard, and softwood). This classification is recommended when cuttings are used to perform the radiosensitivity test (*see Note 7*).
5. Cut scions to ten nodes (meristems) per cutting (*see Note 8*).
6. Tie 10–15 cuttings with a rope into a pack (or put in a transparent plastic bag). Package volume should not exceed the irradiator chamber.
7. Place plant material in Petri dishes or appropriate containers for irradiation (*see Note 9*).

2.3.2 *In Vitro Material*

1. Prepare Petri dishes with two wet Whatman filter papers.
2. Prepare one-node cuttings from freshly micropropagated plantlets (subculture intervals of 3 weeks).
3. Place cuttings into Petri dishes with wet filter paper (20–30 cuttings per 94 × 16 mm Petri dish).
4. Seal Petri dishes with Parafilm to avoid contamination outside the tissue culture laboratory.
5. Prepare the regeneration media (MS medium supplemented with growth regulators).
6. Collect the plant material for mutagenesis (rescued mature embryos, shoot tips from micropropagated cultures, leaves, meristems, and cotyledons).

2.3.3 *Mutagenesis by Chemical Agents*

2.3.3.1 EMS Mutagenesis of In Vivo Material (*See Note 10*)

1. Mix the required volumes of distilled water and 2 % (v/v) DMSO and autoclave at 120 °C for 15 min. Let the mixture cool to room temperature (RT) (*see Note 11*).
2. Prepare decontamination solution consisting of 10 % (w/v) sodium thiosulfate (*see Note 4*).
3. Prepare the concentration series of EMS in water-DMSO mixture (Table 2.1), including a pure water control treatment.
4. Use a sterile syringe and a 0.2 µm filter to add the required volume of EMS solution to the sterile water-DMSO mixture (*see Note 12*). Commence with the lowest concentration.
5. Shake the EMS-DMSO solution vigorously to form a homogeneous emulsion, and decant approximately equal volumes into each of bottles (for seeds, *see Note 16*) and flat containers (for stem cuttings) labeled with the tissue type, EMS concentration, and incubation time (*see Note 17*).

Table 2.1 Example of EMS concentrations chosen for mutagenic treatment (T) of seeds and stem cuttings of *J. curcas* (*see Note 13*)

Plant material	T 1	T 2	T 3	T 4	T 5	T 6
Seeds	–	–	0.8 %, 1.5 h	1.6 %, 1.5 h	0.8 %, 3 h	1.6 %, 3 h
Stem cuttings	0.4 %, 1.5 h	0.4 %, 3 h	0.8 %, 1.5 h	1.6 %, 1.5 h	0.8 %, 3 h	1.6 %, 3 h

Incubation times were 1.5 h and 3 h (*see Note 14*). Each treatment should include a control (water-DMSO) (*see Note 15*)

6. Note the time and immerse a separate treatment batch of material into each solution (0 %, 0.4 %, 0.8 %, and 1.6 %) for the selected incubation time (*see Note 18*).
7. After 1.5 h of incubation, quickly but carefully decant each of the 1.5 h treatment batches, and rinse thoroughly with tap water (at least 3–5 times). Capture the residual EMS solution and any wash water for disposal in a hazardous waste container labeled as “Hazardous waste.”
8. Continue incubations of the treatment material selected for 3 h incubation time.
9. After 3 h of incubation, quickly but carefully decant each of the 3 h treatment batches, and rinse thoroughly with tap water (at least 3–5 times). Capture the residual EMS solution and any wash water for disposal in a hazardous waste container labeled as “Hazardous waste.”
10. Detoxify the waste and all unused EMS solution by adding 10 % (w/v) sodium thiosulfate in a 3:1 ratio by volume. Pour into a designated container (marked with “Hazardous waste”) and let stand for at least six half-lives (*see Note 19*).
11. Decontaminate the working area with sodium thiosulfate, and dispose EMS-contaminated items according to laboratory safety rules.
12. Wash plant seeds or stem cuttings in the glasshouse.
13. Mutated seeds and stem cuttings can be immediately planted.
14. Collect data on the germination rate (seed) or sprouting of cuttings and the survival rate after transplants on a weekly basis.
15. Reduce chimerism by producing M₂ or higher mutation populations.
16. Screen mutant populations by either phenotypic or genotypic evaluation (Fig. 2.1.).

2.3.3.2 EMS Mutagenesis of In Vitro Material (*See* Notes 10 and 20 and Fig. 2.2)

1. Prepare containers able to accommodate stem cuttings and bottles for seed mutagenesis. Label them with the incubation time and EMS concentration.
2. Prepare decontamination solution consisting of 10 % (w/v) sodium thiosulfate (*see Note 4*).
3. Calculate the final volume of EMS solution needed (Table 2.2, *see Note 13*). Mix the required volumes of water and 2 % (v/v) DMSO (*see Note 11* and step 1 of Sect. 2.3.3.1.).
4. Prepare the concentration series of EMS in water-DMSO mixture, including a control water treatment (*see Note 12*, and step 3 of Sect. 2.3.3.1.).
5. Shake the solution vigorously to form a homogeneous emulsion, and decant approximately equal volumes into each beaker/bottle labeled with the tissue type, EMS concentration, and incubation time.
6. Note the time. Immerse separate treatment batches of material into each of the four solutions (0 %, 0.4 %, 0.8 %, and 1.6 %) for three incubation times.

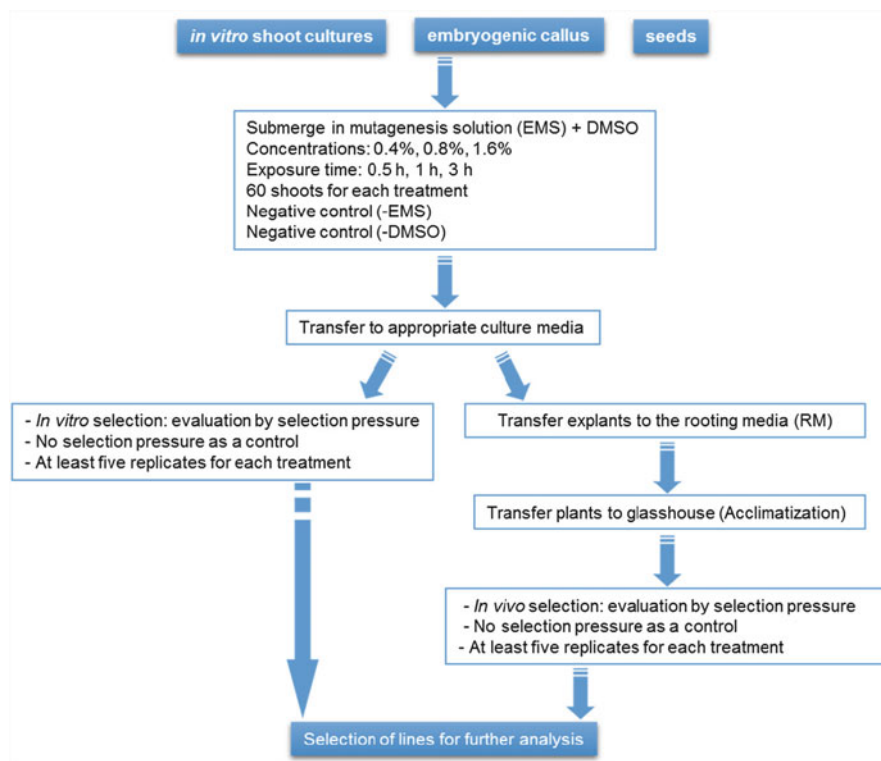


Fig. 2.2 Schematic representation of EMS mutagenesis of in vitro material

Table 2.2 Example of EMS concentrations chosen for mutagenic treatment of seed, embryogenic callus, and in vitro cuttings of *J. curcas* (see Note 13)

Plant material	T 1	T 2	T 3	T 4	T 5	T 6	T 7	T 8
Seeds	–	–	–	–	0.8 %, 1.5 h	0.8 %, 3 h	1.6 %, 1.5 h	1.6 %; 3 h
Embryogenic callus	0.4 %; 0.5 h	0.4 %; 1.5 h	0.8 %, 0.5 h	0.4 %, 3 h	0.8 %, 1.5 h	0.8 %, 3 h	–	–
In vitro shoot cultures	0.4 %, 0.5 h	0.4 %, 1.5 h	0.8 %, 0.5 h	0.4 %, 3 h	0.8 %, 1.5 h	0.8 %; 3 h	–	–

The chosen incubation times of 1.5 h and 3 h resulted in four treatment (T) combinations for each type of mutagenized tissue (see Note 14). Each treatment includes control (water-DMSO) (see Note 15)

- After 0.5 h of incubation, quickly but carefully decant each of the treatment batches labeled with the 0.5 h incubation time, and rinse thoroughly with sterile water (at least 3–5 times). Capture the residue EMS solution and any wastewater for disposal in a hazardous waste container labeled as “Hazardous waste.”

8. Continue the same way with longer incubation times of 1.5 h and 3 h.
9. Detoxify the waste and unused EMS solution by adding 10 % (w/v) sodium thiosulfate in a 3:1 ratio by volume. Pour into a designated container (marked with “Hazardous waste”) and let stand for at least six half-lives (*see Note 19* and step 9 of Sect. 2.3.3.1.).
10. Decontaminate working area with sodium thiosulfate, and dispose of EMS according to laboratory safety rules.
11. After all treatment batches are dispensed, arrange the treated material in each Petri dish/magenta box containing MS media.
12. Take the cultures to the incubation room with 28 °C and 12 h light.
13. Transfer mutagenized tissue to culture media. After 24 h transfer it again to a fresh media.
14. Transfer cultures weekly into fresh media to reduce possible accumulation of phenolic compounds due to the stress of mutagenesis.
15. Thirty days after the treatment, record survival rates of the mutagenized population.
16. Subculture the growing embryogenic callus and in vitro cutting cultures for chimera dissolution.
17. Transfer plants to rooting media for acclimatization phase and subsequently to the glasshouse for further mutant evaluation (Fig. 2.2).

2.3.4 Mutagenesis by Physical Agents

2.3.4.1 Gamma Irradiation of In Vivo Material (See Notes 21–22)

1. Pack seeds in paper envelopes (*see Note 23*), or tie cuttings with a rope into a pack (optionally put in a transparent plastic bag) (*see Note 7*).
2. Calculate the exposure time based on the dose rate of gamma cell irradiator that will be used (Gy/s or Gy/min) (*see Note 24*).
3. Label envelopes according to the dose required (Table 2.3) and genotype when handling more than one genotype. An untreated bag (control) with same number of seeds will be held in the same conditions as the treated ones.
4. Place the packed seeds in a vacuum desiccator (with 60 % glycerol) for moisture equilibration.
5. Keep the seeds in vacuum desiccator for 3–7 days. This equilibrates the seed moisture content to approximately 12–14 %, which is the ideal moisture for efficient induction of mutation (*see Note 25*).
6. Apply the required dose (Table 2.3) by placing bags into irradiator chamber for the exposure time to produce the dosage (*see Note 7*).
7. Start gamma cell irradiator (*see Note 26*).
8. Open the lead shielding collar (when gamma cell is at the loading stage).

Table 2.3 Example of 139 Gy/min gamma irradiator doses chosen for mutagenic treatment (T) of seeds and stem cuttings of *J. curcas*

Plant material	T 1	T 2	T 3	T 4	T 5
Seeds	100 Gy/40s	200 Gy/83 s	300 Gy/126 s	400 Gy/169 s	500 Gy/212 s
Stem cuttings	15 Gy/3 s	20 Gy/5 s	25 Gy/7 s	30 Gy/9 s	35 Gy/12 s

Each treatment also included untreated samples

9. Open the sample chamber door.
10. Place the plant material into the irradiator chamber (Fig. 2.3).
11. Close the sample chamber door and the lead shielding collar.
12. Set the exposure time to produce the required dose (*see* **Notes 24 and 27** and Table 2.3). When the applied dose for the genotype is unknown, a radiation test may be performed (*see* **Note 7**).
13. Put the chamber at the irradiation stage by lowering the elevator.
14. Start the countdown of exposure time.
15. Raise the chamber to the loading stage when exposure time is completed.
16. Open the lead shielding collar and then the sample chamber door.
17. Remove the irradiated plant material (*see* **Note 28**).
18. If necessary, repeat the treatment at defined time intervals to reach the required mutation induction dose.
19. Irradiated seeds and stem cuttings can be planted immediately.
20. Collect data on a weekly basis on the germination rate of seeds, sprouting of cuttings, and the number of survival.
21. Reduce chimerism by producing M₂ or higher mutant populations.
22. Screen mutant populations by performing either phenotypic or genotypic evaluation (Fig. 2.1.).

2.3.4.2 Gamma Irradiation of In Vitro Material

1. Label each Parafilm-sealed Petri dish containing plant material with the selected dose (*see* **Note 29**).
2. Transfer Petri dishes to physical mutagen source laboratory.
3. Apply the required dose for mutation induction (*see* **Notes 7 and 30**).
4. Transfer irradiated samples to tissue culture laboratory.
5. Surface-sterilize each Petri dish with 70 % ethanol before removing the Parafilm (*see* **Note 31**).
6. Transfer the irradiated material into an appropriate tissue culture media.
7. Take the cultures to the incubation room (28 °C and 12 h light).
8. Record survival rates of the mutagenized population 30 days after treatment.
9. Subculture growing embryogenic callus or in vitro shoot cultures for chimera dissolution.

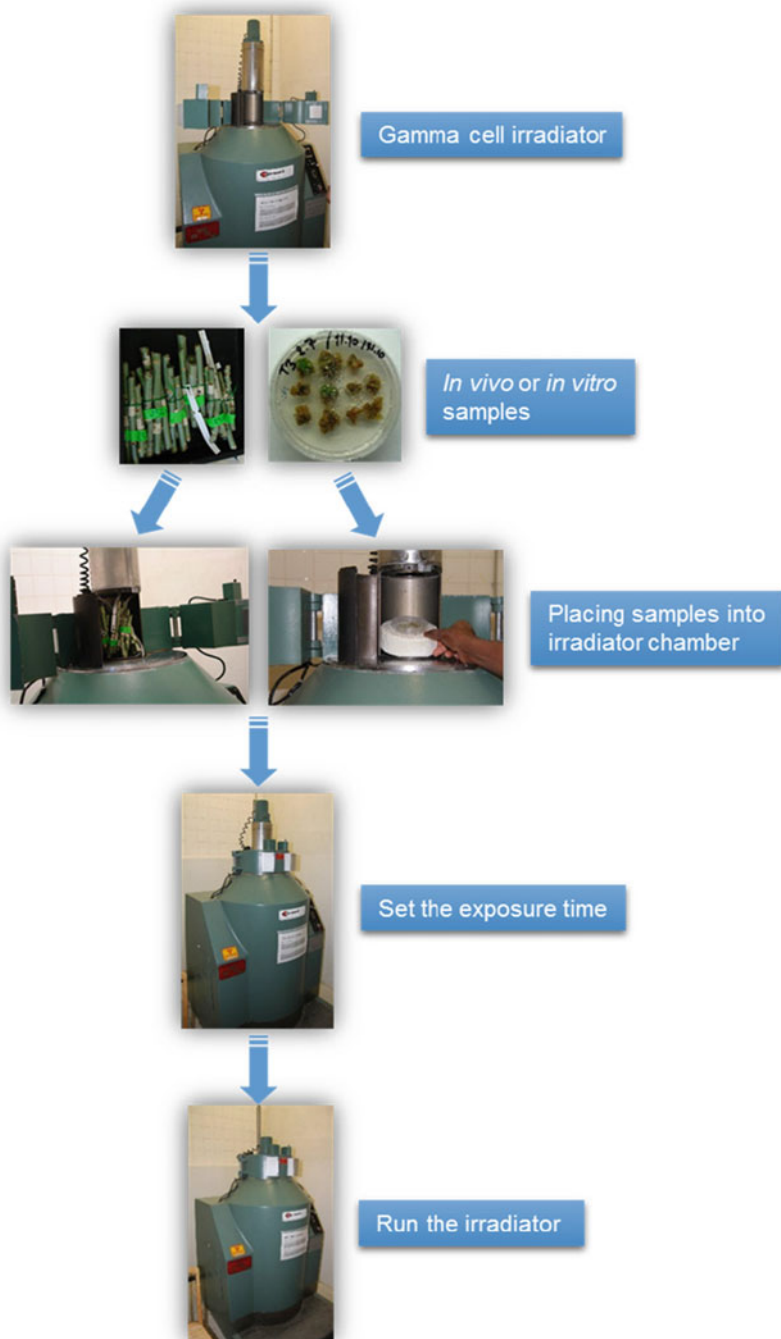


Fig. 2.3 Process of irradiation using gamma cell irradiator cobalt-60 source

10. Transfer plants to rooting media for acclimatization and subsequent transplant to the glasshouse for further mutant evaluation (Fig. 2.2).

2.3.4.3 X-Rays (See Note 32, Fig. 2.4)

1. Label each Petri dish containing plant material with the selected dose.
2. Place samples into containers and fix them with brackets and move into the canister.
3. Place canister with adaptors into the center of the irradiator.
4. Create a vacuum by filling the empty space with calibration samples (i.e., rice).
5. Close the canister with the lid.
6. Open the shielding window of the irradiator chamber.
7. Take out the sample platform.
8. Place the canister in one of the five canister holders (make sure that all canisters are present including the sample canister).
9. Place the platform at the optimal position.
10. Close the shielding window of irradiator chamber.
11. Set the exposure time by putting amount (kW) needed to produce the required dose (see Note 33).
12. Run the irradiator until the monitor displays zero kW.
13. Open the shielding window of the irradiator chamber.
14. Take out the canister containing the irradiated samples.
15. Take off the canister lid.
16. Remove the vacuum samples by dumping the instant rice and do not disturb the adaptor holding plant material.
17. Remove the samples from the canister and adaptors (see Note 34).
18. Take the plant material to appropriate culture media, soil, or storage conditions.

2.4 Further Analyses

The second generation (and higher) after chimera dissolution of *in vivo* and *in vitro* plants can be screened for the selection of candidate genes based on phenotypes or genotypes (Figs. 2.5 and 2.6). Mutations can be detected with various direct and indirect methods such as denaturing high-performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis (DGGE), temperature gradient capillary electrophoresis (TGCE), heteroduplex analysis (HD), the analysis of single-stranded DNA conformation polymorphism (SSCP), chemical or enzymatic cleavage of mismatches (CECMs), and Targeting Induced Local Lesions in Genome (TILLING) (Till et al. 2007). Methods such as whole genome sequencing,

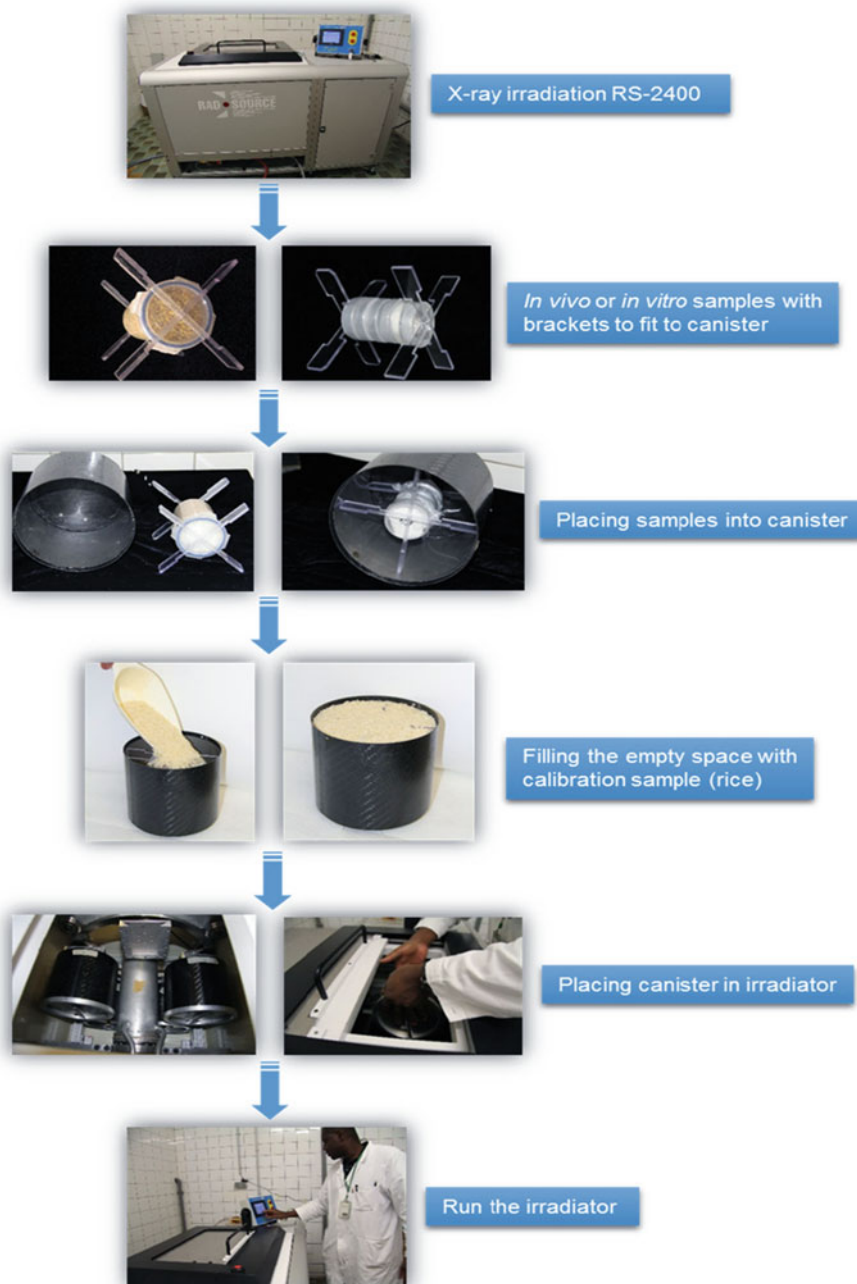


Fig. 2.4 Process of sample preparation for irradiation using X-ray RS-2400 source

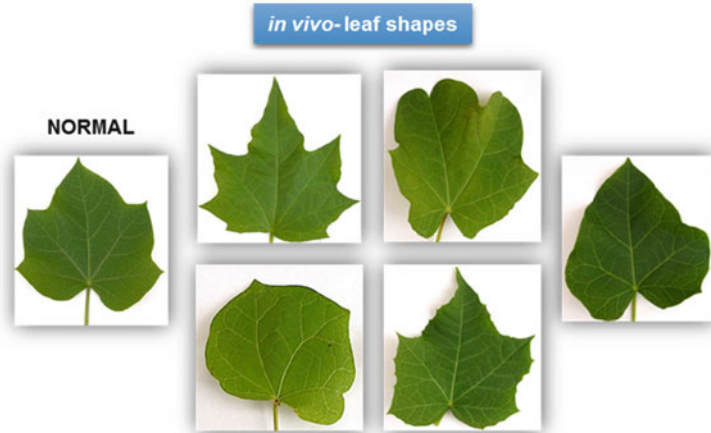


Fig. 2.5 Phenotype analysis of in vivo leaf shapes of mutagenized *Jatropha*

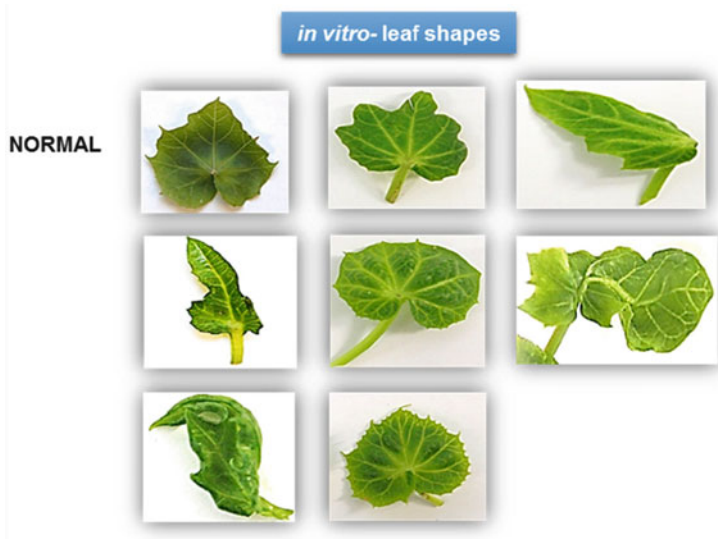


Fig. 2.6 Phenotype analysis of in vitro leaf shapes of mutagenized *Jatropha*

exome capture sequencing, restriction-site-associated DNA (RAD) sequencing, and genotyping by sequencing (GBS) provide the necessary information for mutation confirmation, which cannot be achieved through indirect methods (Maghuly and Laimer 2016).

2.5 Notes

1. Select a population size that assures success of the experiment, bearing in mind that an M_2 population of at least several thousand (5000–10,000) individuals is considered suitable (Kodym and Afza 2003; Van Harten 1998; Brock 1997). Consider that the different explant types, e.g., seeds, in vivo or in vitro cuttings, or embryogenic callus, have different requirements and capacities. This is especially important in the case of long-lived organisms like trees and has consequences at the level of population size, dissolution of chimerism, and frequency of mutation.
2. The mutagenesis should be conducted in the dedicated chemical mutagenesis laboratory.
3. EMS is an irritant and highly carcinogenic compound. Avoid any skin contact and use disposable gloves. All body parts or laboratory coats contaminated with EMS should be washed thoroughly with water and detergent and further neutralized with 10 % (w/v) sodium thiosulfate (*see* step 3 of Sect. 2.2.3).
4. Alternatives to sodium thiosulfate have also been used, such as sodium hydroxide, to inactivate the EMS.
5. Deionized water prevents undesired effect of metallic ions (Kodym and Afza 2003).
6. A germination test should be performed with at least ten seeds.
7. When the applied dose for the genotype is unknown, a radiation test should be conducted. To perform radiosensitivity test on vegetative tissue (e.g., cuttings), select 30 cuttings per dose with a wide range from 0 to 100 Gray (Gy). However, the ranges of 0, 10, 20, 30, 40, 50, and 60 Gy of gamma and X-rays may be sufficient to establish the optimal dose due to the high moisture content in comparison to seeds. The Gy is the unit used to quantify the absorbed dose of radiation (1 Gray = 1 J/kg).
8. Cuttings should not exceed the size of the irradiator's chamber. About 20 cm length per cutting is optimal for the gamma irradiator chamber (Fig. 2.1.). It is important to take meristematic dominance into consideration and thus to perform the mutagenesis with two-node cuttings which allows the production of many putative mutants.
9. Plant materials should occupy the same position along the exposure time. Petri dishes of in vitro material should be wrapped with Parafilm in order to avoid surface contamination.
10. Use only freshly prepared EMS solution.
11. This preparatory step may be carried out in advance.
12. This step and any further steps must be carried out in a laminar flow hood.
13. While reviewing the literature and reports on EMS concentrations used in mutagenesis experiments, both percentage (%) (v/v) and molarity concentrations are listed. Table 2.4 lists the percentage (%), corresponding molarity, and dilutions for an example of 10 ml volume of mutagen. The standard EMS stock from Sigma is 9.7 M.

Table 2.4 Examples of the concentration EMS in molarity or percentage in 10 ml final volume

Molarity (mM)	%	Amount to add to a final volume of 10 ml (in microliters)
9.7	0.10	10
19.4	0.20	20
22.5	0.23	23
25	0.26	25.7
27.5	0.28	28.3
29	0.30	30
48.5	0.50	50

14. Estimate the time needed to conduct the entire experiment with various tissue types. In case you are not confident in doing the mutagenesis on all tissue types, split the plant material into batches for separate days treating one tissue type per day.
15. Some protocols call for the addition of DMSO, which may make the mutagen more miscible.
16. Presoaking seeds increases infusion of the mutagen into the embryo. The time of presoaking depends on the seed coat.
17. The amount of mutagen solution should be enough to cover the whole tissue, to provide a constant concentration of mutagen throughout the treatment, and to expose all samples similarly.
18. If duration of treatment is longer than half-life of the EMS (*see Note 19*), the solution should be replaced with a freshly prepared mutagen solution.
19. The half-life of EMS in water at pH 7 at 20 °C is 93 h and at 30 °C is 26 h (Kodym and Afza 2003). For EMS in a 10 % sodium thiosulfate solution, the half-life is 1.4 h at 20 °C and 1 h at 25 °C.
20. Somatic embryogenesis is an excellent system for plant propagation and mutation induction because somatic embryos originate from single cells. Somatic embryos can be produced on a large scale in cell suspension cultures in Erlenmeyer flasks or in bioreactors. Somatic embryogenesis limits chimeras among in vitro plantlets making embryogenic callus an ideal plant material for mutation induction. Somatic embryos of *Jatropha* can be induced by direct somatic embryogenesis or indirect somatic embryogenesis (Kalimuthu et al. 2007). The difference between the two strategies is whether plants are regenerated directly from embryos or from an intervening callus phase.
21. Gamma ray mutagenesis may be performed using different facilities, such as gamma cell irradiator, gamma phytotron, gamma house, or gamma field. The gamma cell irradiator with cobalt-60 or cesium-137 as radioactive source is the most commonly available equipment worldwide.
22. Radioactivity is mutagenic and carcinogenic. It should be handled by trained personnel in a suitable laboratory. The safety precautions for exposing plant material to a gamma irradiation source must be strictly observed.
23. Perform radiosensitivity test for minimum of ten seeds per bag with at least three replications per dose, or 100 seeds per bag and per dose.

24. Exposure time is equal to the required dose divided by the dose rate of the day.
25. The amount of water has an important impact on the irradiation efficiency. A decreased amount of seed moisture content will increase the mutation frequency. Therefore, it is necessary to equilibrate the seed moisture content before the irradiation process.
26. Take care to observe all safety precautions before exposing tissues to irradiation.
27. For each exposure, dosimetry was performed following the Gafchromic dosimetry system. One 20 by 20 mm (for X-ray) film dosimeter is placed in a small paper envelope labeled with requested dose and then placed in the center of samples before each exposure. The optical density measurement is performed on a radiochromic reader 24 h after irradiation, and the dose was calculated according to the calibration Gafchromic dosimetry film with their optical density. That allows the estimation of the absorbed dose by the sample exposed to X-ray.
28. The irradiated samples are safe to be held in hands because the sample chamber isolates the plant material from the source and there is no surface contamination.
29. A Petri dish with untreated cuttings (control) has to be prepared and kept in the same conditions as the treated samples.
30. Incubate somatic embryos at the appropriate conditions according to direct or indirect techniques.
31. Observe general rules for plant tissue culture practices.

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Chapter 3

Chemical Mutagenesis and Chimera Dissolution in Vegetatively Propagated Banana

Joanna Jankowicz-Cieslak and Bradley J. Till

Abstract Random mutagenesis has been widely used for forward-genetics and crop breeding since the application of ionising radiation on cereals described in the late 1920s. The development of high-throughput and accurate mutation discovery technologies has enabled reverse-genetic screening of mutant populations in the twenty-first century. The majority of mutation-based approaches in crops have involved seed-propagated species. Large bodies of data are available on the spectrum and density of induced mutations for some mutagens. It is well established that genetic chimerism caused by random accumulation of different mutations in different cells is resolved by sexual propagation and that by the second-generation post-mutagenesis (termed the M_2), plants are no longer genetically mosaic. Vegetatively propagated species, however, are quite different as they primarily undergo mitotic propagation. In the absence of meiosis, procedures must be implemented to remove mosaicism and generate plant material that is genotypically homogeneous and suitable for forward- and reverse-genetic screening and breeding. We have previously developed a Targeting Induced Local Lesions IN Genomes (TILLING) platform for the vegetatively propagated triploid banana to investigate the density and spectrum of induced mutations and mechanisms by which tissue culture materials become genotypically homogeneous. Here we provide a detailed protocol for meristematic isolation, mutation induction and dissolution of chimeric sectors focusing on the use of chemical mutagen ethyl methanesulfonate (EMS).

Keywords Chemical mutagenesis • *Musa* • Mutation stability • Diplontic selection • Somatic genome • Polyploidy

J. Jankowicz-Cieslak (✉) • B.J. Till

Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria

e-mail: j.jankowicz@iaea.org

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3.1 Introduction

The nutrition of a billion people relies on crops which are not propagated through seeds, particularly in tropical and Neotropical regions of Africa, Asia, Latin America and the Caribbean. Plants that have completely lost the ability for sexual propagation are called obligate, while those for which meiosis is still possible are called facultative vegetatively propagated plants (van Harten 1998). In a natural setting, genetic diversity is increased in vegetatively propagated crops via the accumulation of spontaneous mutations. Researchers and breeders can further alter the genetics of vegetatively propagated crops through methods including inducing mutations, transgenics, cell fusion hybrids and various genome-editing approaches (Jankowicz-Cieslak and Till 2015). Inducing mutations in combination with other methods such as *in vitro* techniques is advantageous for the improvement and diversification of vegetatively propagated germplasm. This is because recombination and sexual assortment of characters cannot be achieved in mitotically propagated crops, and so inducing mutations is a major way to introduce novel diversity. Moreover, new developments in the field of molecular biology can enable a fast and accurate identification of changes on the genomic level allowing a more precise use of induced mutations.

There are several constraints when working with vegetatively propagated material. The first difficulty appears while performing the mutagenesis experiments. Mutagenesis of vegetative propagules requires pre-mutagenesis preparation of material and oftentimes extensive post-mutagenesis physical manipulations. In many cases, sterile techniques are required for both pre- and post-treatment handling of the material as well as during the mutation steps. Vegetatively propagated crops are usually heterozygous. Mutagenic treatment may uncover otherwise recessive traits that would not express in homozygous material. However, on account of their asexual character and lack of meiotic sieve (the process in which deleterious alleles can be expunged from a genome through genetic recombination and independent assortment), unwanted background mutations are difficult to remove (van Harten 1998). Another result of the mutagenesis performed on *in vitro* material is the generation of chimeric tissues resulting from the fact that each cell treated with a mutagen will accumulate a different collection of mutations. Genotypic heterogeneity can be lessened through several cycles of vegetative *in vitro* regeneration of the mutagenised vegetative propagule (Roux et al. 2001; Jankowicz-Cieslak et al. 2012). However, this has not been extensively studied on the level of genome sequence.

Producing a mutagenised population suitable for forward- or reverse-genetic studies requires careful consideration of several practical experimental design factors. This includes the genetic structure of the target population, choice of the best mutagen, dosage and pre- and post-treatment handling of material. Mutagens that induce point mutations with high density are desirable for reverse-genetic approaches. Chemical mutagens have a known effect on the DNA sequence and have predominated in reverse-genetic projects. This is in part due to the fact that

chemical mutagens producing point mutations are highly predictive in their effect on protein function and there have been decades of development of technologies for mutation discovery (Ng and Henikoff 2003; Colbert et al. 2001; Tsai et al. 2011). The most commonly used chemical mutagen is ethyl methanesulfonate (EMS) which in many species produces almost exclusively G:C to A:T transition mutations (Kurowska et al. 2011). Other mutagens such as gamma and fast neutron irradiation can produce a broader spectrum of mutations ranging from SNPs and small indels to deletions greater than one million base pairs (Jankowicz-Cieslak and Till 2015). While phenotypic consequences of large indels and rearrangements may be greater, data sets on the spectrum and density of such mutations are currently much smaller than that of EMS, and therefore predictability of optimal population size required to recover desired alleles is much lower.

To date there has been limited success in producing new mutant banana varieties via physical irradiation. Three mutant varieties have been officially registered in the mutant variety database (MVD 2016). All were produced using gamma irradiation. The number is far lower than rice, which is the crop with the most officially released varieties (over 800 in total). The reasons are likely manifold. Many fewer people are actively involved in banana breeding compared to rice. The logistics of banana mutation breeding including tissue culture propagation, chimerism, polyploidy, heterozygosity and field space required to find rare favourable mutations also makes banana less tractable than rice. One other possibility may be that mutations are not easily induced in banana at a high density. This may be specific to specific mutagens or dosages previously used. Compounding this was the lack of tools to accurately count induced mutations so that the mutagenesis treatment could be optimised.

Mutagenesis is usually applied in a manner that produces some level of lethality in the treated population. It is hypothesised that physiological differences underlie the enhanced sensitivity of certain species to lethality caused by alkylating agents (Comai and Henikoff 2006). This may be due either to direct cytotoxic effects or differences in the efficiency of DNA repair (Hoffman et al. 2004). Other factors affect the total number of mutations that accumulate in a plant genome. For example, phenotypic and genotypic studies show a higher accumulation of mutations as ploidy levels increase in wheat (Stadler 1929; Uauy et al. 2009).

Prior to the advent of TILLING, direct measurement of the density and spectrum of induced mutations was not carried out in many plant species. This resulted in sometimes contradictory advice on achieving a well-mutagenised population. For example, a range of different recommendations by various research groups for chemical mutagenesis of banana tissue cultures has been reported (Omar et al. 1989; Bhagwat and Duncan 1998). These relied on post-mutagenesis observations of the level of damages on plant growth. One observable trend is that longer periods of exposure to EMS at low concentrations may be desirable because they produce the least amount of visible damage (Savin et al. 1968). The first evaluation of the genomic effect of mutagen on the vegetatively propagated material was conducted by Jankowicz-Cieslak et al. (2012). Tests of incubation time and EMS concentrations for the optimal production of mutagenic populations in banana were

done using traditional TILLING methods employing enzymatic mismatch cleavage and fragment analysis using fluorescence detection. A spectrum of >99 % G:C to A:T transition changes was reported (Jankowicz-Cieslak et al. 2012). The density of one mutation per 57 kb was higher than previously published diploid plants but lower than tetraploid and hexaploid wheat. The results fit the expectation of mutation accumulation first described in the late 1920s (Stadler 1929). Based on this work, we present here a protocol for EMS mutagenesis of isolated banana shoot tips suitable for forward- and reverse-genetic studies, e.g. TILLING or TILLING by sequencing in vegetatively propagated plants.

3.2 Materials

3.2.1 Culture Medium (S-27)

1. Thiamine hydrochloride.
2. 6-Benzylaminopurine (BAP).
3. L-Cysteine HCl.
4. Sucrose (grade1).
5. Murashige and Skoog (MS) basal salt with minimal organics (e.g. Sigma Cat. No. M-6899).
6. Tissue culture grade water.
7. Gelling agent (e.g. Gelrite).
8. 0.22 µm cellulose acetate (CA) sterilising, low-binding filters.
9. Vacuum pump.
10. Sterile tubes (50 ml).
11. Analytical balance.
12. Weighing trays.
13. Spatula.
14. Magnetic stir bar.
15. Hot plate.
16. pH meter.
17. NaOH.
18. KOH.
19. HCl.
20. Media dispenser.
21. Culture tubes.
22. Closures for culture tubes.
23. Erlenmeyer flasks.
24. Aluminium foil.

3.2.2 *Chemical Toxicity Test*

1. Chemical mutagen laboratory equipped with fume hood and flow bench (*see Notes 1 and 2*).
2. Personal protective equipment (dedicated laboratory coat, eye protection/goggles, shoe protection, nitrile gloves).
3. Hazardous liquid waste receptacle (collection vessels for EMS waste solution).
4. Box for dry hazardous material disposal.
5. High-quality, disease-free in vitro plantlets (25 propagules per treatment).
6. Sterile S-27 liquid culture medium. Dispense 12 ml of the culture medium per flask and autoclave.
7. Dimethyl sulfoxide (DMSO).
8. 10 % (w/v) sodium thiosulfate.
9. Ethyl methanesulphonate (EMS) AR grade, M.W. 124.2.
10. Sterile distilled water.
11. Sterile membrane for filtering EMS solution: 25 mm diameter, 0.2 μm pore size.
12. Syringe.
13. Needle.
14. Petri plates (94 mm and 145 mm diameters).
15. Bottles (100 ml, 500 ml).
16. Beakers (500 ml and 1000 ml).
17. Sieves (metal, 70 mm diameter, 10–100 μm pore size).
18. Forceps.
19. Scalpels.
20. Scalpel blades.
21. Parafilm.
22. Orbital shaker.
23. Disposable pipettes (5 ml, 25 ml).

3.2.3 *Calculation of Growth Reduction (GR)*

1. Analytical balance.
2. Sterile Petri plates for weighing in vitro material.
3. Forceps.
4. Sterile S-27 liquid culture medium.
5. Standard spreadsheet software, e.g. Microsoft Excel.

3.2.4 Bulk Mutagenesis

1. All materials as listed in Sect. 3.2.1.
2. High-quality, disease-free in vitro plantlets (e.g. 1000 propagules per treatment).

3.2.5 Chimera Dissolution

1. Tissue culture laboratory equipped with sterile flow benches (equipped with gas).
2. Growth rooms with light and temperature control (light regime 65 $\mu\text{mol}/\text{m}^2/\text{s}$, e.g. Cool White fluorescent tubes, Philips TLP 36/86; temperature regime of $22^\circ \pm 2^\circ \text{C}$).
3. Sterile S-27 liquid culture medium.
4. Sterile S-27 solid culture medium.
5. Ethanol for surface and tools sterilisation.
6. Forceps.
7. Scalpels.
8. Scalpel blades.
9. Petri plates (94 mm and 145 mm diameters).
10. Orbital shaker.

3.3 Methods

3.3.1 Preparation of Liquid Culture Medium

1. Prepare stock solutions of thiamine (1 mg/ml), BAP (0.23 mg/ml) and L-cysteine (4 g/l).
2. Filter sterilise stock solutions.
3. Dispense into 50 ml batches and freeze (-20°C) for further use. Store the working solution at 4°C .
4. For 1 l of the liquid culture media, use the following: 4.4 g of Murashige and Skoog basal salt with minimal organics, 40 g sucrose, 10 ml L-cysteine, 20 ml BAP and 1 ml of thiamine stock solutions. Use double distilled water.
5. Place the media on the mixer and let it mix properly.
6. Calibrate the pH meter as per manufacturer instructions.
7. While stirring, adjust medium to pH 5.8 using NaOH, HCl or KOH.
8. Dispense 12 ml of the culture medium per Erlenmeyer flask.
9. Close each Erlenmeyer flask tightly over the top with aluminium foil.
10. Autoclave for 20 min at 120°C .
11. Allow the medium to cool.

12. Store the medium for up to a week in a cold room.

3.3.2 Preparation of Solid Culture Medium

1. For 1 l of solid medium cook 1.8 g of Gelrite and 40 g sucrose in 400 ml of tissue culture grade water.
2. In a separate beaker containing 400 ml water, mix 4.4 g of Murashige and Skoog basal salt with minimal organics, 40 g sucrose, 10 ml L-cysteine, 20 ml BAP and 1 ml of thiamine stock solutions.
3. Place the media on the mixer and let it mix properly.
4. Calibrate the pH meter as per manufacturer instructions.
5. While stirring, adjust the pH to 5.8 using NaOH, HCl or KOH.
6. Heat the solution.
7. Add cooked Gelrite/sucrose and continue stirring and heating until the solution is homogenous.
8. Dispense 8 ml of the medium into culture tubes.
9. Sterilise the medium for 20 min at 120 °C.
10. Allow medium to cool prior to use.
11. Store for up to a week in a cold room.

3.3.3 Chemical Toxicity Test

1. Review safety procedures of the chemical mutagenesis laboratory (*see Note 2*).
2. Autoclave all non-disposable materials (e.g. sieves, forceps).
3. Choose appropriate concentrations of EMS solution and incubation times. Examples of dilutions for EMS concentrations are given in Table 3.1.
4. Dispense required volumes of distilled water and autoclave at 120 °C for 15 min. Let the mixture cool to room temperature.
5. Add DMSO using sterile pipette tip.

Table 3.1 Concentrations chosen for the toxicity test in banana (for two incubation times at 2 and 4 h)

EMS concentration	H ₂ O	DMSO	EMS
10 % (stock solution)	88 ml	2 ml	10 ml ^a
0.25 %	95.5 ml	2 ml	2.5 ml ^b
0.5 %	93 ml	2 ml	5 ml ^b
1 %	88 ml	2 ml	10 ml ^b
1.5 %	83 ml	2 ml	15 ml ^b
0 % control	98 ml	2 ml	–
0 % control (<i>without</i> DMSO)	100 ml	–	–

^aPrepare fresh immediately before experiment

^bPrepare fresh from the 10 % EMS stock solution before experiment

6. Prepare meristematic cuttings of banana plantlets (e.g. 25 propagules per treatment including controls). Store in a sealed Petri plate until processed.
7. Prepare a fresh 10 % EMS stock solution by adding the required volume of EMS solution to the sterile water-DMSO mixture. Use a sterile syringe and a 0.2 μm filter for this step. Place syringe and filter into a beaker containing 100 mM sodium thiosulfate to inactivate EMS before placing in hazardous waste.
8. Prepare the concentration series of EMS using 10 % EMS stock solution (*see* Table 3.1). Commence with the lowest concentration. Shake the solution vigorously (*see* Note 3). Decant approximately equal volumes into each bottle labelled with the appropriate treatment code (concentration and incubation time). This step and any further steps must be carried out in a laminar flow cabinet.
9. Place meristematic cuttings (e.g. 25 each) into each treatment bottle. Place closed bottles on a rotary shaker set at 180 rpm and start incubation. Examples in this protocol are provided for incubation times of 2 and 4 h (*see* Figs. 3.1 and 3.2 and Table 3.2).

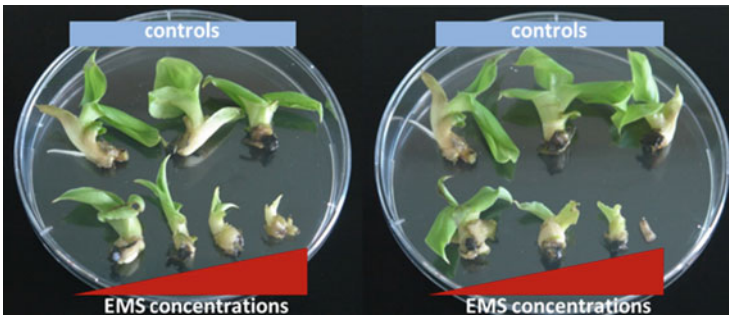


Fig. 3.1 Example of chemical toxicity test performed on the *in vitro* banana material. Grand Naine plantlets were exposed to four concentrations of EMS (from left to right: 0.25 %, 0.5 %, 1 % and 1.5 % EMS) and controls (from left to right: water, DMSO and untreated) and incubated for 2 (*left panel*) and 4 (*right panel*) hours. Material is shown after 30-day post-treatment growth in S-27 liquid media

Fig. 3.2 Reduction in fresh weight of mutagenised *in vitro* plantlets of Grand Naine as a percentage of the control (*Y axis*) against EMS concentrations (*X axis*). Incubation times of 2 and 4 h are presented. *Red bars* show estimation of mutagen concentrations where 50 % growth reduction is achieved

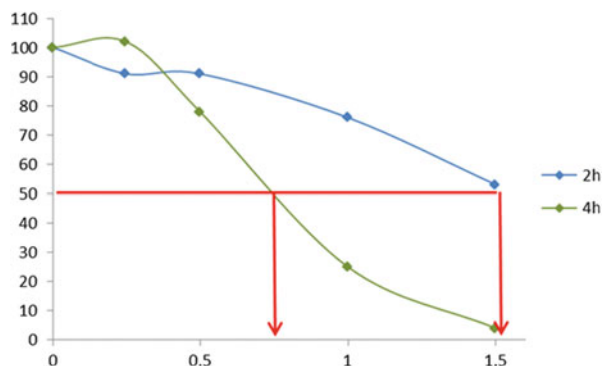


Table 3.2 Average fresh weights of 30-day-old Grand Naine in vitro plantlets exposed to varying concentrations of EMS for 2- and 4-h incubation periods

	2-h incubation time		4-h incubation time	
	^a Fresh weight (g)	Percentage of control (%)	^a Fresh weight (g)	Percentage of control (%)
0 TIME	1.60	100	1.60	100
0.25 % EMS	1.46	91	1.63	102
0.5 % EMS	1.46	91	1.25	78
1 % EMS	1.21	76	0.40	25
1.5 % EMS	0.84	53	0.07	4

^aAverage of 25 plantlets weighed separately under sterile conditions

10. After the incubation time, quickly but carefully decant each of the treatment batches and rinse with sterile water (approximately 1 l of dH₂O per treatment).
11. Collect all the liquid waste in a dedicated bucket labelled as hazardous waste (*see Note 4*). Dispose of toxic waste according to local regulations.
12. Transfer explants into Erlenmeyer flasks containing fresh MS medium (five explants per flask) and incubate in the growth chamber for 24 h.
13. After 24 h transfer explants into fresh MS medium to remove any residual EMS and DMSO.
14. Incubate material in the liquid media for the next 30 days (rotary shaker 60 rpm with a continuous light 65 $\mu\text{mol}/\text{m}^2/\text{s}$; Cool White fluorescent tubes, Philips TLP 36/86 and the temperature of $22^\circ \pm 2^\circ \text{C}$).

3.3.4 Calculation of Growth Reduction (GR)

1. After 30 days of incubation, assess the viability of plants by counting surviving plantlets and measuring the fresh weight of each plant (*see Fig. 3.1*).
2. Place a balance in the laminar bench and weigh each mutated plant separately in a sterile Petri plate.
3. Record the data for each treatment and enter it into a spreadsheet (e.g. Microsoft Excel).
4. Calculate average fresh weights for each treatment and for the control (*see Table 3.2*).
5. Calculate percentage of the fresh weight of the plant in relation to the control (*see Note 5*).
6. Plot percentage of control against mutagenesis treatment (*see Fig. 3.2*).
7. Estimate the mutagen concentration required to obtain 50 % of control (*see Fig. 3.2*, red line).
8. Identify concentrations suitable for bulk mutagenesis of your material.

3.3.5 Bulk Mutagenesis

1. Autoclave all non-disposable materials (e.g. sieves, forceps).
2. Choose appropriate EMS concentration and incubation time based on the results obtained from the chemical toxicity test.
3. See Figs. 3.3 and 3.4 for an overview of the bulk mutagenesis procedure.
4. Dispense required volumes of distilled water and autoclave at 120 °C for 15 min. Let the mixture to cool to room temperature.
5. Add DMSO using sterile pipette tip.
6. Prepare meristematic cuttings (e.g. 1000) per each treatment chosen (see **Note 6**).
7. Place a maximum of 200 cuttings into each autoclaved bottle (labelled with EMS concentration and incubation time).
8. Transfer closed bottles containing in vitro material into the chemical mutagenesis laboratory. Add 100 ml of appropriate EMS mixture into each bottle (see **Note 7**).
9. Place bottles on a rotary shaker and incubate at 180 rpm for the chosen length of time.
10. After incubation, add water into the bottles, mix gently and immediately decant carefully using a sterile sieve. See Fig. 3.4 and **Note 8** on post-mutagenesis procedures.
11. Repeat washing step three times.
12. Transfer micropropagules into Erlenmeyer flasks containing liquid medium (maximum of ten meristematic cuttings per flask).
13. Transfer flasks into the growth room and place on a rotary shaker (60 rpm) with continuous light (65 $\mu\text{mol}/\text{m}^2/\text{s}$; Cool White fluorescent tubes, Philips TLP 36/86) and the temperature of 22° \pm 2 °C.
14. The next day transfer all treated plantlets into fresh liquid medium.
15. Transfer cultures weekly into fresh liquid media to reduce possible accumulation of phenolic compounds due to the stress caused by mutagenesis.
16. After 30 days of incubation, count survival rates and make note of any visible morphological abnormalities.

3.3.6 Chimera Dissolution

1. Grow explants in liquid culture media under constant horizontal rotation at 60 rpm with continuous light (65 $\mu\text{mol}/\text{m}^2/\text{s}$; Cool White fluorescent tubes, Philips TLP 36/86) at 22° \pm 2 °C.
2. After 30 days, calculate survival rates of the mutagenised population.
3. Define each mutated meristem as a source of an individual line (see **Note 9**).
4. Isolate meristematic tips from each of mutated plants.

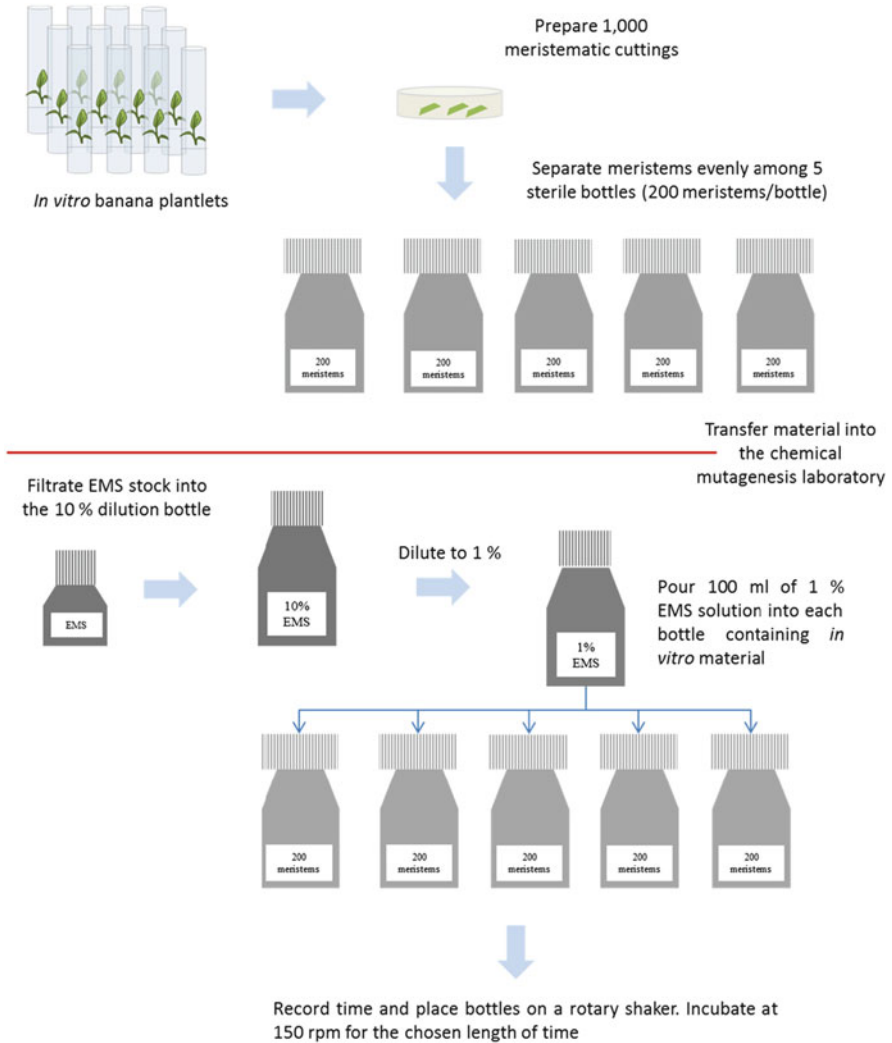


Fig. 3.3 Bulk mutagenesis process of 1000 *in vitro* meristematic cuttings. Cuttings are first placed into bottles under sterile conditions. Bottles are then transferred to the chemical mutagenesis laboratory equipped with a laminar flow bench. Sterile water + DMSO solutions are prepared. Following this a 10% EMS stock is freshly prepared in water/DMSO. This is used to make further dilutions of EMS concentration. A subset of cuttings is then added to different EMS concentrations. Both EMS concentration and incubation times are recorded. In this example, material is subjected to 3-h incubation time

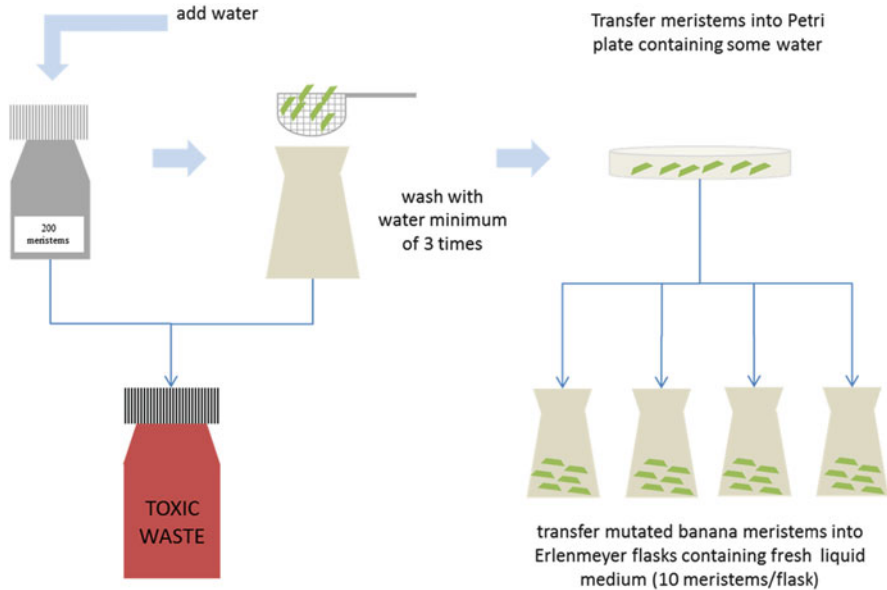


Fig. 3.4 Post-mutagenesis washing of banana in vitro meristematic cuttings. Meristems must be carefully washed after EMS treatment in order to remove the residual EMS. After a minimum of three washes are complete, cuttings are placed on Petri plates, sealed with parafilm and moved back into the in vitro laboratory. Mutated banana explants should be immediately transferred into fresh liquid medium. Attention is paid to avoid EMS contamination of laboratory

5. Transfer every single isolated meristem into a separate Erlenmeyer flask containing fresh liquid media.
6. In order to remove potential chimeric sectors, propagate shoot tip meristems through longitudinal division into two propagules using a scalpel (*see* Fig. 3.5).
7. Assign a unique number to every flask. This number corresponds to the mutant line that will be generated from this particular individual meristem.
8. Incubate cultures for 4–5 weeks.
9. Repeat the process in order to make the population of M_1V_3 individuals (*see* Fig. 3.5 and **Notes 10** and **11**).
10. At each meristematic division, transfer the material into fresh culture media. Remember to follow the nomenclature assigned for each line.
11. Transfer plants into the solid media for a long-term storage (culture media supplemented by 1.8 % Gelrite).
12. Maintain cultures at $22^\circ \pm 2^\circ$ under stationary incubation and 12-h light cycle for the duration of the study.
13. Screen mutagenised population using reverse- or forward-genetic methods (*see* **Note 12**).

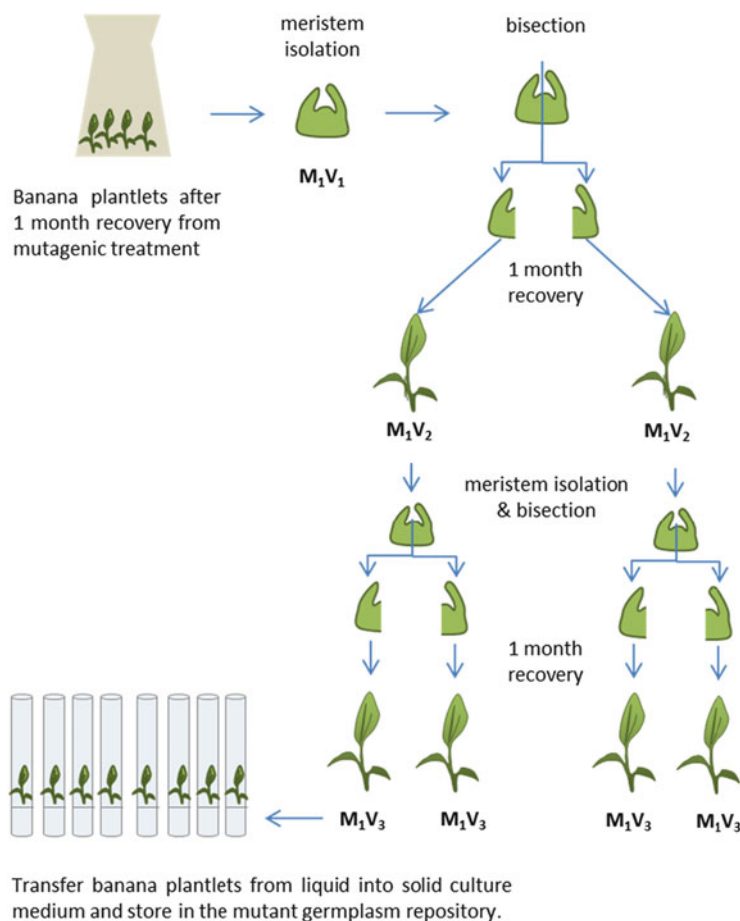


Fig. 3.5 Dissolution of chimeras in bananas. After the mutagenic treatment, plants are allowed to grow for 30 days in a liquid culture media at a constant rotary shaking (60 rpm). Each mutated plant is given a unique line number and assigned a population stage starting with M_1V_1 . Chimeras may exist after 30 days recovery period if surviving meristematic cells harbour different mutations. In order to dissolve potential chimeras, meristems are isolated and divided into two parts through a longitudinal cut which results in most cases in generation of two daughter plants. These are allowed to recover and grow for another 30 days. The procedure can be repeated up to three times. At the M_1V_3 plants are transferred into the solid culture media for long-term storage. Maintain cultures at $22^\circ \pm 2^\circ\text{C}$ under stationary incubation and 12-h light cycle for the duration of the study

3.4 Notes

1. All the mutagenesis experiments should be conducted in a dedicated chemical mutagenesis laboratory equipped with a ducted fume hood and toxic waste disposal and should have decontamination procedures.

2. Read the material safety data sheet (MSDS) of materials being used and follow the recommendation of the manufacturer. Pay careful attention to the information for EMS and what to do in case of exposure. It is very important to wear personal protective equipment (gloves must be compatible with chemical mutagens, for instance, PVC or neoprene gloves): safety glasses with side shields or chemical goggles, lab coat, closed-toe shoes, shoe protections and full-length trousers. A double-glove system is advised. EMS can be inactivated by treatment with sodium thiosulfate. Keep beakers of sodium thiosulfate (100 mM) on hand during laboratory procedures to inactivate any spills and to clean tips and other consumables prior to disposing in hazardous waste.
3. EMS is immiscible in water. DMSO is added as a carrier agent to improve miscibility. Thorough shaking of the liquid mixture also facilitates this. Prior to shaking, test bottles and caps with water to ensure there is no leakage. Shake the mixture up and down vigorously approximately 20–30 times.
4. Care should be taken not to splash liquid when decanting. Choose a bucket or beaker and practise first with water. It is also possible to decant directly into a beaker containing 100 mM sodium thiosulfate. This inactivates the EMS prior to disposal in toxic chemical waste container.
5. This calculation is made by dividing the weight of the mutated material (numerator) by the weight of the control material measured at the same time (denominator) multiplied by 100. For example, the weight of material treated for 2 h at 0.25 % is 1.46 g. The control material is 1.6 g. The percentage is then $1.46/1.60 \times 100 = 91.25 \%$.
6. *Caution:* If this is being prepared a day before, place in vitro cuttings in a Petri plate with sufficient water. Seal Petri plate with parafilm. The optimal population size depends on the spectrum and density of induced mutations and to a lesser extent on the application (forward- or reverse-genetics). We describe a population size of 1000 because this is easily accommodated in medium size laboratories, and for polyploid bananas, this may be considered suitable in reverse-genetic approaches to recover mutations in most target genes (Jankowicz-Cieslak et al. 2012). For forward-genetic screens, a larger population size may be required because the chance of uncovering useful traits in polyploids in the M_1 generation is reduced due to heterozygous state of induced mutation.
7. *Caution:* Conduct all the steps under the fume hood.
8. *Caution:* The washed out solution is still highly mutagenic and must be carefully discarded following the toxic waste disposal rules and regulations of the respective institution.
9. Ideally a line would represent a pedigree of clonally related material that begins with the first non-chimeric individual such that all subsequent material produced from this progenitor inherits the same mutations. Because the exact state where mutant in vitro plantlets are no longer chimeric is not easily determined, defining a line this way is not always possible. We therefore suggest defining a line simply as the pedigree of all material resulting from a single progenitor-mutated plantlet. Materials in a line therefore may inherit the same mutations as

was observed in Jankowicz-Cieslak et al. (2012) but may also inherit different mutations if chimerism exists in meristematic cells at the time the progenitor is subcultured to produce daughter plantlets.

10. The first population after mutagenic treatment is referred to as M_1V_1 whereby V_1 signifies the first vegetative generation after mutagenic treatment. Increasing numbers following V represent successive vegetative generations, and increasing numbers after M indicate meiotic propagation. This allows tracking of generations in both facultative and obligate vegetatively propagated species.
11. We recommend three subculturing cycles to ensure the dissolution of chimeric materials. If too few cycles are performed, the resulting plants may still be chimeric, and mutations may not be stably passed from one to another vegetative generation. Previous reports suggest that plants may be free of chimeras as early as M_1V_2 (Jankowicz-Cieslak et al. 2012). We recommend that extra propagation cycles after plants are no longer chimeric should be avoided. If done, extra work is performed but no additional genetic variation is produced (Jankowicz-Cieslak et al. 2012).
12. Procedures for TILLING in banana were described in Jankowicz-Cieslak et al. (2012). Tests will need to be performed to determine the degree of clonal relationship within each line. Throughput can be increased with different mutation discovery platforms (Tsai et al. 2011). However, care should be taken when performing three-dimensional pooling as the same mutation may be present in more than one individual making assignment of mutations to individuals difficult. Forward-genetic (phenotypic) screens can also be considered.

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Chapter 4

Mutation Induction Using Gamma Irradiation and Embryogenic Cell Suspensions in Plantain (*Musa* spp.)

Jorge López, Aymé Rayas, Arletys Santos, Víctor Medero, Yoel Beovides, and Milagros Basail

Abstract Bananas and plantains (*Musa* spp.) are among the world's most important crops and are considered as a poor man's crop in tropical and subtropical countries. For this reason, they play a very important role in tropical regions' food security. Several pathogens throughout the tropical areas of the world have become a threat for production, including "black sigatoka" (*Mycosphaerella fijiensis* Morelet) which is the most damaging disease for *Musa* plantations in Cuba. It is present in all areas of the country, and it replaced "sigatoka disease" (*Mycosphaerella musicola*) as the main threat. Chemical control and cultural practices reduce damages, but they are not solving the problem. Genetic variations are the basic tools to develop new cultivars with better traits, such as tolerance against various environmental stresses, resistance against pests and diseases, and improved yield and quality. Tissue culture techniques offer the opportunity for variation induction, handling of large plant populations using established selection methods, and rapidly cloning selected variants. All these strategies can increase the efficiency of mutagenic treatments and subsequent screening of mutant materials. Somatic embryogenesis (SE) is also an excellent system for clonal propagation and mutation induction. The present chapter describes a protocol on how to use embryogenic cell suspensions (ECS) in plantain (*Musa* spp.) using both in vitro gamma irradiation and plant regeneration in order to achieve genetic improvement. The process involves a series of steps to properly select ECS for irradiation and the posttreatment handling for plant regeneration as well as mutant selection during acclimatization phase and under field conditions.

Keywords Breeding • In vitro mutagenesis • Radiosensitivity testing • Somatic embryogenesis

J. López (✉) • A. Rayas • A. Santos • V. Medero • Y. Beovides • M. Basail
Instituto de Investigaciones de Viandas Tropicales (INIVIT), Apdo 6, Santo Domingo,
Villa Clara CP 53000, Cuba
e-mail: lab.cell.biotech@inivit.cu

4.1 Introduction

Bananas and plantains (*Musa* spp.) are important staple crops. Over 400 million people throughout tropical and subtropical areas of the developing countries depend on these crops as a major source of calories and as an important commodity for local and international trade. Several pathogens throughout the tropical regions have become a threat for banana and plantain (*Musa* spp.) production (Suprasanna et al. 2012). In Cuba, this is a high-priority crop as part of the national food system because of its ability to produce all year round, its high per capita consumption rate, and the diversity of its uses. However, the country faces persistent low yields which are mainly attributed to attacks of “black sigatoka” disease caused by the fungal pathogen *Mycosphaerella fijiensis*. Because of disease damages and poor-quality planting materials, plantains (AAB group) are being gradually replaced by other more resistant variants, such as cooking bananas of the ABB *Musa* group and tetraploid hybrids (López et al. 2013).

Breeding banana and plantain for resistance to diseases and pest is urgently needed by small-scale and commercial producers. However, these crops have very low clonal multiplication rates, and most of the commercial varieties are polyploid, with extremely poor seed production. These barriers impede sexual hybridization and consequently slow down genetic improvement of the crop (Bakry et al. 2009; Okole 2010). The heterozygotic status is expected to be Aa in loci of diploid cultivars, while the triploids of A genomic types can exist in either Aaa or AAa forms. For interspecific hybrids, the heterozygotic constitution could be AaB, Aab, AAb, ABb, aBb, or aBB. Mutation induction may uncover a recessive phenotype by mutating, inhibiting, or deleting the corresponding dominant allele (Jain and Swennen 2004).

The success of any *in vitro* mutagenesis programme depends on the establishment of reproducible *in vitro* plant regeneration procedures, optimization of mutagenic treatments, and efficient screening of the mutagenized populations for desired variations (Jain 2007; Xu et al. 2012). Somatic embryogenesis (SE) is an excellent system for clonal propagation and mutation induction. The fact that somatic embryos originate from a single cell prevents chimaeras among regenerated plants and makes them an ideal subject for mutagenesis (Jain 2010). It has been shown that somatic embryos arising from banana cell suspensions most “probably” have a single-cell origin (Roux et al. 2004). Thus, the main advantage of using the ECS for mutagenesis would be either in obtaining non-chimaeric populations or the quick dissociation of the chimaeric sectors if any (Roux et al. 2001).

4.1.1 Somatic Embryogenesis in *Musa* spp.

SE is a process in which bipolar structures resembling a zygotic embryo develop from a non-zygotic cell without vascular connections with the original tissue. It is

an important system where multiplication can be done at enormous rates (Arnold et al. 2002). During their development, SE passes through four consecutive phases: (1) induction of SE, (2) somatic embryo formation, (3) maturation of somatic embryo, and (4) somatic embryo germination and conversion into viable plantlets (Parrott 1993).

SE is a promising method for the establishment of protocols reaching rapid multiplication of new and elite genotypes and synthetic “seed” production. In vitro selection uses various biotic and abiotic stresses in order to study genetic manipulation (Kamle et al. 2011). In *Musa*, SE has been obtained by different ways. They include cultures of zygotic embryos (Cronauer and Krikorian 1988; Escalant and Teisson 1988), leaf tissues and rhizome (Novak et al. 1989a), highly proliferating meristems (Dhed’a et al. 1991; Schoofs 1997), male inflorescences (Ma 1991; Escalant et al. 1994), and female inflorescences (Grapin et al. 1998). The most successful explants were the proliferating meristems and male inflorescences. They allow for the establishment of ECS, the formation of somatic embryos, and their subsequent conversion into plants that undergo field evaluation (Strosse et al. 2003; Aguilar et al. 2008).

Although SE in banana is now a well-established method (Strosse et al. 2003; Aguilar et al. 2008), the initiation of a “genotype-independent” embryogenic cell culture is still far from routine (Schoofs et al. 1999; Kulkarni et al. 2007). It is difficult to have an efficient and repeatable protocol for all *Musa* genomic groups. López (2006) developed a protocol for plantain cultivars that do not have the male inflorescence (Horn and Pseudo-horn type). This protocol is based on the use of shoot apices (explant) from the multiplication of the axillary buds in the presence of ancymidol (0.2–0.4 mg l⁻¹ depending on the cultivar). It also relies on the somatic embryos’ ability to achieve differentiation in semisolid and liquid culture media. The use of shoot apices of axillary buds to induce ECS in AAB group plantain cultivars in Cuba prompted the scaling up of propagation by SE to biofactories (commercial laboratories) that are being established as an alternative for clonal propagation (López et al. 2013) and its application in the banana genetic improvement programme by mutations (López et al. 2007; Sales et al. 2013).

4.1.2 Mutation Induction in *Musa* spp.

Biotechnology, while making use of both mutagenesis and conventional methods, can assist in overcoming hurdles during the development of new and improved banana cultivars for sustainable fruit production. This in turn improves the socio-economic status of growers (Jain and Maluszynski 2004). The most commonly used mutagens so far are physical mutagens such as gamma rays (Roux 2004). The *Musa* mutation induction system based on in vitro techniques to obtain mutant plants and micropropagate desirable mutants was developed by Novak et al. (1989b). Later, Roux (2004) standardized the methodology to provide guidelines to mutation induction programmes in *Musa* spp. Shoot tips were excised from clones

representing different genomic constitutions of the genus *Musa*. Later, they were treated with ten doses from 10 to 100 Gy of a cobalt-60 source at a dose rate of 44 Gy/min. Radiation sensitivity and postirradiation recovery were assessed by measuring survival rate, propagation rate, shoot height, and fresh weight. The different *Musa* accessions showed different responses depending on the ploidy level and genomic constitution. The following ranges of doses were recommended: 10–20 Gy for diploid cultivars (AA and BB), 30–40 Gy for triploid cultivars (AAA and AAB), and 40–50 Gy for triploid cultivars (ABB) (Roux 2004).

In vitro mutagenesis of multicellular meristems of *Musa* spp. can lead to a high degree of chimaerism. The regenerated mutant plants will be potentially unstable in regard to cellular genotypes and expressed phenotypes due to segregation in subsequent vegetative propagated generations. Interestingly, data from chemical mutagenesis of triploid bananas suggests that chimaerism can be quickly resolved and may not be an issue in some cases (Jankowicz-Cieslak et al. 2012). Similar data is not available for gamma irradiation, and therefore several rounds of subculturing are advised to remove chimaeras (*see* Chap. 3 for more on chimaera dissolution in banana). Once subculturing is completed, the rooted plantlets are weaned in the greenhouse and planted in the fields for evaluation (Kulkarni et al. 2007).

The main advantage of using the ECS for in vitro mutagenesis treatment is the instant production of non-chimaeric populations or the quick dissociation of the chimaeric sectors if they are found. Using this process can therefore increase the efficiency of mutagenic treatments (Roux et al. 2001). There are only a few available reports on gamma irradiation for ECS in *Musa* spp. (López et al. 2007; Roux 2004; Kulkarni et al. 2004). The time at which the cells are irradiated is very critical, according to Roux et al. (2004) and Kulkarni et al. (2007). The optimal timing for irradiation is considered to be 4–6 days after subculture because at this time the majority of cells are in the G1 phase. One of the most important steps in mutagenesis experiments is to determine the appropriate dose. Theoretically, LD50 will cause the highest frequency of mutations (Van Harten 1998). Studies on irradiated embryogenic cell suspension from cultivars “CEMSA 3/4” (AAB) and “Calcutta 4” (AA) showed that the LD50 should be calculated during the embryo germination stage (sprouts and roots emission) (López et al. 2007; Sales et al. 2013).

The irradiated ECS survival has been too high and variable. Roux et al. (2004) stated that the ECS of cultivars “Williams” and “Three Hand Planty” grew even at a very high dose of 250 Gy. Kulkarni et al. (2004) observed that a 40 Gy dose in cv. “Gran Nain” was completely lethal. Due to the higher hydration levels, the ECS were more radiosensitive. However, previous reports indicate that gamma irradiation at approximately 70 Gy was completely lethal to the shoot-tip multiples.

When searching for a desired trait, mutation induction protocols should aim to increase the degree of plant’s genetic diversity within the resulting plant population. In order to accomplish this goal, it is necessary to continue integrating the gamma ray mutagenesis processes with selection of in vitro cell lines or tissue cultures using a selection agent (e.g. a medium with a high concentration of salt or

phytotoxin). The present chapter describes a protocol for plant regeneration from ECS in plantain (*Musa* spp.) and its use for in vitro mutagenesis.

4.2 Materials

4.2.1 *Explant Preparation: Shoot-Tip Establishment and Multiplication*

1. Donor plants for in vitro establishment (culture initiation). Materials used for this protocol were taken from the germplasm bank with previous characterization by descriptors for banana *Musa* spp. (INIBAP 1996).
2. Semi-controlled growth environment (shade or screen house).
3. Detergent dish soap in water (20 g for 100 explants).
4. Sodium hypochlorite (NaOCl) 2.5 and 3 % in water.
5. Laminar airflow cabinet.
6. Dissecting instruments (scalpels with removable blades and forceps).
7. Culture tubes (150 × 25 mm).

4.2.2 *Culture Medium and Incubation Materials*

1. Murashige and Skoog mineral salts (MS) (Murashige and Skoog 1962) (see **Note 1** and Table 4.1).
2. Balance.
3. pH meter.
4. Autoclave.
5. Culture tubes (150 × 25 mm).
6. Petri dishes (80 × 15 mm).
7. Erlenmeyer flasks (10 and 100 ml).
8. Plastic mesh with 100 µm holes.
9. Temporal immersion system, type Rita[®] of 500 ml capacity.
10. Laminar airflow cabinet.
11. Dissecting instruments (scalpels with removable blades, and forceps).
12. Stereo microscope and inverted microscope.
13. Rotary shaker (70–100 rpm).
14. Growth chamber or a windowless room with environmental control. Specific details for culture room conditions in each stage of the protocol are given in Table 4.2 (see **Note 2**).

Table 4.1 Composition of culture media (mg l^{-1}) for somatic embryogenesis in plantain (*Musa* AAB)

Source	Culture initiation	Multiplication	Calli and ECS	Embryo formation	Embryo maturation	Embryo germination
	López (2006)	López (2006)	Dhed'a et al. (1991)	Dhed'a et al. (1991)	López (2006)	Gómez et al. (2002)
Macro elements	MS	MS	$\frac{1}{2}$ MS	$\frac{1}{2}$ MS	MS	MS
Micro elements	MS	MS	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS	MS	MS
Ascorbic acid	10	10	10	10	10	10
Myo-inositol	100	100	–	100	100	100
LAA	0.88	0.17	–	–	2	2
2,4-D	–	–	1	–	–	–
6-BAP	1.13	2.25	–	–	–	0.5
Zeatin ^a	–	–	0.22	–	0.22	–
Ancymidol	–	0.2–0.4	–	–	–	–
Sucrose	30,000	30,000	30,000	30,000	45,000	30,000
Phytigel	2300	2300	2300 ^b	2300 ^c	2300 ^c	2300 ^c

^aFilter sterilization is carried out using a filter size of 0.2 μm . ECS embryogenic cell suspensions^bOnly for calli^cOnly for semisolid culture media

Table 4.2 Culture conditions for plant regeneration by somatic embryogenesis in plantain (*Musa AAB*)

Stages	Culture room conditions
<ul style="list-style-type: none"> • Establishment and multiplication of shoot tip in semisolid culture media • Formation of somatic embryos in semisolid culture media • Germination of somatic embryos in temporary immersion system and semisolid culture media 	Cultures are maintained on open shelves in climate-controlled laboratories (25 ± 2 °C). Shelves are lighted with 40 W cool white fluorescent lamps providing $62\text{--}68 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic photon flux density under 16/8 light and dark
<ul style="list-style-type: none"> • Callus formation with embryogenic structures in semisolid culture media • Maturation of somatic embryos in semisolid culture media 	Cultures are maintained on closed shelves in climate-controlled laboratories (25 ± 2 °C) in dark
<ul style="list-style-type: none"> • Establishment and multiplication of embryogenic cell suspensions • Formation of somatic embryos in liquid culture media • Maturation of somatic embryos in liquid culture media 	Explants were put on an orbital shaker at 90 rpm in climate-controlled laboratories (25 ± 2 °C), lighted with 40 W cool white fluorescent lamps providing $62\text{--}68 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic photon flux density under 16/8 light and dark

4.2.3 Acclimatization

1. Regenerated plantlets
2. 3.5 l black polyethylene bags (15 cm diameter, 20 cm high)
3. Substrates: filter press mud or soil to compost mixture (1:1 v/v)
4. Urea
5. Acclimatization phase

4.2.4 Mutation Induction Using Gamma Irradiation

1. Cobalt-60 source
2. Embryogenic cell suspensions
3. 1000 μm pore-size mesh
4. 2.0 ml Eppendorf plastic tubes
5. Petri dishes (80 \times 15 mm)
6. Culture tubes (150 \times 25 mm)

4.3 Methods

4.3.1 *Explant Preparation: Shoot-Tip Establishment and Multiplication*

4.3.1.1 Shoot-Tip Establishment

1. Remove “sword” suckers between 25 and 30 cm in height from healthy flowering donor plants.
2. Grow material under semi-controlled conditions for 45–60 days until vigorous and healthy-looking suckers of 15–25 cm in height with a corm diameter of about 10 cm are observed. Also, smaller buds (sleeping eyes) on the corm can be used as explants for tissue culture initiation as an alternative to increase the seed volume at the initial stage.
3. Remove leaf sheaths from the corm to obtain 4–5 cm diameter sections enclosing the vegetative apex. Wash the tissue cubes under running tap water.
4. Rinse four times with detergent (20 g by 100 explants) and disinfect explants with sodium hypochlorite (3 %) plus 4 ml ethanol (95 %) for 20 min. Swirl the solution frequently (*see Note 3*).
5. Decant the hypochlorite solution. Rinse explants three times during 3–4 min with sterile deionized water and decant.
6. Explants are reduced to 2.5–3 cm high with a square base of about 1.5 cm and transfer (the explants) to a laminar airflow cabinet followed by a second disinfection for 10 min with 2.5 % NaOCl. Decant the hypochlorite solution and wash explants three times with sterile distilled water.
7. Apices are reduced to a rectangular shape of 1.0 × 0.5 cm and are inoculated in culture tubes containing 10 ml of establishment culture medium for 15–18 days (Tables 4.1 and 4.2) (López 2006).

4.3.1.2 Shoot-Tip Multiplication

1. First subculture: The shoot tips are cut longitudinally through the apex after *in vitro* establishment period (15–18 days) and are inoculated in culture tubes containing 10 ml multiplication culture medium for 21–30 days.
2. Second subculture: Shoot tips are decapitated from 2 to 3 mm from the corm base, and sprouts smaller than 15 mm are not separated from each other and are not decapitated. They are inoculated in culture tubes containing 10 ml multiplication culture medium for 21–30 days. At this stage, shoot-tip multiplication is stimulated through multiple subcultures to obtain shoot proliferation.
3. Third subculture: Multiplication is followed as in the second subculture. Through repeated subcultures, the multiplication of propagules follows an exponential increment pattern until the desired number of propagules is reached. From this subculture, shoot tips are used for inducing SE process.

4.3.2 Protocol for Plant Regeneration via Somatic Embryogenesis

This protocol involves a series of sequential stages: obtaining explants (multiplication of shoot-tip cultures, *see* Sect. 4.3.1); callus formation with embryogenic structures; establishment and multiplication of ECS; formation, maturation, and germination of embryos (Tables 4.1 and 4.2); conversion to plants; and field evaluation (López 2006) (Fig. 4.1).

4.3.2.1 Callus Formation with Embryogenic Structures

Shoot tips are excised from axillary buds (2–3 mm) with the aid of a stereoscopic microscope for callus formation with embryogenic structures. They are incubated in culture tubes with 10 ml of culture medium for callus formation during 3–8 months (*see* Note 4).

4.3.2.2 Establishment and Multiplication of Embryogenic Cell Suspensions

1. Use proembryos and embryos in the globular stage obtained from callus with embryogenic structures [0.08–0.1 g fresh weight (gFW)] to establish ECS. Incubation is performed in 10 ml Erlenmeyer flask with 2–3 ml of suspensions culture medium (Santos et al. 2002). Media renewals (50 %) are carried out every 3 days to eliminate the presence of non-embryogenic cell aggregates and died cells. Cultures are placed on an orbital shaker at 90 rpm.
2. After 20–30 days, cellular cultures formed are sieved through filters of 500 μm metallic mesh. At this moment homogeneous cell suspensions are formed.
3. The multiplication of ECS is carried out at 3 % settle cell volume (SCV) and subcultured each 15 days. Incubation is performed in 100 ml Erlenmeyer flasks (López 2006; López et al. 2005a) (*see* Note 5). Culture media and culture conditions are similar to those applied in the establishment of ECS.

4.3.2.3 Formation of Somatic Embryos

ECS from the exponential or linear growth phase is used for somatic embryos formation in semisolid or liquid culture media:

1. Somatic embryo formation in semisolid culture media is obtained by incubating a drop of ECS on Petri dishes. ECS have been previously diluted in 25 % SCV. Four 100 μm pore-size plastic meshes are placed on each Petri dish. Each mesh has a 1 cm^2 size to four one cell drop at 25 % SCV incubate during a 4–6 weeks period.

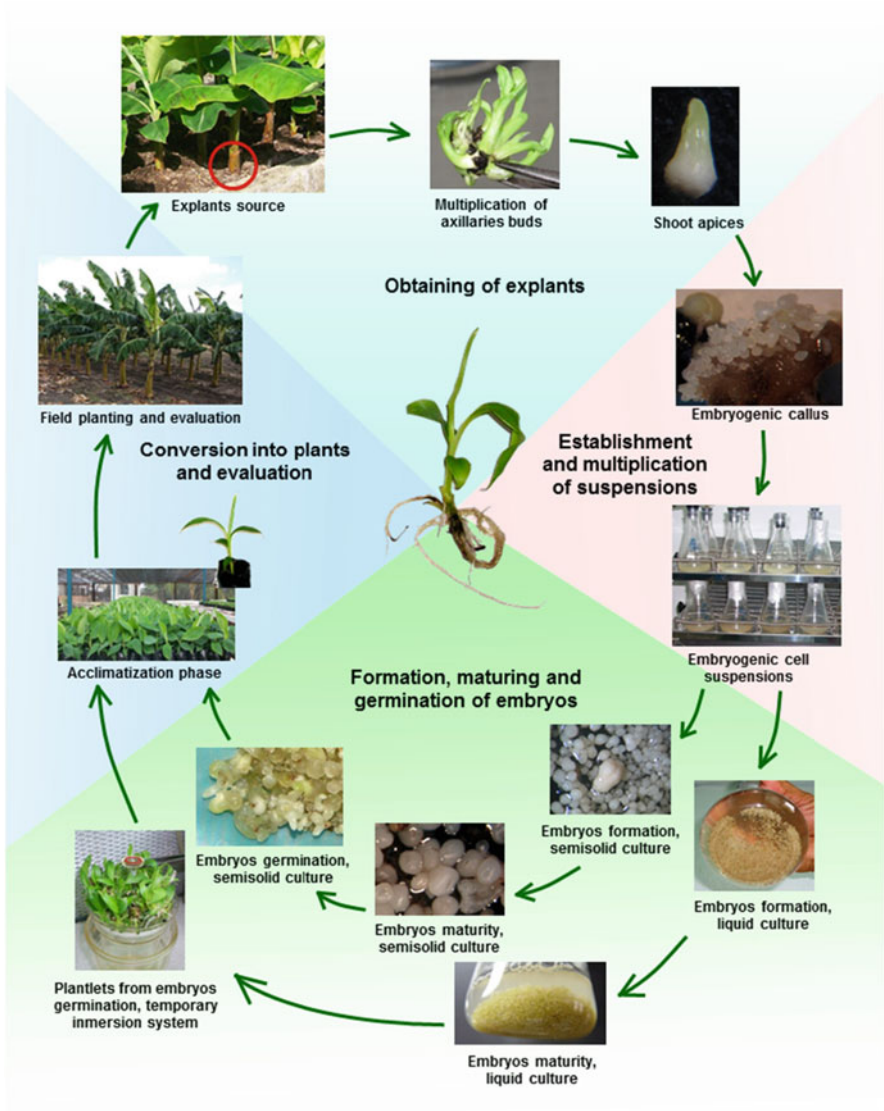


Fig. 4.1 Steps for developing somatic embryogenesis in *Musa* AAB cultivars

2. For somatic embryos formation in liquid culture medium SCV is adjusted to 12 %. Keep culture for 4–6 weeks. Culture medium is renewed every 15 days.

4.3.2.4 Maturation of Somatic Embryos

1. Maturation of somatic embryos in semisolid culture media

Embryogenic mass portions (0.3–0.4 mm) from somatic embryos in the globular stage form in semisolid culture media and are incubated during a month on Petri dish for somatic embryos maturation.

2. Maturation of somatic embryos in liquid culture media

In order to obtain matured somatic embryos: 0.5 gFW of somatic embryos from the globular stage in liquid culture media are incubated on 30 ml of maturing culture medium in 100 ml Erlenmeyer flask during a month. Culture medium is renewed after 15 days of culture.

4.3.2.5 Germination of Somatic Embryos

1. Germination of somatic embryos in semisolid culture media

Mature somatic embryos with visible invagination at a stereo microscope are incubated for a month on Petri dish containing semisolid culture medium for somatic embryo germination.

2. Germination of somatic embryos in temporal immersion system, type Rita[®] (see Note 6)

The temporal immersion system, type Rita[®] [developed by Teisson and Alvard (1995) with 200 ml culture medium for germination], is used for germination of somatic embryos according to López (2006). The immersion frequency was three times daily, during 1 min each according to Escalant et al. (1994). For every temporal immersion system, use an inoculant density of 0.5 g fresh weight of somatic embryo (gFW). Incubate during a 25–30 days period.

4.3.2.6 Acclimatization Phase of Somatic Embryos, Conversion into Plants

In order to carry out somatic embryo conversion into plants, the following conditions must be guaranteed for plantlets coming from in vitro:

1. Keep relative humidity above 80 % within the first 10 days after transplanting to acclimatization phase.
2. Protect plants from direct sunlight incidence with zaran to allow light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. Gradually, these conditions must be reduced to achieve full plant adaptation to field conditions.

For somatic embryos conversion into plants, the following procedures are followed:

1. Plantlets are removed from the culture vessel.

2. Wash the plantlets carefully to remove residual culture media and separate them into individual plants. Classify plants by size for planting (large: more than 5 cm high; medium: 3–5 cm; and small: less than 3 cm).
3. Transplant plantlets into polyethylene bags containing filter press mud or soil compost mixture (1:1 v/v).
4. Fertilize with foliar urea 5 g l^{-1} after 15 days and every 7 days until transplanting to field conditions.
5. Plantlets are kept in the acclimatization phase for 45–60 days until they reach a height of around 15 cm. At this time, they are suitable to plant out in the field.

4.3.3 Mutation Induction Using Gamma Irradiation

Mutation induction is achieved by exposing ECS to gamma irradiation. Mutant selection occurs both during the acclimatization phase and under field conditions (López et al. 2007; Sales et al. 2013) (Fig. 4.2). The whole process for irradiation of ECS is detailed below:

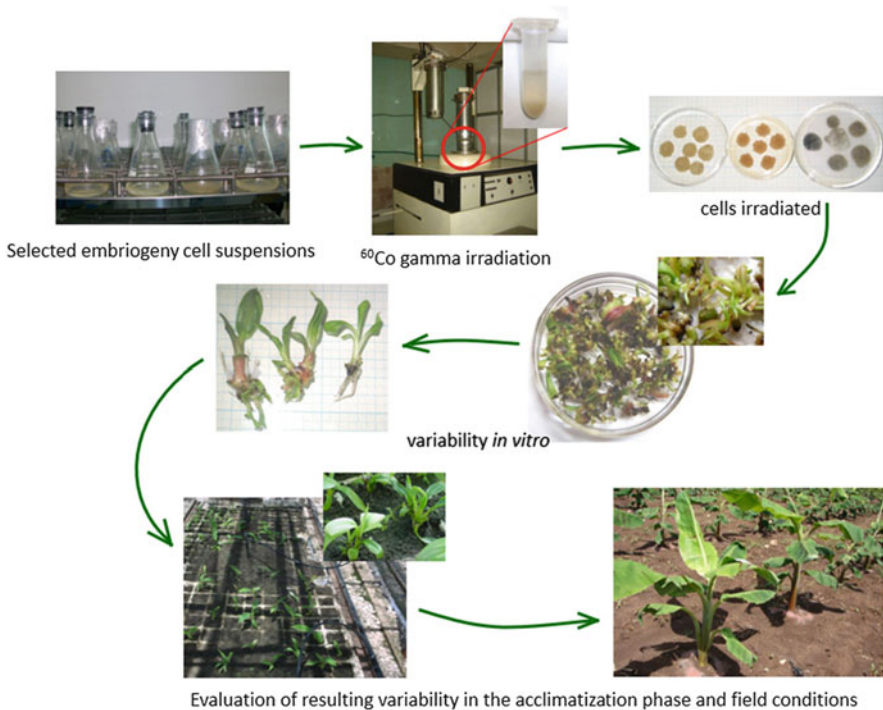


Fig. 4.2 Use of embryogenic cell suspensions (*Musa* AAB) for *in vitro* mutagenesis

1. Selected ECS with stable growth and proven ability to regenerate plants are taken 4 days after subculture and are sieved through 1000 μm pore-size mesh.
2. Add 0.4 ml cells to each 2.0 ml Eppendorf plastic tubes (three per each irradiation dose) to irradiate using a cobalt-60 gamma source (0–100 Gy, in steps of 10 Gy).
3. Incubate irradiated ECS and the nonirradiated control in semisolid medium for somatic embryos formation during 30–40 days.

Incubation of each treatment to promote somatic embryo formation occurs as follows: Each Eppendorf plastic tube contains four plantings coming from a single Petri dish. Each study treatment includes three Eppendorf tubes with a total of three Petri dishes per treatment.

4. Transfer somatic embryos formed to a semisolid maturing medium for somatic embryos maturation. Keep them in maturing medium for 30 days.
5. Transfer matured somatic embryos to a semisolid medium in order to promote germination. Keep them in semisolid medium for 30 days. At this stage, calculate the LD50 for germinated embryos (*see Note 7*).
6. Transfer germinated embryos (plantlets) to the acclimatization phase to promote plant conversion. Survival of plantlets from irradiated ECS and phenotypic changes are evaluated and compared with plantlets obtained from nonirradiated ECS (control) according to the methodology proposed by Sandoval et al. (1997) and López et al. (2005b).

The following parameters are tested 60 days after transplantation:

- Survival rate
 - Plant height (cm)
 - Petiole length of the second leaf (cm)
 - Length of the second leaf (cm)
 - Width of the second leaf (cm).
 - Distance between the second and third leaf (cm)
7. Transfer plantlets from irradiated ECS, plantlets from nonirradiated ECS (control), and corm buds (also as control) to field conditions in order to evaluate the resulting variability and possible mutants according to Sandoval et al. (1997) and López et al. (2005b).

The following parameters are tested 6 months after planting and during harvest:

- Pseudostem circumference at a meter height (cm)
- Plant height (cm)
- Number of active leaves
- Flowering date (weeks)
- Number of fingers/bunch
- Number of hands/bunch
- Bunch weight
- Length of the central finger in the second hand (cm)

- Length of the central finger in the next to the last hand (cm)
 - Length of the central finger in the last hand (cm)
 - Incidence of “black sigatoka” at the flowering time according to some criteria for the Technical Guidelines 7 from INIBAP (Carlier et al. 2003).
8. Multiply and evaluate possible mutants selected from the first cycle during a second field crop (INIBAP 1996).

4.4 Notes

1. Mineral salts from Murashige and Skoog medium (Murashige and Skoog 1962) are very suitable for banana and plantain shoot-tip culture and SE (Table 4.1). Culture media are prepared by dissolving all media components in a flask with distilled water. pH values are adjusted to 5.8 in all media by using HCl and KOH. For medium solidification, 2300 mg l⁻¹ of Phytigel is added to and dissolved in the medium by heating it on the stirring/hot plate. The medium is dispensed into tissue culture vessels, and then it is sterilized for 15–30 min depending on the size of culture vessels (121 °C × 1.2 kg cm⁻²).
2. A windowless room having a controlled environment is necessary to keep *in vitro* cultures. An air conditioning unit is suitable for temperature control, and the photoperiod is also controlled with artificial lighting.
3. It is important to have enough active substance, capable of reacting with the surface of the vegetable material. To accomplish this, the amount of NaOCl added for disinfection needs to be two to three times higher than the number of apices disinfected.
4. The formation of embryogenic cultures is observed after 3 months. It is characterized by yellow meristematic globules (nodular calli) with and without embryo formation in the meristematic globule surface similar to those described by Dhed’a et al. (1991) and Schoofs (1997).
5. During the multiplication stage, cell suspensions are characterized by many spherical cells in active division. Cell aggregates might be heterogeneous, irregular, translucent, and non-translucent. The abovementioned cell characteristics are considered indicators of embryogenic conditions for cell suspensions (Dhed’a et al. 1991).
6. Temporal immersion system shows better performance than semisolid culture media (Escalant et al. 1994; López 2006).
7. LD50 calculations are preferably completed during the germination phase due to the low synchronization of the plant regeneration system during embryo histodifferentiation (López et al. 2007). It is also important to consider that the closest irradiation dose (lower and upper) to the LD50 is more likely to produce favorable mutants (Kulkarni et al. 2007).

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Chapter 5

Optimisation of Somatic Embryogenesis in Cassava

Kenneth E. Danso and Wilfred Elegba

Abstract Somatic embryogenesis, an efficient regeneration system that is being used successfully in genetic transformation, can be coupled with mutation induction to breed for cassava varieties with desired traits. The embryogenesis system requires the initiation of totipotent cells which can be used as targets for mutagenic treatments. However, initiation of these totipotent cells is low, highly genotypic dependent and has low plant conversion rate. Thus, for its successful application in mutation induction, the system needs to be optimised to overcome these drawbacks. To optimise the system, totipotent cells are initiated on embryo initiation medium (EIM) which consisted of Murashige and Skoog (Physiol Plant 15:473–497, 1962) basal salts supplemented with picloram and 2,4-dichlorophenoxy acetic acid (2,4-D) to produce callus. After 21 days, calli produced are cultured for embryo maturation on maturation medium (EMM) or can also be treated with mutagenic agents for mutant induction. The resulting primary embryos are recycled three consecutive times, each by fragmentation of the cotyledons followed by culture on embryo initiation medium to produce more matured embryos. Somatic embryos produced are then successfully converted into plants by abscisic acid pretreatment in embryo maturation medium or by air desiccation under the laminar flow hood. The methodology described offers optimised, reproducible procedures of somatic embryogenesis for its incorporation into mutation breeding programmes in cassava.

Keywords Somatic embryos • Cyclic embryogenesis • Picloram • 2,4-dichlorophenoxy acetic acid • Abscisic acid • Embryo desiccation

5.1 Introduction

Cassava has the potential to remain a food security crop for millions of people living in the tropics while also serving as starch substrate for many industrial applications. This duality of roles requires that cassava varieties targeting different

K.E. Danso (✉) • W. Elegba

Ghana Atomic Energy Commission, Biotechnology and Nuclear Agriculture Research Institute, P.O. Box LG 80, Legon, Accra, Ghana

e-mail: kaedanso@hotmail.com

end uses must be bred from existing landraces which are well adapted to the traditional farming practices. Conventionally, cassava is bred through sexual hybridisation. The crop is highly heterozygous, and cross- and self-pollination occur naturally. Consequently, there is high sexual hybridisation leading to high genetic diversity and long breeding cycle which are only useful for heritable traits. For traits lacking sources of heritable variation, recombinant DNA techniques or induced mutations might provide alternate means for introgression of desired traits into existing varieties.

Both recombinant DNA and mutation induction require the generation of totipotent cells or tissues that could act as targets for insertion of genes of interest or exposure to mutagenic agents. Of the several regeneration methods, only somatic embryogenesis (SE) has been identified as an efficient regeneration system for the development of such totipotent tissues in cassava (Feitosa et al. 2007; Puontis-Kaerlas 1997; Raemakers et al. 1997; Zainudin et al. 2012). These totipotent cells can be used for large-scale clonal propagation of genetically uniform elite clones (Verdreil Jean-Luc et al. 2007) or treated with mutagens (physical and chemical) to produce mutants with traits of interest (Joseph et al. 2004). The production of an intermediate embryogenic calli in indirect somatic embryogenesis can also serve as targets which can also be treated with mutagens (physical or chemical) to obtain mutants with useful traits.

Of the over 3000 mutant varieties in the IAEA database, only two cassava mutants have been documented (Forster and Shu 2012). These are *Tekbankye* and *Fuxuan* which were released in 1997 and 2005 in Ghana and China, respectively. Both mutants were obtained through the irradiation of stem cuttings using gamma rays (Asare and Safo-Kantanka 1995). The choice of gamma rays might be due to the bulky nature of the stem cuttings used for the irradiation. The alternate use of chemical mutagens may be limited by the bulky stem cuttings that do not allow easy absorption of the mutagens (Leitão 2012). Somatic embryos or friable embryogenic calli may therefore provide ideal propagules for mutation induction in the crop.

In spite of its usefulness, the somatic embryogenic system is highly genotype dependent often making protocols for embryo production non-reproducible and also has low plant conversion rate. Therefore, for its application in mutation induction, the procedures for embryo production needs to be optimised, made more efficient and reproducible.

Somatic embryos can be induced either directly or indirectly through a callus-phase intervention. The production of embryos via callogenesis involves three distinct steps. These are initiation of embryogenic competent cells from a suitable explant on embryo initiation medium amended with auxin (**Step 1**), maturation of somatic embryos on embryo maturation medium amended with (low auxin) or without auxin (**Step 2**) (Stamp and Henshaw 1987) (Fig. 5.1) and embryo conversion into plants on a standard cassava culture media for shoot development (**Step 3**).

Several authors have used different explants for primary somatic embryo induction. The most commonly used explants include cotyledons and embryonic axis from zygotic embryos (Konan et al. 1994; Stamp and Henshaw 1987), immature leaf lobes (Li et al. 1998), meristem and shoot tips (Puontis-Kaerlas 1997) and

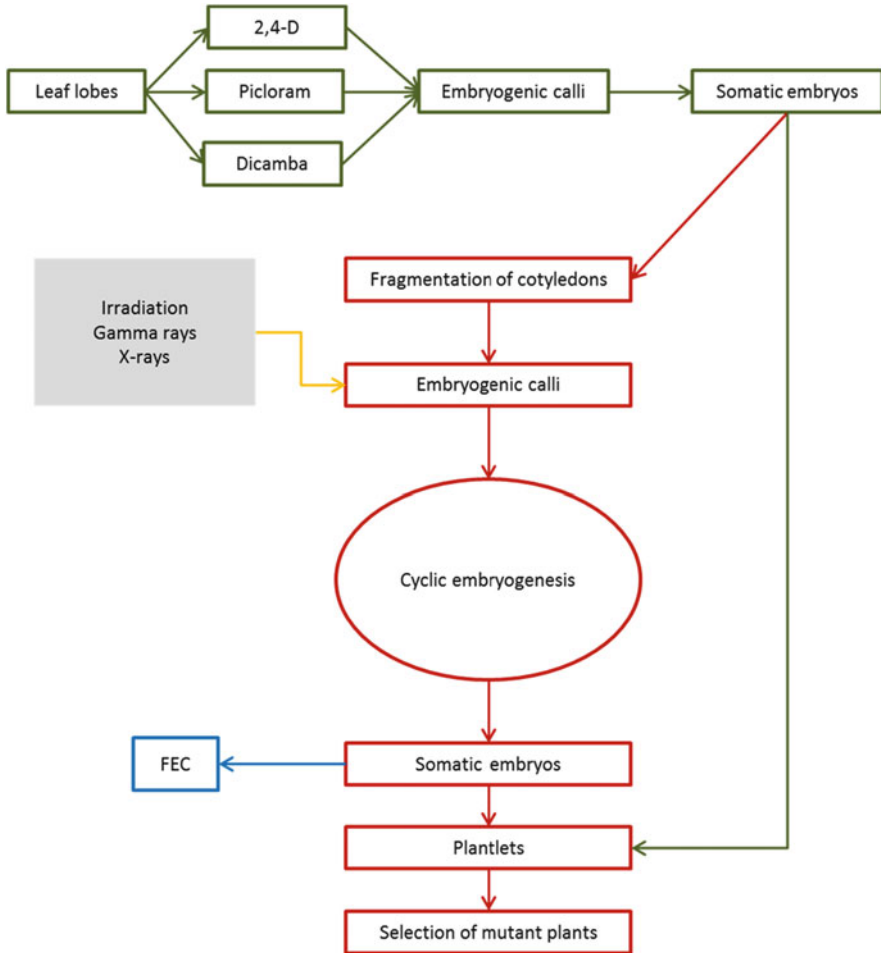


Fig. 5.1 Flow diagram illustrating production of primary, cyclic embryos and intervening calli in cassava which can be incorporated into mutation breeding programmes

BAP-enlarged axillary buds (Nyaboga et al. 2013). High embryogenic competence is, however, restricted to meristematic and embryonic tissues (Puontis-Kaerlas 1997). In cassava, the most commonly used explant is immature leaf lobes (Raemakers et al. 1997). Other factors including genotype, culture medium as well as growth regulators have been found to influence primary embryo production (Taylor et al. 2001).

5.1.1 Production of Cyclic Embryos

The frequency of primary somatic embryos production in cassava is very low (Raemakers et al. 1997). It is also a genotype-dependent process thereby making efficiency of the somatic embryogenesis system especially for mutation induction limited. In most embryogenic systems, primary embryos are often used as source of explant to scale up embryo production through recycling (Danso and Ford-Llyod 2004; Raemakers et al. 1993). In cyclic embryogenesis, cotyledons of matured primary embryos are fragmented, cultured on embryo initiation medium (solid or liquid MS medium supplemented with auxin) to induce embryogenic calli. The embryogenic calli induced contain globular embryos, which develop to cotyledonary stage on embryo maturation medium usually containing a cytokinin (0.1 mg/L BAP). Repetitive subculture of mature embryos every 21 and 30 days, respectively, leads to cyclic embryogenesis (Sofiari et al. 1997). The frequency of embryogenic competence decreases after three or four repetitive subcultures, and induction of new primary embryos is recommended to maintain competence.

In micropropagation, cyclic embryogenesis can be used to scale up the frequency of embryo multiplication through repeated subculture of matured embryos which are allowed to develop into organised somatic embryos (OSE) for conversion into plants (Fig. 5.1). The cyclic embryogenic technique is also useful in plant breeding as the intervening friable embryogenic calli (FEC) can be treated with physical (gamma or X-rays) or chemical mutagens (e.g. ethyl methanesulphonate, EMS) for production of valuable mutants (Joseph et al. 2004). The system can also be coupled with all cassava genotypes that are amenable to transformation. Successful production of somatic embryos via cyclic embryogenic system depends on the type of growth regulator in the culture medium and the number of repetitive cycles of subculture (Sofiari et al. 1997).

5.1.2 Influence of Growth Regulators on Primary Embryo Induction

A critical factor influencing somatic embryogenesis is the type of auxin used for embryo initiation. For several years, indirect somatic embryo production in cassava has relied on the use of synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D) for embryogenic calli initiation prior to transfer to embryo maturation medium (Stamp and Henshaw 1987; Taylor et al. 2001). The use of 2,4-D for embryo initiation often results in low frequency of primary embryo production as well as poor conversion into plants due to lack of root primordium especially when duration of calli production on the initiation medium is prolonged (Raemakers 1993). More recently, many authors have reported of the successful use of other synthetic auxins such as picloram (4-amino-3,5,6-trichloropiconic acid) and dicamba (3,6-dichloroanisic acid) (Mongomake et al. 2015). The application of new

generation of growth regulators such as oligosaccharides, jasmonate, polyamines, brassinosteroids (George et al. 2008) and phloroglucinol (Teixeira da Silva et al. 2013) has also proved to be useful for primary somatic embryos induction in many plant species. Therefore to improve the embryogenic system for mutation induction, the use of synthetic auxins such as picloram or dicamba as well as jasmonates and brassinosteroids is worth investigating particularly in cassava landraces whose embryogenic competence is not known.

High frequency of somatic embryo production can also be achieved through the manipulation of the culture medium. It has been reported that a switch from Murashige and Skoog (1962) basal medium (MS) to DKW or DCR medium increased the production of somatic embryos even from older leaf explants which hitherto were considered non-embryogenic (Driver and Kuniyuki 1984). It has also been reported that additional copper sulphate or silver nitrate in the culture medium can also have positive effect on embryo production (Danso and Ford-Llyod 2004).

5.1.3 Somatic Embryo Conversion into Plants

In vitro culture of cassava via organogenesis or somatic embryogenesis can be deployed in mutation breeding programmes if the efficiency of plant conversion is optimised. It has been shown that the efficiency of somatic embryo maturation and plant conversion can be improved via the use of abscisic acid (ABA) which is known to synchronise embryo maturation (Groll et al. 2002; Quainoo and Dwomon 2012), confer desiccation tolerance (Torres et al. 2001) and enhance plant conversion (Quainoo and Dwomon 2012). Also, desiccation of somatic embryos prior to culture on standard cassava medium has been used to enhance plant conversion (Matthews et al. 1993). The protocol described below aims to optimise cassava somatic embryo induction at both the primary and the cyclic stage using the synthetic auxins 2,4-D and picloram. The effect of these two auxins on cyclic embryogenesis as well as the influence of ABA on embryo maturation and plant conversion of cassava accession is also worth investigating.

The somatic embryogenesis technique will be more beneficial in mutation induction if the duration and frequency of embryo production as well as plant conversion from somatic embryos are optimised to make it more efficient and applicable to all cassava genotypes. In the following protocols, we describe an optimised reproducible embryogenic system for Ghanaian cassava landraces for mass production of somatic embryos which can be treated with physical or chemical mutagens in mutation breeding programmes. The protocols can be adapted for other farmer- or industry-preferred cassava cultivars.

5.2 Materials

5.2.1 Chemicals and Equipment

1. 1 M HCl.
2. 1 M NaOH.
3. Laminar flow hood (NuAire Biological Safety Cabinet, UK).
4. Stereomicroscope (Leica Zoom 2000, China).
5. Magnetic hotplate stirrer.
6. Autoclave (Astell Scientific, UK).
7. Oven (Gallenkamp Hotbox, Sanyo).

5.2.2 Culture Media

Culture media was prepared from Murashige and Skoog (1962) basal salts powder (Sigma Aldrich). Gamborg B5 vitamins were prepared from stock solutions (*see Note 1*). The composition of the basal medium and Gamborg B5 solutions are shown below.

5.2.3 Shoot Initiation Medium (*see Notes 2–4*)

Shoot initiation medium was prepared using Murashige and Skoog (1962) basal salts powder and Gamborg B5 vitamins (Table 5.3).

5.2.4 Embryo Initiation Medium

Embryo initiation medium was prepared using Murashige and Skoog basal salts and vitamins and Gamborg B5 vitamins. The composition of the medium is shown below. Sucrose and phytigel were added (*see Notes 2–5*).

5.2.5 Embryo Maturation Medium

Embryo initiation medium was prepared using Murashige and Skoog basal salts and vitamins and Gamborg B5 vitamins. The composition of the medium is shown below. Sucrose and phytigel were added (*see Notes 2–5*).

5.2.6 Somatic Embryo Conversion Medium

Embryo initiation medium was prepared using Murashige and Skoog basal salts and vitamins and Gamborg B5 vitamins. The composition of the medium is shown below. Sucrose and phytigel were added (*see* **Notes 2–6**).

5.3 Methods

5.3.1 Preparation of Shoot Initiation Medium

1. For the shoot initiation medium (SIM), mix together MS basal medium (MS) powder and Gamborg B5 vitamins (100 mg/L myo-inositol, 1.0 mg/L nicotinic acid, 1.0 mg/L pyridoxine-HCl, 10 mg/L thiamine-HCl and 2 mg/L glycine) modified with 30 g/L sucrose, 0.5 mg/L benzylaminopurine (BAP), 0.02 mg/L naphthalene acetic acid (NAA) and 0.5 mg/L GA₃ (Tables 5.1, 5.2 and 5.3).
2. Adjust the medium to pH 5.8 using 1 M NaOH or 1 M HCl.
3. Add 3.5 g/L of Phytigel for solidification (*see* **Note 3**).
4. Sterilise the medium in an autoclave at 121 °C for 15 min and a pressure of 15 psi.
5. Keep sterilised media at room temperature prior to usage or store in a fridge at 4 °C.

5.3.2 Collection and Sterilisation of Donor Plants

1. Collect cassava stakes from the field grown plants and transfer into the laboratory (*see* **Note 7**).
2. Prepare cuttings of 10–15 cm long each bearing two to three nodes.
3. Plant stakes in black polyethylene pots filled with loamy soil in a heat chamber at a temperature of 35–45 °C provided by six bulbs each of 200 W to enhance virus elimination.
4. Water planted stakes every other day using watering can in order to monitor water requirements and thereby provide sufficient water without water logging.

Table 5.1 Composition of Murashige and Skoog (1962) basal salts

Compound	Concentration (mg/L)
Ammonium nitrate (NH ₄ NO ₃)	1650
Potassium nitrate (KNO ₃)	1900
Calcium chloride (CaCl ₂ · 2H ₂ O)	440
Potassium hydrogen phosphate (KH ₂ PO ₄)	170
Magnesium sulphate heptahydrate (MgSO ₄ · 7H ₂ O)	370
Boric acid (H ₃ BO ₃)	6.2
Sodium molybdate (NaMoO ₄ · 2H ₂ O)	0.25
Cobalt chloride hexahydrate (CoCl ₂ · 6H ₂ O)	0.025
Copper sulphate pentahydrate (CuSO ₄ · 5H ₂ O)	0.025
Zinc sulphate heptahydrate (ZnSO ₄ · 7H ₂ O)	8.6
Manganese sulphate (MnSO ₄ · 4H ₂ O)	22.3
Potassium iodide (KI)	0.83
Na ₂ EDTA	37.25
FeSO ₄	27.85

Table 5.2 Composition of Gamborg B5 vitamins and growth regulators (*see Note 2*)

Myo-inositol	100 mg/L
Nicotinic acid	1.0 mg/L (×1000)
Pyridoxine-HCl	1.0 mg/L (×1000)
Thiamine-HCl	1.0 mg/L (×1000)
Glycine	2.0 mg/L (×1000)
<i>Growth regulators</i>	
BAP	0.5 mg/L (×1000)
NAA	0.02 mg/L (×1000)
GA ₃	1.0 mg/L (×1000)

Table 5.3 Composition of shoot initiation medium

MS medium	4.4 g/L
Myo-inositol	100 mg/L
Nicotinic acid	1.0 mg/L
Pyridoxine-HCL	1.0 mg/L
Thiamine-HCL	1.0 mg/L
Glycine	1.0 mg/L
Benzylaminopurine	0.05 mg/L
Naphthalene acetic acid	0.02 mg/L
Sucrose	30 g/L
Phytigel	3.5 g/L

5.3.3 Sterilisation and Culture for Shoot Initiation

1. Harvest 14–20 days old sprouted shoots into a honey jar bottle and wash under running tap water for 1 h in the laboratory.

2. Sterilise shoot tips under the laminar airflow hood (NuAire Biological Safety Cabinet, UK).
3. Wash them with sterilised distilled water mixed with Tween 20 for 30 min (*see Note 8*).
4. Wash shoot tips again with three changes of sterilised distilled water.
5. Shoot tips are then first immersed in 70 % (v/v) aqueous ethanol for 5 min.
6. Washed shoot tips again with sterilised distilled water. This procedure is repeated three times to complete the sterilisation regime.
7. Dissect the shoot tips under stereomicroscope by gradual removal of leaves using sterilised forceps and scalpel blades which have previously been sterilised in an oven at temperature of 110 °C for 2 h.
8. Excise meristematic dome/tissues (0.2 mm in diameter) with one or two leaf primordia from the dissected shoot tips (*see Note 9*).
9. Culture meristematic tissue on the shoot initiation medium (Table 5.3).

5.3.4 Culture Incubation

Maintain the cultures under growth room conditions at a temperature of 26 ± 1 °C, 16/8-h light/dark photoperiod with light provided by white fluorescent tubes at intensity of 3500 lux.

5.3.5 Initiation of Primary Somatic Embryos

1. Excise young leaf lobes (1–3 mm) from 8-week-old in vitro-grown plantlets under a stereomicroscope using forceps and scalpel (*see Note 10*).
2. Culture excised leaf lobe on embryo initiation medium which consisted of modified MS basal salts and Gamborg B5 vitamins supplemented with 8 mg/L 2,4-D or 16 mg/L picloram (*see Table 5.3 and Notes 2, 4 and 11*).
3. Place the leaf lobes with their adaxial surfaces in contact with the medium (*see Note 12*).
4. Incubate cultures in a dark box (or wrap in aluminium foil) for 21 days to enhance calli formation.
5. Examine leaf lobe explants for embryogenic calli formation after 21 days (*see Note 13*).
6. Transfer into embryo maturation medium (Table 5.4).
7. Transfer Petri dishes containing the embryogenic calli into growth room lighting conditions for embryo maturation.
8. Count the number of embryos with well-distinct cotyledons 14 days after culture on embryo maturation medium. Mature embryos have well-developed cotyledons with distinct hypocotyl (Fig. 5.2).

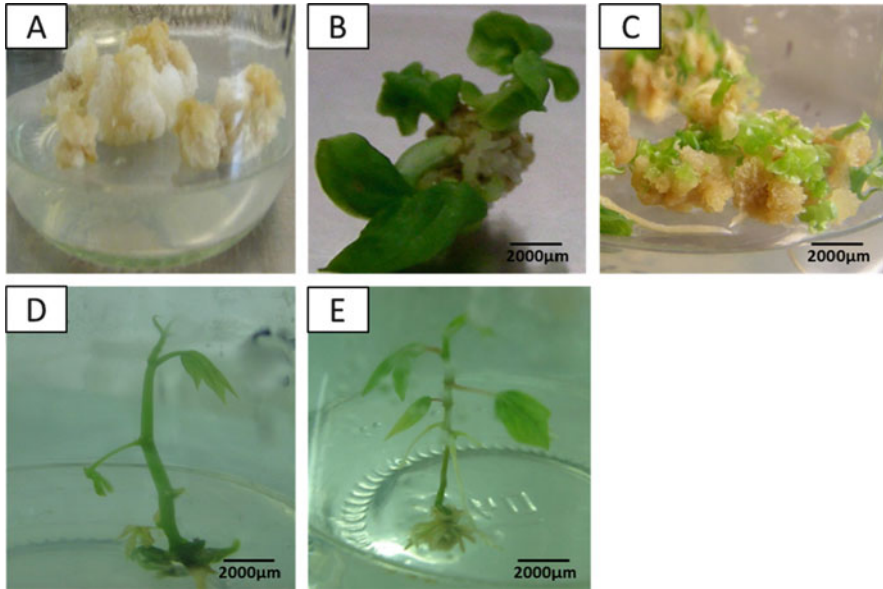


Fig. 5.2 (a) Callus initiated on EIM supplemented with picloram. (b) Primary somatic embryos obtained from leaf lobes. (c) Cyclic embryos obtained from fragmented cotyledons. (d) Plantlet obtained from desiccated embryo. (e) Plantlets obtained from ABA pretreated somatic embryos

5.3.6 Cyclic Embryo Initiation and Production

1. Isolate matured primary somatic embryos (*see* Figs. 5.1 and 5.2 and **Note 14**).
2. Fragment cotyledons in Petri dishes with a scalpel blade under the laminar flow hood (*see* **Note 15**).
3. Transfer 0.2 g of somatic embryos to embryo initiation medium (Table 5.5).
4. Incubate the cultures in dark box or wrap in aluminium or an incubator without light for 21 days to enhance calli development.
5. Examine cultured fragmented embryos for embryogenic calli formation.
6. Transfer cultures into embryo maturation medium (Table 5.5)
7. Transfer into growth room lighting conditions (16/8 h day/dark photoperiod, 26 °C light intensity of 3500 lux) for maturation of somatic embryos.
8. Isolate matured somatic embryos after 14 days.
9. Fragment isolated embryos again and culture on primary embryo initiation medium (Table 5.5) for continuation of cyclic embryogenesis (Fig. 5.1).
10. Repeat the procedure for three subcultures (*see* **Note 16**).

Table 5.4 Composition of embryo maturation medium

MS powder	4.4 g/L (Table 5.1)
Gamborg B5 vitamins	(Table 5.2)
Sucrose	30 g/L
Phytigel	3.5 g/L
CuSO ₄	2 µM
BAP	0.1 mg/L

Table 5.5 Composition of embryo initiation medium

MS powder	4.4 g/L (Table 5.1)
Gamborg 5 vitamins	(Table 5.1)
Phytigel	3.5 g/L
Sucrose	30 g/L
2,4-dichlorophenoxyacetic acid (2,4-D)	8 mg/L
Picloram (4-amino-3,5,6-trichloropicolinic acid)	16 mg/L
Copper sulphate (CuSO ₄)	2 µM

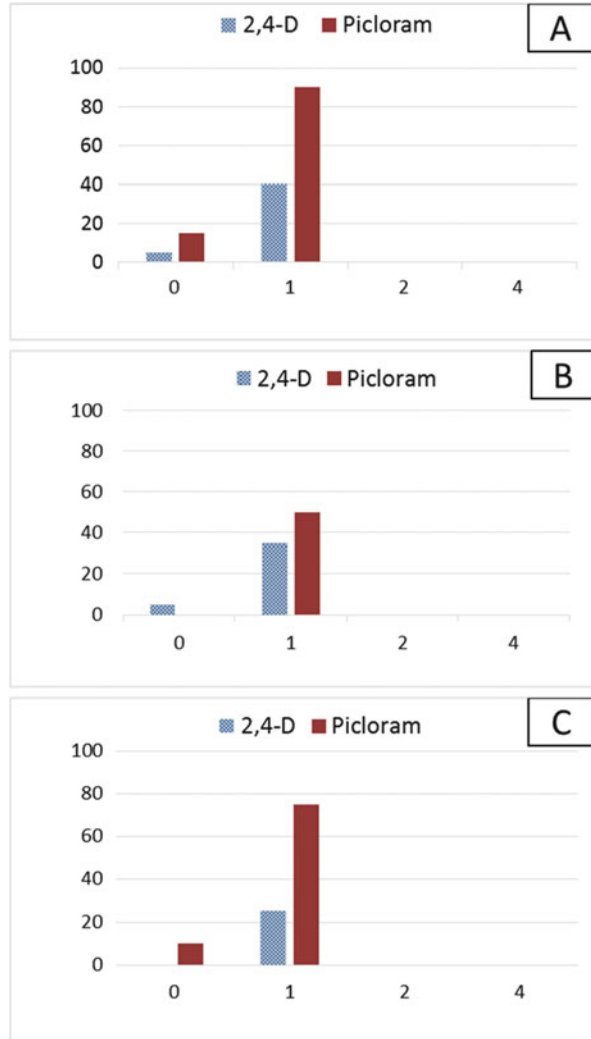
Table 5.6 Composition of plant conversion medium

MS powder	4.4 g/L (see Table 5.1)
Gamborg B5 vitamins	(see Table 5.2)
Sucrose	30 g/L
Phytigel	3.5 g/L
CuSO ₄	2 µM
BAP	0.1 mg/L
ABA	1.0 mg/L

5.3.7 *Abscisic Acid Effect on Conversion of Somatic Embryos into Plant*

1. Weigh 0.2 g of embryogenic calli induced on embryo initiation medium (Table 5.5).
2. Transfer cultures to embryo maturation medium (Table 5.6) and add 1 mg/L ABA. The concentration of ABA has influence on plant conversion from somatic embryos (*see* Fig. 5.3 and Note 6).
3. Transfer cultures to the growth room conditions (16/8 h day/light photoperiod, temperature of 26 °C) for incubation for 30 days.
4. Isolate matured embryos from the embryogenic calli.
5. Transfer isolated embryos to standard cassava culture medium for plant conversion (Table 5.3).

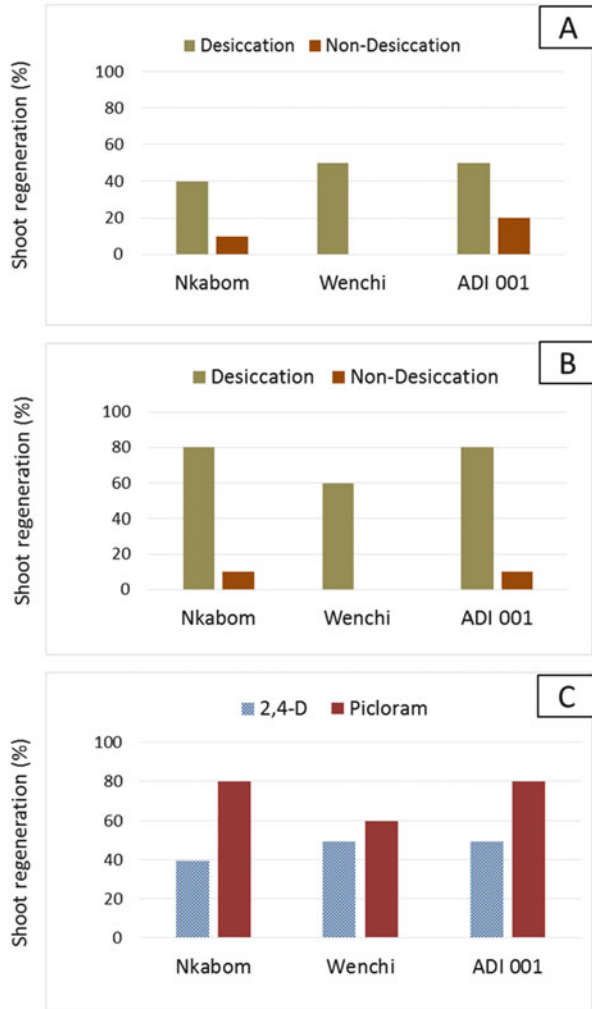
Fig. 5.3 Effect of abscisic acid on 2,4-D and picloram-induced somatic embryos from (a) Nkabom, (b) Wenchi and (c) ADI 001 cassava landraces



5.3.8 Desiccation of Embryos for Plant Conversion

1. Transfer matured somatic embryos (14 days old) induced on either 8 mg/L 2,4-D or 16 mg/L picloram to sterile Petri dishes.
2. Weigh 0.6 g embryos and desiccate by keeping them in a sealed Petri dish under growth room conditions for 4 days. Desiccation reduces the moisture loss which results in high frequency of plant regeneration. When desiccated embryos are cultured on picloram amended medium plant regeneration is improved (see Fig. 5.4 and Note 17).

Fig. 5.4 The effect of desiccation on shoot regeneration from (a) 2,4-D, (b) picloram-induced somatic embryos and (c) comparison of 2,4-D and picloram-induced somatic embryos in three cassava landraces (Nkabom, Wenchi, ADI 001) 8 weeks after culture



3. Change Petri dishes every other day to remove condensed moisture on the lid of the Petri dish.
4. Reweigh the embryos after 4 days of partial desiccation to determine the final weight.
5. Calculate the moisture loss in somatic embryos in percentage using the following formula:

$$\% \text{Moisture loss} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100$$

6. Transfer desiccated embryos to shoot initiation medium (Table 5.2).

5.4 Notes

1. All culture media described in this protocol consisted of Murashige and Skoog (Murashige and Skoog 1962) basal salts powder (Sigma Aldrich) and Gamborg B5 vitamins. Growth regulators were added to suit particular experiments.
2. Stock solutions of vitamins and growth regulators were prepared and aliquoted into 1.5 mL Eppendorf tubes and stored in the refrigerator at 4 °C. They were used as and when required but not more than 8 weeks. Vitamins and growth regulators were filter sterilised and added to the basal salts. In the absence of filter steriliser, they may be added to the medium and autoclaved together.
3. Thirty grammes (30 g) of sucrose was added to the media and stirred to dissolve on a hotplate. pH was adjusted to 5.8 using 0.1 M sulphuric acid and 0.1 M sodium hydroxide.
4. Phytigel (3.5 g) was dissolved in 200 mL of sterile distilled water and was heated to melt in a microwave and then added to the culture media and the solution made up to 1 L prior to pH adjustment.
5. CuSO_4 was added to enhance somatic embryo initiation from leaf lobe explants. Silver nitrate may also be added. Both compounds suppress ethylene suppression in culture medium and improve somatic embryo production.
6. ABA enhances embryo maturation and shoot conversion from somatic embryos. It also makes somatic embryos tolerant to desiccation which also improves plant regeneration.
7. Cassava accessions ADI 001, Nkabom and Wenchi used for development of this protocol are landraces originally grown by farmers in the Brong Ahafo Region of Ghana. They have very good poundability; thus they are good for making *fufu*, a traditional meal. All the accessions mature after 12 months of planting. The stakes were collected from the Crop Science Department of the Kwame Nkrumah University of Science and Technology (KNUST) farms and were transported to Biotechnology and Nuclear Agriculture Research Institute (BNARI). Stakes were collected from plants with visually no symptoms of cassava mosaic virus diseases (CMVD). To proof for the absence of viral diseases, the plants should be screened using molecular techniques.
8. Tween 20 was added to improve efficiency of sterilisation with the sterilants.
9. The size of the meristem should not be more than 0.2 mm to avoid transmission of cassava mosaic viruses. It must also not be too small to make shoot regeneration very difficult.
10. In cassava somatic embryogenesis, young leaf lobes are ideal for primary somatic embryo induction. Older leaves are seldom used because they may only dedifferentiate into callus on culture medium without producing somatic embryos.
11. 2,4-D can initiate primary embryos even if the embryogenic competence of the explant is low. However, the frequency of matured embryo production is low on a 2,4-D-amended medium. Contrarily, picloram enhances embryogenic

- competence in explants but has low frequency of primary embryo production. It also induces somatic embryos earlier with high frequency of embryo production if the explant is embryogenically competent. Both auxins are however removed at the maturation stage of somatic embryo induction (**Step 2**).
12. The orientation of the leaf lobes has influence on somatic embryo induction. When the adaxial surface is in contact with the medium, somatic embryo development is enhanced compared to when the abaxial surface is in contact.
 13. Somatic embryos mature with well-developed hypocotyl and cotyledons within 14–21 days.
 14. Of all the explants used for embryo induction, primary embryos have high embryogenic competence.
 15. Fragmentation increases the frequency of embryo production because each fragment is highly embryogenically competent and can develop into full mature embryo.
 16. Serial subculture in cyclic embryogenesis should end at three or four cultures as long subcultures reduce embryogenic competence in the subsequent cycle. Longer serial subculture may also result in somaclonal variation among plants regenerated.
 17. Desiccation reduces the moisture content of the somatic embryos and thus mimics natural process of seed maturation in planta. The reduction in moisture content enhances germination. A combination of desiccation and ABA pretreatment improves somatic embryo germination.

5.5 Conclusion

Somatic embryogenesis can be coupled with mutation induction to overcome the limitations associated with conventional breeding of cassava. The embryogenetic system must therefore be optimised to reduce genotypic dependence. This can be achieved through indirect somatic embryogenesis which can provide both friable embryogenic calli and organised somatic embryos which are totipotent tissues that can be used as targets for mutagenic treatment (Fig. 5.2). Picloram-amended culture medium resulted in early embryogenic calli development, higher primary embryo production and more plant recovery from leaf lobe explants compared to 2,4-D. Recycling of the primary embryos on picloram-amended medium also led to higher embryo production. The presence of abscisic acid as well as air desiccation also resulted in high plant recovery. The optimised somatic embryogenesis protocol described can be coupled with mutation induction for production of useful traits in cassava breeding programmes.

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Chapter 6

Creation of a TILLING Population in Barley After Chemical Mutagenesis with Sodium Azide and MNU

Iwona Szarejko, Miriam Szurman-Zubrzycka, Malgorzata Nawrot, Marek Marzec, Damian Gruszka, Marzena Kurowska, Beata Chmielewska, Justyna Zbieszczuk, Janusz Jelonek, and Mirosław Maluszynski

Abstract Since the development of the Targeting Induced Local Lesions in Genome (TILLING) strategy, it has been applied in both plants and animals in many studies. The creation of an appropriate population is the first and most crucial step of TILLING. The goal is to obtain a highly mutagenized population that allows many mutations in any gene of interest to be found. Therefore, an effective method of mutation induction should be developed. A high mutation density is associated with saving time, costs, and the labor required for the development of a TILLING platform. The proper handling of the mutated generations, the establishment of a seed bank, and the development of a DNA library are essential for creating a TILLING population. The database in which all of the data from the molecular and phenotypic analyses are collected is a very useful tool for maintaining such population. Once developed, a TILLING population can serve as a renewable resource of mutations for research that uses both forward and reverse genetic approaches. In this chapter, we describe the methods for the development and maintenance of a TILLING population in barley.

Keywords Chemical mutagenesis • Alkylating agents • MNU • Sodium azide • TILLING • Barley • Phenotyping • Database

I. Szarejko (✉) • M. Szurman-Zubrzycka • M. Nawrot • M. Marzec • D. Gruszka • M. Kurowska • B. Chmielewska • J. Zbieszczuk • J. Jelonek • M. Maluszynski
Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Jagiellonska 28, 40-032 Katowice, Poland
e-mail: iwona.szarejko@us.edu.pl

6.1 Introduction

Targeting Induced Local Lesions in Genomes (TILLING) is a strategy of reverse genetics that takes advantage of classical mutagenesis and high-throughput methods of mutation identification. TILLING was originally developed for *Arabidopsis* as an alternative to insertional mutagenesis (McCallum et al. 2000). Since then, TILLING has successfully been applied in a broad spectrum of plant species, including agronomically important crops such as barley, wheat, or maize (Kurowska et al. 2011). The objective of TILLING is to find individuals that carry mutation(s) in the gene of interest and, through the phenotypic analysis of the identified mutants and their progeny, assign a function to the analyzed gene. Furthermore, TILLING may also be performed for genes that have a known function in order to produce a series of alleles that result in different modifications in a trait of interest. The newly identified alleles can be used as a valuable resource in breeding programs. There are many successful examples of using TILLING for crop improvement, for example, in *Triticum aestivum* and *Triticum turgidum* subsp. *durum* for obtaining a better starch quality (Slade and Knauf 2005); in *Hordeum vulgare* for the manipulation of spike morphology (Gottwald et al. 2009), starch content (Sparla et al. 2014), and waterlogging tolerance (Mendondo et al. 2016); in *Cucumis melo* (Dahmani-Mardas et al. 2010) and *Solanum lycopersicum* (Okabe et al. 2011) for enhanced shelf life; and in *Sorghum bicolor* (Xin et al. 2008) and *Avena sativa* (Chawade et al. 2010) for increased digestibility.

The first step of the TILLING strategy is the creation of a sufficiently large mutagenized population, which is called the TILLING population. Before performing mutagenesis, some crucial aspects should be considered. The first one is the material for the mutagenic treatment—because of their technical ease for seed propagated crops, such as barley, the seeds are usually treated. The second is the choice of a mutagen. Although physical mutagens such as gamma rays, thermal neutrons, and X-rays have contributed greatly to the release of mutant varieties in more than 175 plant species, including many important crops (<http://mvd.iaea.org/>), they have very seldom been used for the creation of TILLING populations (Sato et al. 2006). In most cases, chemical mutagens—ethyl methanesulphonate (EMS), *N*-methyl-*N*-nitrosourea (MNU), or sodium azide (NaN₃, SA)—have been used for TILLING treatments in plants.

Chemical mutagens cause mainly point mutations that are irreversible and are produced in relatively high densities. Alkylating agents belong to the most commonly applied chemical compounds in plant mutagenesis. They directly alter the structure and properties of the DNA bases. These mutagens cause every type of point mutations—transitions, transversions, deletions, and frameshifts and a relatively low frequency of the chromosome breakages that cause various chromosomal rearrangements (Maluszynski et al. 2003). In most cases, chemical mutagens generate an allelic series at any target locus, which may result in a reduction in the activity, specificity, or loss of the protein function. These types of changes are very valuable in assigning a gene a function.

The optimization of a mutagen dose before the commencement of a large-scale experiment is crucial for the successful mutagenic treatment. Simple pot tests for seedling emergence and growth reduction are routinely used to select a critical dose, i.e., a dose that results in a 50–70 % growth reduction at the seedling stage (Maluszynski et al. 2003). Such a dose causes a very high degree of sterility and lethality of M_1 plants, and therefore, for plant breeding projects that are aimed at improving only one or two characters in a well-adapted variety, doses of a mutagen that cause less than a 30 % growth reduction should be applied (Maluszynski et al. 2009). Too high a concentration of a mutagenic agent leads to a high lethality in the M_1 populations, whereas too low a concentration of the mutagen may result in a low density of mutations. A lower frequency of mutations requires a larger mutagenized population, which is associated with higher costs and labor. Therefore, in treatments that are used for the creation of TILLING populations, doses that induce the highest frequency of point mutations in M_2 plants, regardless of the somatic effects in M_1 , are sometimes applied (Martin et al. 2009). Nevertheless, the somatic effect of the mutagen should be known before a large-scale treatment in order to estimate the size of the M_1 population required for development of a TILLING population.

Alkylating agents such as EMS and MNU mainly induce GC>AT transitions (Till et al. 2003; Maluszynski et al. 2003); however, inversions and translocations have also been observed at low frequencies after MNU treatment (Szarejko and Maluszynski 1980). These mutagens cause DNA damage by transferring a methyl ($-\text{CH}_3$) group to the oxygen and nitrogen atoms of the nucleotide bases. A wide spectrum of lesions can be obtained, with the biological effect of these lesions ranging from less harmful to those that lead to cell death. It has been proven that methylation at the O^6 position of guanine has the strongest mutagenic property, as the O^6 -meG mispairs with T, and after DNA replication, the transition of G/C to A/T occurs (Richardson et al. 1986; Kleibl 2002; Warren et al. 2006). The alkylation of guanine in a nontranscribed (sense) DNA strand leads to its mispairing with thymine and after replication to its replacement by adenine (G>A transition). The alkylation of guanine in the transcribed (antisense) strand results in the C>T transition.

MNU is considered to be a very strong chemical mutagen, which is sometimes called a “supermutagen” (Maluszynski et al. 2009). A detailed characterization of mutation types and frequencies induced by MNU in barley genome was reported by Kurowska et al. in 2012. The majority (63.6 %) of the MNU-induced nucleotide changes were transitions, with a similar rate of G>A and C>T substitutions. This indicates a lack of bias in the repair of the O^6 -methylguanine lesions between DNA strands. However, a strong specificity of the nucleotide surrounding the O^6 -meG at the -1 position was observed. Purines formed 81 % of the nucleotides identified at the -1 site (Kurowska et al. 2012). In studies performed on rice, G>A and C>T transitions were predominant and accounted for 50 % and 42 % of all mutations detected, respectively (Suzuki et al. 2008). Similarly, in soybean, 90 % of the observed nucleotide changes formed these two types of transitions (Cooper et al. 2008). A similar share of G>A and C>T transitions that has been reported in barley, rice, and soybean indicates a lack of bias in the repair of the O^6 -meG lesions between DNA strands.

Another chemical—sodium azide (NaN_3 , SA)—is a potent mutagen in microorganisms and a very efficient mutagen in barley as well as in some other crop species; however, it is marginally mutagenic in mammalian systems and not at all mutagenic in *Neurospora* sp., in *Drosophila melanogaster*, and in *Arabidopsis thaliana* (Gruszka et al. 2012). The mutagenicity of sodium azide is mediated through the production of an organic metabolite of azide—the β -azidoalanine moiety [$\text{N}_3\text{-CH}_2\text{-CH(-NH}_2\text{)-COOH}$]. The mutagenic effect of NaN_3 depends on the acidic pH of the treatment solution (Nilan et al. 1973). The doses of NaN_3 that are routinely used for the mutagenic treatment of barley seeds are usually within the range of 0.5–4 mM for 3–5 h (Nilan et al. 1973; Maluszynski et al. 2003), although a dose as high as 10 mM for 2 h was applied for the creation of a TILLMore population of cv. ‘Morex’ (Talamè et al. 2008). Almost all of the mutations that were identified in this population were G/C>A/T transitions, while in the study performed by Olsen et al. (1993), transitions made up 86 %, and transversions constituted 14 % of the substitutions that were induced by NaN_3 . In this study, the frequency of A/T>G/C transitions was about three times higher than G/C>A/T. Deletions and mutation hot spots were not found after NaN_3 treatment. The high frequency of mutations that were induced by this chemical, the low frequency of chromosome aberrations, and its low toxicity for human health (Kleinhofs et al. 1978) make sodium azide a particularly efficient mutagen that is very useful for practical barley breeding purposes (Gruszka et al. 2012; Salvi et al. 2014).

Barley TILLING populations have been established for different cultivars—‘Optic’, ‘Morex’, ‘Barke’, ‘Lux’, and ‘Sebastian’ (Caldwell et al. 2004; Talamè et al. 2008; Gottwald et al. 2009; Lababidi et al. 2009). These populations were developed after being treated with EMS (Caldwell et al. 2004; Gottwald et al. 2009) and sodium azide (Talamè et al. 2008; Lababidi et al. 2009) or a double treatment with sodium azide and MNU. The average mutation density varied between the populations (Table 6.1).

M_1 plants that are grown from treated seeds have a chimeric structure, since the multicellular tissue (seed) has been treated with mutagen. Only mutations that have been induced in germline cells are inherited, i.e., transferred to the next generation. Because of the chimeric structure of M_1 plants, screening for mutations at the molecular level is carried out in the M_2 generation. Usually only one M_2 plant is grown from each M_1 plant in order to avoid the reappearance of the same mutations. DNA is individually isolated from each M_2 plant, and then DNA samples are pooled together prior to genotypic screening to increase the throughput of mutation discovery (usually five- to eightfold DNA pools are prepared). All M_2 plants are basically phenotyped and harvested, and a seed bank is established for M_3 seeds. It is beneficial to create a database in order to store information about the mutated generations, the phenotypes of the M_2 individuals and M_3 lines, the gene sequences analyzed, the types of mutations detected, the availability of M_2 and M_3 seeds, etc.

There are many different methods for mutation identification (e.g., see Chaps. 17, 18, 19, and 20 of this book). After the identification of an M_2 plant carrying a mutation in the gene of interest, it is important to develop a homozygous line for phenotyping. If the M_2 plant carries a mutation in the homozygous state, it

Table 6.1 Mutation density in different barley TILLING populations

Cultivar	Mutagen	Mutation density	References
Optic	EMS	1/1000 kbp	Caldwell et al. (2004)
Morex	NaN ₃	1/374 kbp	Talamè et al. (2008)
Barke	EMS	1/500 kbp	Gottwald et al. (2009)
Lux	NaN ₃	1/2500 kbp	Lababidi et al. (2009)
Sebastian	NaN ₃ and MNU	1/477 kbp	This paper

is possible to perform phenotypic analysis directly in the M₃ progeny and further generations. However, if the mutation is in the heterozygous state in the analyzed M₂ plant, it is necessary to select plants that carry the mutation in the homozygous state in M₃ generation. A large number of seeds are required for many phenotyping experiments, and therefore it is always recommended that the material be multiplied before analysis.

Phenotypic analysis of allelic mutants identified through TILLING may reveal the function of the gene of interest. However, it has to be borne in mind that after effective mutagenesis, many mutations are generated in a plant genome. For example, in barley, whose genome size is 5.1 Gbp (IBGSC 2012), each M₂ plant may carry circa 10,000 mutations, assuming that the mutation density in the TILLING population is 1/500 kb. The majority of these mutations are in intergenic regions or in introns, but there is a possibility that an M₂ plant carrying a mutation in the gene of interest may also carry other mutations (in different genes) that influence the phenotype. To clean the genetic background of the mutant from unwanted mutations, backcrosses with the parent variety should be carried out. However, in order to confirm that the altered phenotype is a consequence of the identified mutation, the co-segregation analysis of the mutation and a specific phenotypic trait should be performed.

The creation of a TILLING population is strictly associated with the production of a large amount of data that has to be safely stored and made available for population designers and for future users of the developed TILLING platform. Most of the available databases contain data on 5000–13,000 plants of the M₂ population (reviewed by Kurowska et al. 2011). The planning and creation of a TILLING population have to be preceded by a decision about how to provide the generated data to the recipients. It should be noted that the availability of a database containing detailed information about a TILLING population on the Internet significantly increases the impact of the studies being performed (Coronel and Morris 2016). In this manual, we present a version of a database that can be shared on the Internet.

There are many different ways to establish databases for a TILLING population, and various programs can be used to design them, including freeware and commercial software. For the Internet-available databases, phpMyAdmin is a useful program that allows simple and sophisticated databases to be developed that are easily accessed via the WWW. In combination with Internet platforms created using HTML and PHP languages, there are enormous possibilities for presenting,

storing, and searching data without increasing the costs of the development of a TILLING population (Miller 2014).

In this chapter, we describe the creation of a TILLING population for barley after combined treatment with two chemical mutagens: sodium azide and MNU. The application of a double mutagenic treatment with a period of inter-incubation germination of seeds between treatments gave a high frequency of point mutations in barley (Szarejko and Maluszynski 1999) and rice (Till et al. 2007). The TILLING population that was developed in barley after the combined treatment with sodium azide and MNU has been successfully used for the functional analysis of the genes involved in the control of DNA repair (Stolarek 2015a, b), waterlogging (Mendiondo et al. 2016), and strigolactone perception (Marzec et al. 2016).

6.2 Materials

6.2.1 Mutagenesis

1. Seeds (*see Note 1*)
2. Sodium azide (SA, NaN_3) (e.g., Sigma—Cat. No. S2002)
3. *N*-methyl-*N*-nitrosourea (MNU) (e.g., Oakwood Chemical—Cat. No. 098373)
4. KH_2PO_4 and H_3PO_4 for the preparation of a phosphate buffer
5. Distilled water
6. Equipment:
 - Laboratory fume hood
 - Laboratory balance (0.001–100 g)
 - Laboratory balance (0.01–2000 g)
 - Beakers (0.5 L, 1 L, 2 L)
 - Measuring cylinders (different sizes)
 - Trays with filter paper

6.2.2 Handling of Mutated Population

1. Soil and vermiculite
2. Pots (e.g., square—13 × 13 × 13 cm pots)
3. Water-soluble 22:5:10 fertilizer (N–P–K)
4. Equipment:
 - Vacuum packer (e.g., Tepro PP5.4)
 - Freezers for seed storage
5. Greenhouse or a growth chamber

6.2.3 DNA Isolation

1. Leaf fragments of M₂ plants (2–3 fragments, 2 cm long)
2. Silica gel (e.g., Sigma—Cat. No. 10087)
3. Glass beads (diameter, 3 mm) (e.g., Sigma—Cat. No. Z265926)
4. CTAB extraction buffer containing 2 % CTAB, 100 mM TrisHCl, 20 mM EDTA, 1.4 M NaCl, and 1 % β-mercaptoethanol (the last ingredient should be added to the appropriate volume of buffer immediately before use) (*see Note 2*)
5. Solution of chloroform-isoamyl alcohol (in 24:1 ratio)—should be kept in dark
6. 96 % and 70 % ethanol
7. TE buffer containing 10 mM TrisHCl and 1 mM EDTA
8. RNase solution containing 1 % RNase A, 9 mM sodium acetate, and 90 mM TrisHCl
9. Equipment:
 - Laboratory fume hood
 - Electric mill (e.g., Retsch MM 200, Retsch MM 301) or tissue homogenizer (e.g., FastPrep[®]-24, MP Biomedicals)
 - Water bath or thermomixer
 - Centrifuge for Eppendorf tubes that enable spinning at 18,000 × g at 4 °C
 - Vacuum concentrator (e.g., SpeedVac, Savant)—optional
 - Spectrophotometer (e.g., NanoDrop ND-1000)

6.2.4 Creation of a Database

1. A server connected to the Internet for a database that is available in the WWW (*see Note 3*)
2. Program for designing a database (*see Note 4*)

6.3 Methods

6.3.1 Mutagenesis

6.3.1.1 General Remarks

1. Mutagens are dangerous; take appropriate safety precautions (*see Note 5*).
2. Carefully plan out experiments in advance (*see Notes 6–8*).

6.3.1.2 Mutagenic treatment

1. Before a large-scale mutagenic treatment, evaluation of a critical dose of mutagen is recommended. Use 4–5 doses of mutagen, e.g., 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, and 3.0 mM sodium azide and 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM MNU; duration of treatment—3 h (*see Note 8*). Use simple laboratory tests for seedling emergence and growth reduction in order to establish the critical dose of a mutagen.
2. Calculate the amount of seeds needed for treatment. For laboratory tests, use 300 seeds (3 replications \times 100 seeds) plus a few seeds for eventual loss. For a large-scale treatment, use the number of seeds estimated on the basis of evaluation of somatic effects of the mutagen (*see Note 9*).
3. Put the seeds into a labeled glass container.
4. Presoak seeds in an excess of dH₂O for 8–12 h (*see Note 10*). The amount of water used in presoaking should be at least two to three times the volume of dry seeds. The beakers with presoaked seeds should be gently shaken a few times to remove air bubbles, which can block access of mutagen to embryos.
5. Calculate the amount of solutions needed for all of the treatment combinations. For small grain seeds, such as barley, use 0.5 ml of solution per one seed.
6. For treatment with sodium azide, prepare phosphate buffer, pH = 3.0 (*see Note 11*). Use 54.436 g KH₂PO₄ and add 3.67 ml H₃PO₄ per 1 L buffer. The buffer will be needed for all of the treatment combinations including the control.
7. Prepare the mutagenic solutions starting from the basic solution (the highest concentration used for treatment). Dissolve sodium azide in the buffer while MNU in distilled water. Leave a part of this solution for treatment, and dilute the rest to the required concentrations according to the calculations done. You can use a formula: $C_1 \times V_1 = C_2 \times V_2$, where C_1 is the concentration of the basic solution, V_1 volume of the basic solution, C_2 concentration of the solution required, and V_2 volume of the solution required. Use a fume hood for the preparation of mutagenic solutions (*see Note 5*).
8. Before treatment, pour out the dH₂O from the containers with seeds and rinse the seeds twice in tap water. Be careful to completely remove the water after rinsing.
9. Perform the treatment, i.e., pour the mutagen solutions into the beakers with the presoaked and rinsed seeds. Use a fume hood for treatment. Keep the same order of combinations during the whole procedure, i.e. presoaking, rinsing, treatment, and rinsing after treatment. Perform the mutagenic treatment at room temperature.
10. After 3 h of treatment, pour out the mutagenic solutions and rinse the seeds thoroughly in running tap water, minimum three to four times (*see Note 12*). Be careful not to lose any seeds (*see Note 13*).

11. For a double treatment with two mutagens, a 5–6-h period of inter-incubation germination is applied. After 3-h treatment with sodium azide (the first applied mutagen) and thorough rinsing, put the seeds into trays with a few layers of a filter paper, cover them with a wet sheet of the filter paper and keep for 6 h at room temperature. Then transfer seeds into the labeled beakers and pour the mutagenic solution with the second applied mutagen, i.e., MNU. Treat the seeds for 3 h and then rinse them in running tap water.
12. Plant M_1 seeds. If you perform a pot test for seedling emergence and growth reduction, see protocol below (Sect. 6.3.1.3). If you treat the seeds for a large-scale experiment to obtain M_1 generation for TILLING, plant the seeds into pots as described in Sect. 6.3.2. If you cannot plant M_1 seeds immediately after treatment, completely dry the seeds on a filter paper and store them until sowing in plastic bags at 4 °C. The treated seeds can be stored in a refrigerator for a few weeks.

6.3.1.3 Evaluation of a Critical Dose of Mutagens

1. Mix the soil with the vermiculite (2:1); wet the mixture.
2. Fill the pots (13–15 cm diameter) with the mixture to 3 cm from the top.
3. Label the pots (genotype, mutagen concentration (dose), replication).
4. Plant 100 seeds per replication into each labeled pot.
5. Cover with a 3-cm layer of wet sand.
6. Place the pots randomly into the growth chamber at temperature 22 °C/20 °C day/night, 16/8 photoperiod, and light intensity at least 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and grow them till the emergence of the second leaf (Fig. 6.1).
7. Seven to ten days after the mutagenic treatment, cut all of the seedlings near the surface of the sand, count their number, and measure their length (separately for each variety, dose, and replication).
8. Calculate the mean \pm SD of the seedling number and length separately for each mutagenic combination.
9. Calculate the seedling emergence reduction and seedling growth reduction for each combination. Use the following formulas:

Seedling emergence reduction (%) = $1 - \text{Average number of seedlings in a treated combination} / \text{Average number of seedlings in the control} \times 100\%$

Seedling growth reduction (%) = $1 - \text{Average length of seedlings in a treated combination} / \text{Average length of seedlings in the control} \times 100\%$

10. Select one to two doses for a large-scale treatment (*see Note 9*).

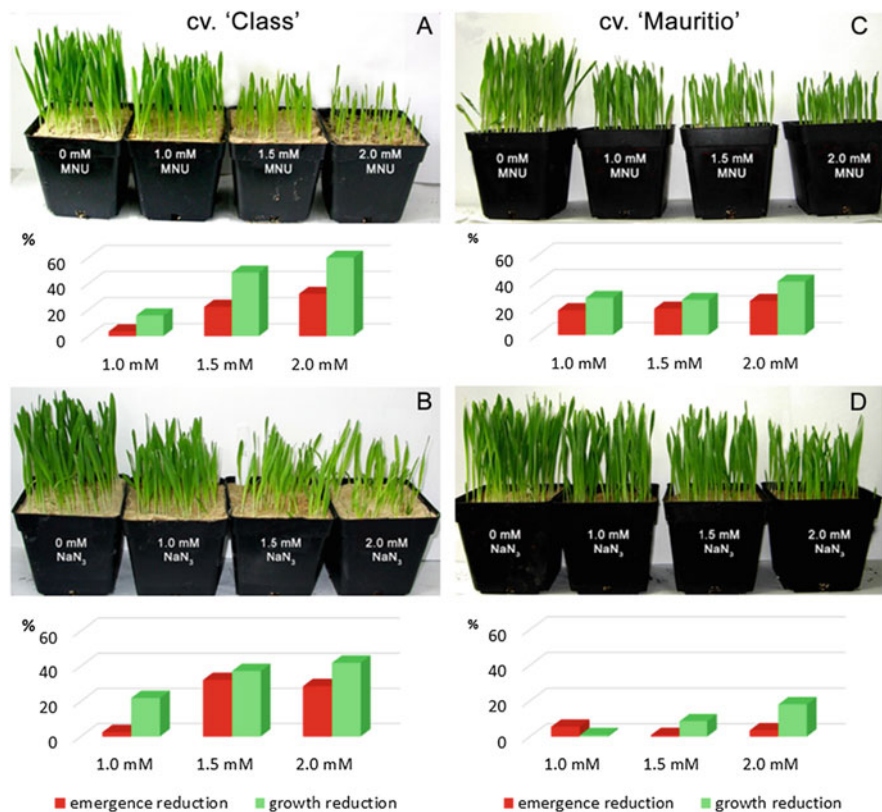


Fig. 6.1 Examples of different sensitivity of barley cultivars to mutagenic treatment. Laboratory tests for seedling emergence and growth reduction after mutagenic treatment of barley cv. 'Class' (A, B) and 'Mauritio' (C, D) with MNU (A, C) and NaN₃ (B, D)

6.3.2 Handling of the Mutated Generations and the Basic Phenotyping of M₂ Plants and M₃ Lines

1. Prepare a substrate—mix soil and vermiculite at a 2:1 ratio.
2. Plant the mutagenized seeds (M₁) of spring cultivars into pots (13–15-cm diameter) and the seeds of winter genotypes into flats or small pots (5 cm diameter) filled with the substrate. Two weeks after sowing, the seedlings of the winter genotypes are vernalized at 4 °C, 16/8 h photoperiod, light intensity 100 μmol m⁻² s⁻¹ for 8–10 weeks, after which they are transferred to bigger pots and grown under the same conditions as the spring genotypes in a greenhouse or a growth room (22/20 °C day/night, 16/8 photoperiod, light intensity 350–450 μmol m⁻² s⁻¹). (see **Notes 14** and **15**).
3. Assign a number to each M₁ plant. Optimally, develop approximately 10,000 M₁ plants. Remember to water and fertilize the plants regularly.

4. Harvest M_1 plants and establish a seed bank. Vacuum pack the dried M_2 seeds from each M_1 plant separately and store them in a freezer or cold chamber (at 4–5 °C, optimally –20 °C).
5. Sow one seed from each M_1 plant into a 13–15-cm pot. Only one M_2 plant derived from each M_1 individual is grown in order to avoid the repetition of the same mutations. Optionally, two seeds from the same plant can be sown in order to save time in the event that one of them does not germinate or is a chlorophyll mutant. If two M_2 seedlings emerge, discard one of them. There should only be one M_2 plant per pot.
6. Grow the M_2 generation in a greenhouse or a growth room under optimal conditions (temperature 22/20 °C day/night, 16/8 photoperiod, light intensity 350–450 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Remember to water the plants regularly. Fertilize them with 22:5:10 (N:P:K) fertilizer two to three times during vegetation. Assign the appropriate number to each M_2 plant—for example, if the M_2 seed was taken from M_1 plant number 333, name the M_2 plant 333/01.
7. When the plants begin to tiller, collect leaf samples for DNA isolation. Isolate DNA from each M_2 plant separately (*see* Sect. 6.3.3).
8. Observe the M_2 plants for chlorophyll mutations at seedling stage (*see* **Note 16**) and any morphological changes during growth. Input the information about them into a database.
9. Before harvesting, perform a basic phenotype analysis of M_2 plants. Collect the information on, e.g., plant height, culm length, spike length, spike morphology, awn length/shape, number of tillers, fertility/partial sterility, number and weight of M_3 seeds, and any visible alterations. Input these data into a database.
10. Collect M_3 seeds from each M_2 plant separately.
11. Put the seeds of M_3 and further generations into the seed bank. The seeds should be cleaned, dried, vacuum packed, and stored at a low moisture content (below 12 %—the moisture content can be monitored using a grain moisture meter) at a temperature below 10 °C, (–20 °C is the best option, *see* **Note 17**). Each package should be described with information on the plant ID, year of harvest, and optionally with the number and weight of the seeds. A label should be placed inside the package and printed out using non-bleaching ink (Fig. 6.2).
12. After the identification of a particular M_2 plant carrying a mutation in the gene of interest (for methods of mutation identification, *see* Chaps. 17, 18, 19, and 20 of this book), use the seeds of its progeny for phenotyping (reverse approach). Only plants carrying the mutation in a homozygous state should be analyzed (*see* **Note 18**). It is important to multiply the homozygous material prior the detailed phenotypic analysis.
13. If a large number of M_3 seeds from a particular M_2 plant have been obtained, you can sow 15–20 M_3 seeds in a field in order to check for any morphological traits and their segregation. M_3 lines with positive traits may be used in breeding programs (forward approach). All data should be archived in the database.
14. Perform a backcross of an identified mutant with the parent variety in order to clean the genetic background of the mutant from other mutations. In the BCF_2



Fig. 6.2 Vacuum packer and seeds prepared for storage in a seed bank

generation, identify plants carrying a homozygous mutation in the analyzed gene. Use the BCF₂ homozygous mutants for phenotyping, including a detailed evaluation of traits of interest.

6.3.3 DNA Isolation: Creating the M_2 DNA Library

6.3.3.1 Isolate DNA from the M_2 Plants According to the Modified Micro-CTAB Method (Doyle and Doyle 1987 and see Note 19)

The DNA of each M_2 plant must be isolated and stored individually. Alternative DNA extraction methods can be considered (for example, see Chap. 14).

1. Dry leaves in silica gel for a minimum of 3 days.
2. Put dry fragments of leaves into 2.2-ml Eppendorf tubes together with three glass balls.
3. Grind the leaf tissue in an electric mill (45 min in a Retsch MM 200 or 301; 40 s in a FastPrep[®]-24 homogenizer) in order to disrupt the tissue structure and cell walls.
4. Add 1 ml of a CTAB extraction buffer heated to 60 °C to each tube in order to lyse the cell membranes.
5. Incubate samples in a water bath or thermomixer at 60 °C for 0.5 h.
6. Add 800 μ l of chloroform-isoamyl alcohol (24:1) to each tube to denature the proteins and separate the aqueous and organic phases.
7. Centrifuge at 18,000 $\times g$ at 4 °C for 20 min.
8. Transfer the upper aqueous phase into a new tube; discard the lower organic phase.
9. Add 600 μ l of chloroform-isoamyl alcohol (24:1) to each tube.
10. Centrifuge at 18,000 $\times g$ at 4 °C for 10 min.
11. Transfer the upper aqueous phase into a new tube; discard the lower organic phase.
12. Add 1 ml of 96 % ethanol to each tube to precipitate DNA.
13. Centrifuge at 18,000 $\times g$ at 4 °C for 30 min.
14. Remove supernatant.
15. Add 1 ml of 70 % ethanol to each tube.
16. Centrifuge at 18,000 $\times g$ at 4 °C for 5 min.
17. Remove supernatant.
18. Repeat **steps 15–17**.
19. Dry the pellet, e.g., using a SpeedVac (Savant).
20. Add 100 μ l of a TE buffer to each tube to dissolve DNA.
21. Incubate samples at 37 °C for 20 min (or 4 °C overnight).
22. Add 1 μ l of an RNase solution to each tube.
23. Incubate samples at 37 °C for 45 min.
24. Measure the DNA concentration in each sample using a NanoDrop ND-1000.
25. Dilute DNA to a concentration of 100 ng/ μ l.
26. Store the DNA (both, stocks and dilutions) at -20 °C.

6.3.3.2 Prepare Pools of DNA to Identify Plants Carrying Mutations Within the Gene of Interest

Different pooling strategies are used for different mutation discovery methods (for example see Chaps. 18 and 20 of this book)

6.3.4 Creation of a Database

6.3.4.1 General Information

1. A database is composed of tables containing an unlimited number of columns that describe specific traits/information and an unlimited number of rows that represent individuals (plants, genes, mutations, etc.).
2. The structure of the tables can be modified at each stage of database creation.
3. The number of tables in a database is unlimited.
4. It is convenient to design individual tables for each generation or for each part of the presented studies.
5. Each table should contain a column with an ID number that allows information about a specific individual that is collected in different tables to be connected. This could be the name/number of a plant or an additional ID.
6. Each column in the tables should be defined in regard to data type (in numeric format, text format, data, etc.) and in regard to the symbol number that is used to describe this feature. This will allow space on the server to be saved and will ensure the efficient operation of the database.
7. Database users should never have direct access to the tables that contain the data. All operations, including adding, modifying, removing, and searching the data, should be done using forms that are prepared by the administrator. The forms, which are usually prepared in the HTML and PHP languages, can be created using special programs (web page editors, *see Note 4*) or using any text editor.
8. All operations during the creation of database and its administration can be done on files that are saved on the server, but it is more convenient to test all of the implemented solutions on a local computer and then to transfer them to the server. A freeware software such as a WampServer can be used to create a virtual server on a local computer (<http://www.wampserver.com>).

6.3.4.2 Creation of a Database: An Example

Information about a basic version of a database for a barley TILLING population is presented below. This database contains tables that describe the plants of the M_1 , M_2 , and M_3 generations, any identified mutations, and a large-scale screening analysis that were carried out in the developed population (*see Note 19*):

1. Create Table “m1,” which contains information about the plants of the M_1 generation, such as the number of seeds collected from the main spike, the number of all of the seeds collected, the number of seeds that were planted for the development of M_2 , and the information about the mutagenesis when different treatments were used (Fig. 6.3a).
2. Create Table “m2,” which contains columns that allow the data that describes the isolated DNA (quantity, quality, storage location), the basic phenotype of the M_2 plants (*see* Sect. 6.3.2), other phenotypic changes, the number of M_3 seeds produced by the M_2 plants, and the current availability of M_3 seeds (part of seeds might have been used up in experiments) to be stored (Fig. 6.3b).
3. Create the “mutations” table for the descriptions of the mutations identified in the M_2 generation of the TILLING population. This table should contain information about the names/numbers of the M_1 and M_2 individuals, information about the genes that were analyzed (the name and sequence), information about the length of analyzed amplicon, and detailed information about mutations—position in the analyzed sequence, mutation type, information about the homo- or heterozygosity of mutation, allele name (Fig. 6.3c).
4. The TILLING population can be used for large-scale screening analysis in M_3 or further generations. In this case, each individual plant should be identified by at least three columns: “m1_name,” “m2_name,” and “m3_name.”
5. Regular backups of each table should be stored on a local computer.
6. After adding a large amount of data, the modified tables should be checked and optimized.

Notes

1. Selection of a cultivar or line for mutagenesis is a very important step. It depends on the goal of creation of a TILLING platform. The batch of seeds chosen for mutagenesis should be homozygous or near homozygous and should have a high germination capacity. Any seeds that are injured, are abnormal, or have symptoms of a disease should be discarded.
2. DNA isolation procedure should be performed under the fume hood, due to the usage of β -mercaptoethanol and chloroform. β -Mercaptoethanol should be added under the fume hood because of its extensive smell.
3. Most scientific institutions provide space on the institution’s server for their employees. Otherwise, commercial services that provide the security and maintenance of the server can be used. It is also possible to develop a database on a server that is free of charge. However, this solution is not recommended due to the problems with data security and with unwanted advertisements being displayed while using a database. The space needed for the development of database on a server depends on the format of the data that will be stored. Pictures and all graphical files occupy much of the available space and

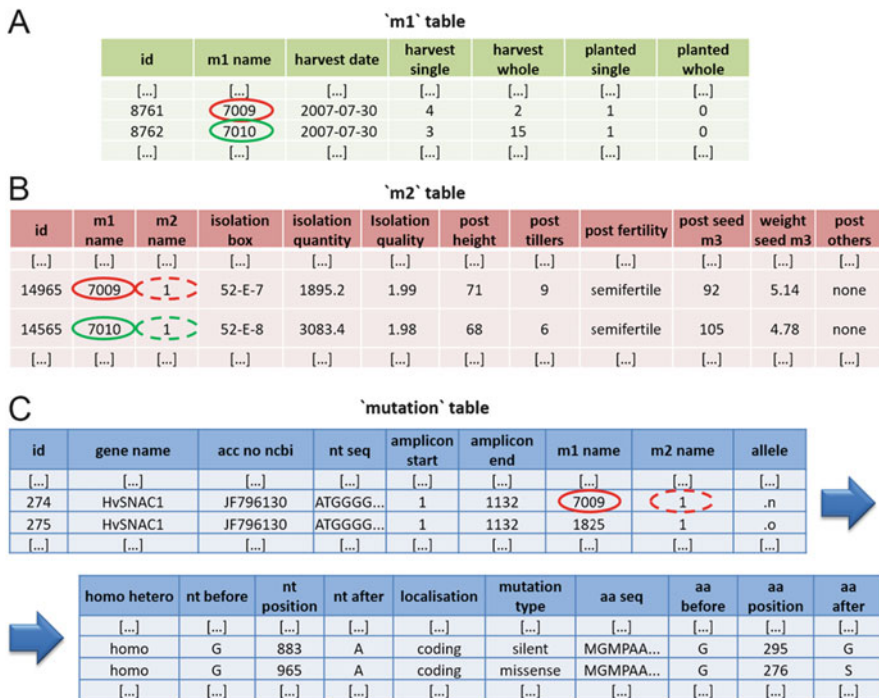


Fig. 6.3 Examples of database tables with information about the M₁ (A) and M₂ (B) generations and the mutations identified in the analyzed genes (C). The scheme shows three independent tables; red and green circles indicate how information from different tables can be related using the names of the M₁ plants. Columns: “m1_name,” “m2_name” contain information about the specific names of the analyzed plants; “harvest single” and “harvest whole,” number of seeds from the apical spike and other spikes; “planted single” and “planted whole,” number of seeds already used; “isolation box,” storage location of the isolated DNA; “isolation quantity” and “isolation quantity,” describe the quantity (ng/μl) and quality (260/280 absorbance) of isolated DNA; “post height,” height of mature plants; “post tillers,” number of tillers; “post seed m3” and “seed weight m3,” number and weight of the collected M₃ seeds; “gene name,” name of an analyzed gene; “acc no ncbi,” NCBI accession number of the gene; “nt seq,” nucleotide sequence of gene; “amplicon start” and “amplicon end,” position of the analyzed amplicon in the sequence from the previous column; “allele,” name of the identified allele; “homo hetero,” mutation zygosity; “nt before,” nucleotide in the original sequence; “nt position,” position of the identified mutation; “nt after,” mutated nucleotide; “localization,” information about mutation position in the coding or noncoding sequence; “mutation type,” type of the identified mutation; “aa seq,” amino acid sequence; “aa before,” amino acid in the original sequence; “aa position,” position of the aa substitution in the protein; “aa after,” the changed amino acid

significantly increase the transfer rate needed to use the database. For a simple database that contains data in a text file, at least 0.5 GB of space is recommended.

4. For the creation of a database and the individual tables, we recommend the freeware phpMyAdmin, which is installed by default on most servers. phpMyAdmin may also be downloaded from <http://www.phpmyadmin.net>.

However, any other program for database creation and management can be used, such as Aptana Studio (<http://www.aptana.com>), Komodo Edit (<http://www.komodoide.com>) or Eclipse (<https://www.eclipse.org>).

5. It should be stressed that most chemical mutagens are also strong carcinogens. For this reason, all steps of mutagenic treatment should be carried out under a biohazard fume hood. Disposable gloves and a lab coat should be used when performing treatments and dealing with treated seeds. Taking these precautions is especially important during treatment with MNU—a strong mutagen and carcinogen.
6. There are four main steps in mutagenic treatment of seeds with chemical mutagens:
 - Presoaking the seeds in distilled water (*see* also **Note 10**)
 - Treatment with the mutagen
 - Posttreatment rinsing in tap water
 - Planting the M_1 seeds
7. When a double treatment with two mutagens is performed, the regular protocol of mutagenic treatment is followed with the addition of the 5–6-h inter-incubation germination period between treatments during which the seeds are incubated on a wet filter paper.
8. The concentration of the mutagen should be considered together with duration of the treatment. A shorter treatment time with a higher concentration of mutagen can increase somatic effects and could be insufficient to penetrate equally all cells in the plant material.
9. In addition to DNA lesions in the nucleus and cytoplasmic organelles, mutagens can generate damages in all components of cytosol and disturbances of the cell cycle. Therefore, mutagenic treatment can impair metabolism of cells in various tissues and organs and influence the growth and development of M_1 plants. These effects, called “somatic effects” of mutagen, are manifested by delay in seed germination, reduction of plant emergence, growth reduction, appearance of chlorophyll defects, reduction of fertility, and plant survival. The size of the M_1 generation should be calculated taking into account the lethality and sterility of M_1 plants in order to guarantee enough seeds for M_2 generation. It is worthwhile to organize a pilot experiment to help compare the somatic and genetic effects induced by a range of doses. The pilot experiment will extend the whole procedure but it helps in a proper selection of the dosages for the large-scale treatment.
10. The presoaking in distilled water should be applied to activate seeds physiologically before treatment with mutagen. Eight hours of presoaking at room temperature (20–24 °C) is the optimal time for barley, but for convenience the seeds can be presoaked overnight. The presoaking reduces the somatic effect of chemical mutagen.
11. Sodium azide is a very efficient mutagen in several species if applied at low pH (<4). For barley mutagenesis, sodium azide is dissolved in a phosphate buffer at pH=3, and this solution is used for treatment.

12. The extensive posttreatment rinsing in tap water is necessary to stop the action of the mutagen and to remove its residues from the surface of the seeds. To facilitate sowing, the treated seeds can be dried on a filter paper under a fume hood. However, too intensive drying, especially at increased air temperature, can enhance somatic effects of the mutagen.
13. If a net is placed over the incubation beaker, that would protect seed from falling out.
14. If the appropriate space in the greenhouse or the growth room for growing M_1 generation is not available, the M_1 plants can be grown in an experimental field, but the size of the population should be increased.
15. Optionally, M_1 seeds of spring cultivars may be planted into small pots filled with the same substrate and kept for two weeks in a cold growth chamber ($4\text{ }^\circ\text{C}$, 16/8 photoperiod, light intensity $100\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) for hardening. After this period, the seedlings may be transplanted into bigger pots (13–15 cm) and grow them under the greenhouse conditions until harvesting.
16. The analysis of chlorophyll mutations is based on visual observation. There are several types of chlorophyll mutations that can be observed at seedling stage in barley. The most common are: *albina* (white), *xantha* (yellow), and *viridis* (pale green). The other categories include *alboviridis* (partially white and pale green), *tigrina* (with transverse yellow or necrotic stripes), and *striata* (with longitudinal, narrow white stripes).
17. The seed bank for seed storage is a crucial element of a TILLING platform. Proper maintenance of seeds will allow a TILLING platform to be a long-lasting, renewable source of mutations for studies. Nevertheless, after several years of storing, the germination capacity of M_3 seeds should be checked out, and when it is low, the remaining seeds should be multiplied. M_3 plants should be harvested individually and the track of M_3 and M_4 origin from each individual M_2 plant should be kept. Optionally, the whole M_3 progeny derived from the same M_2 plant can be bulked, but in this case, the identification of plants carrying mutation in the homozygous state might be more difficult.
18. Most of mutations are recessive; therefore, it is crucial to develop homozygous lines of mutants before phenotyping.
19. The database that is established on a server can be used as a local variant without publication on the Internet or as a public version. In both cases, the forms (HTML + PHP) should be used to manage the database. Never allow users to work on database with access to the administrator rights.

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Chapter 7

Site-Directed Mutagenesis in Barley by Expression of TALE Nuclease in Embryogenic Pollen

Maia Gurushidze, Stefan Hiekel, Ingrid Otto, Götz Hensel, and Jochen Kumlehn

Abstract In this chapter, a detailed protocol for the induction of targeted mutations in barley using customized transcription activator-like effector nucleases (TALENs) is provided. As explant for the introduction of TALEN expression constructs, embryogenic pollen cultures are used which consist primarily of haploid cells able to be converted via pollen embryogenesis and genome duplication into fertile doubled haploid plants. Thanks to the haploid nature of these target cells, a mutation induced by TALE nucleases in just one *gfp* allele can be readily detected in vitro and thus genetically fixed primary mutant plants obtained in one generation.

Keywords Customizable endonuclease • Doubled haploids • Embryogenesis • Homozygosity • Microspore • Targeted knockout

7.1 Introduction

7.1.1 Site-Directed Mutagenesis in Plants

The continuously growing demand of the world population for food is a tremendous challenge for modern agriculture, plant breeding, and biotechnology (Griggs et al. 2013). To rapidly produce new plant varieties with improved gene variants and traits, more efficient, flexible, and reliable methods such as site-directed mutagenesis are needed. In addition, these techniques will also have a big impact on basic science by helping to further investigate gene function and regulation.

Site-directed mutagenesis enables researchers to alter the DNA sequence at a predefined location in the genome of a plant cell. Such mutations can be generated by the use of customized endonucleases for target sequence-specific double-strand

M. Gurushidze • S. Hiekel • I. Otto • G. Hensel • J. Kumlehn (✉)
Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)
Gatersleben, Corrensstrasse 3, 06466 Seeland, OT Gatersleben, Germany
e-mail: kumlehn@ipk-gatersleben.de

break (DSB) induction. The repair of DSBs via nonhomologous end-joining is error-prone to some extent and therefore can lead to nucleotide deletions or insertions (indels) at the breakage site (Waterworth et al. 2011). Depending on the type of mutation, this can result in functional modification, complete disruption of gene function, or removal of whole gene clusters from the plant genome.

For application of this technique in model and crop plant species, the following customizable endonuclease platforms with different benefits and drawbacks are available: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided endonucleases (RGENs); the latter derive from a clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) microbial defense system [reviewed in Fichtner et al. (2014)].

TALENs are created by the fusion of a customized TALE DNA-binding domain (DBD) and the FokI restriction-endonuclease domain (FokI-R) (Christian et al. 2010). Primarily, a TALE-DBD from plant pathogenic bacteria *Xanthomonas* sp. consists of a variable number of nearly identical amino acid repeats. Each repeat has a length of 33–35 amino acids and binds preferentially to a certain nucleotide of the target DNA sequence (Boch et al. 2009; Moscou and Bogdanove 2009). Different binding specificity of distinct repeats can be explained by variations in amino acids 12 and 13, which are therefore called repeat variable diresidues (RVDs). The amino acid pairs most commonly used in RVDs of TALE-DBDs are NI (binding to adenine), NG (thymine), NN (guanine), and HD (cytosine). Given the opportunity of rearrangement of these repeats, it is possible to assemble a TALE-DBD for virtually any DNA target sequence of choice.

Such customized TALEN-DBD in combination with the 25 kDa FokI-R domain, with the latter not having any own DNA-binding specificity (Li et al. 1992), TALENs represent a universal tool for DSB induction. However, FokI-R needs to form dimers to be catalytically active (Bitinaite et al. 1998). Therefore, two TALEN subunits binding to the desired target sequence in inverse orientation to each other are required. This obligatory dimerization decreases the probability for off-target mutations but also doubles the effort to produce an effective TALEN. TALEN-coding DNA subunits can be highly efficiently assembled via Golden Gate cloning [e.g., Cermak et al. (2011)], PCR-based methods [e.g., Yang et al. (2013)], or FLASH assembly (Reyon et al. 2012).

TALENs are more cost effective and much easier to handle than zinc-finger nucleases (Beumer et al. 2013). Therefore, TALENs were successfully applied for site-directed mutagenesis in a number of model and crop plants like *Arabidopsis* (Christian et al. 2013), tobacco (Zhang et al. 2013), *Nicotiana benthamiana* (Mahfouz et al. 2011), *Brachypodium distachyon* (Shan et al. 2013), rice (Li et al. 2012), *Brassica oleracea* (Sun et al. 2013), soybean (Haun et al. 2014), wheat (Wang et al. 2014), maize (Liang et al. 2014), and barley (Wendt et al. 2013; Gurushidze et al. 2014).

7.1.2 *Haploid Technology*

The development of immature barley pollen at the vacuolated microspore or early bicellular stage can be switched from the normal process of pollen maturation to an embryogenic pathway (Sunderland et al. 1979). The embryogenic structures formed can be regenerated into haploid and doubled haploid, instantly homozygous plants. As each doubled haploid individual originates from a different gametophytic cell produced via meiotic recombination, a population of doubled haploid plants represents a collection of genetically unique and fixed individuals. For this reason, haploid technology has become a valuable tool in functional genomics as well as in modern plant breeding. The genetic transformation of such haploid target cells followed by whole genome duplication opened up the unique opportunity to immediately generate plants homozygous with regards to the integrated transgene (Kumlehn et al. 2006). Similarly, the ability to mutagenize haploid cells in a targeted manner via expression of customized endonucleases (in the present case TALENs) permits the generation of instantly homozygous mutants, which makes this approach highly advantageous compared to previously published methods of gene targeting.

7.2 Materials

7.2.1 *Donor Plants*

1. The protocol is optimized for the winter type cultivar “Igri” released by Saatucht Ackermann (Irlbach, Germany) in 1976. The target chosen in the example demonstrated here is the reporter gene encoding green fluorescent protein (GFP), since this provides a simple bioassay for knockout events. Accordingly, the transgenic “Igri” lines PV 89 and BPI 09 were used, which each carry a single copy of *gfp* in homozygous state.
2. The germination substrate is a 3:1:2 mixture of garden mulch/sand/white and black peat (Substrate 2, Klasmann, Germany).
3. The soil substrate used after vernalization is a 2:2:1 mixture of compost/Klasmann Substrate 2 and sand. Osmocote is used as a fertilizer containing 19 % N, 6 % P, and 12 % K.
4. *Petuniensubstrat* (Klasmann, Germany) is used for the acclimation of in vitro regenerants to grow on soil substrate. After vernalization, the substrate is refreshed as described above.

7.2.2 Stock Solutions and Culture Media

7.2.2.1 Stock Solutions

1. Major mineral salts:

K macro (20×): 1.6 g/L NH_4NO_3 , 40.4 g/L KNO_3 , 6.8 g/L KH_2PO_4 , 8.8 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.9 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

K4N macro (20×): 6.4 g/L NH_4NO_3 , 72.8 g/L KNO_3 , 6.8 g/L KH_2PO_4 , 8.8 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.9 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

2. Trace mineral salts:

K micro (1000×): 8.4 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 3.1 g/L H_3BO_3 , 7.2 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 120 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 25 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 24 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 170 mg/L KI.

3. Individual mineral salts:

Ferric sodium ethylenediaminetetraacetate (NaFeEDTA; 75 mM, 27.5 g/L): filter-sterilized and stored at 4 °C.

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1 M, 147 g/L): filter-sterilized and stored at 4 °C.

KH_2PO_4 (1 M, 136 g/L): filter-sterilized and stored at room temperature.

K_2HPO_4 (1 M, 174 g/L): filter-sterilized and stored at room temperature.

Phosphate buffer (1 M, pH 5.0): 97.5 % 1 M KH_2PO_4 , 2.5 % 1 M K_2HPO_4 , filter-sterilized and stored at 4 °C.

Phosphate buffer (1 M, pH 5.9): 90 % 1 M KH_2PO_4 , 10 % 1 M K_2HPO_4 , filter-sterilized and stored at 4 °C.

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (10 mM, 2.5 g/L): filter-sterilized and stored at 4 °C.

4. Carbohydrates:

Mannitol (0.4 M, 72.9 g/L): autoclaved and stored at 4 °C.

Maltose·H₂O (1 M, Sigma Grade I, 360 g/L): filter-sterilized and stored at room temperature.

Maltose·H₂O (1 M, standard quality, 360 g/L): filter-sterilized and stored at room temperature.

Maltose·H₂O (0.55 M, standard quality, 198 g/L): filter-sterilized and stored at 4 °C.

5. Complex organics:

KM organics (100×, Sigma K-3129): 2 mg/L *p*-aminobenzoic acid, 200 mg/L L-ascorbic acid, 1 mg/L D-biotin, 100 mg/L D-calcium pantothenate, 2 mg/L cyanocobalamin, 40 mg/L folic acid, 10 g/L myo-inositol, 100 mg/L nicotinamide, 100 mg/L pyridoxine·HCl, 1 mg/L retinol, 20 mg/L riboflavin, 100 mg/L thiamine·HCl, stored at -20 °C.

Gamborg B5 organics (1000×): 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine·HCl, 10 mg/L thiamine·HCl, filter-sterilized and stored at -20 °C.

6. Growth regulators:

2,4-Dichlorophenoxyacetic acid (2,4-D, 1 mM): 221 mg/L dissolved in few drops of 50 % ethanol by heating gently, made up to the final volume with hot H₂O, filter-sterilized and stored at 4 °C.

6-Benzylaminopurine (BAP, 1 mM): 224 mg/L dissolved in 30 ml hot H₂O and 3–5 drops 1 M NaOH, made up to the final volume, filter-sterilized and stored at 4 °C.

7. Selective agents:

Bialaphos (41 mg/ml): filter-sterilized and stored at –20 °C.

Spectinomycin (100 mg/ml): filter-sterilized and stored at –20 °C.

Tetracycline (10 mg/ml): dissolved in few drops of 50 % ethanol, filter-sterilized and stored at –20 °C.

Timentin (150 mg/ml): filter-sterilized and stored at –20 °C.

8. Gelling agent:

Phytagel (2×, 6 g/L): suspended in cold water (at best 1.5 g Phytagel per 250 ml unit), autoclaved and stored at room temperature.

9. Other working solutions:

Acetosyringone (1 M, 196 mg/ml): dissolved in dimethyl sulfoxide (DMSO) and stored at –20 °C.

Ethanol (70 %): stored at room temperature.

Glycerol (15 % w/v): autoclaved and stored at room temperature.

L-glutamine (37 mg/ml): dissolved by adding a few drops of 0.1 M KOH and heating in a water bath, filter-sterilized and stored at –20 °C.

Morpholinoethanesulfonic acid (MES, 212.2 g/L, pH 5.0, pH 5.5, and pH 5.9): dissolved in 40 ml water, the pH adjusted with KOH (few pellets), stored at room temperature overnight, and the pH readjusted with either 1 M KOH or 1 M HCl, made up to the required final volume, filter-sterilized and stored at 4 °C (*see Note 1*).

7.2.2.2 Medium for *Agrobacterium Tumefaciens*

CPY medium for *A. tumefaciens* strain LBA4404: 0.1 % (w/v) yeast extract, 0.5 % (w/v) pancreatic peptone, 0.5 % (w/v) sucrose, 2 mg/L MgSO₄·7H₂O (pH 7); add 1.2 % w/v Bacto Agar to produce solid medium.

7.2.2.3 Media for Plant Cell Culture

1. Barley pollen culture (KBP) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 12 ml/L L-glutamine, 4 ml/L BAP, and 250 ml/L maltose (1 M, Sigma Grade I) stocks, pH adjusted to 5.9 and stored at 4 °C.

2. Coculture (CK) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, Sigma Grade I), 2 ml/L BAP,

- 0.5 ml/L acetosyringone, 10 ml/L MES (pH 5.9), and 50 ml/L phosphate buffer (pH 5.9) stocks, stored at 4 °C.
3. AgroStop (ASt) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, Sigma Grade D), 10 ml/L MES (pH 5.0), 25 ml/L phosphate buffer (pH 5.0), 7 ml/L CaCl₂, 4 ml/L L-glutamine, 10 ml/L 2,4-D, 2 ml/L BAP, 0.5 ml/L acetosyringone, 750 µL/L bialaphos, and 1.3 ml/L Timentin stocks, stored at 4 °C.
 4. Selection medium (S3): 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, standard quality), 10 ml/L MES (pH 5.5), 12 ml/L L-glutamine, 1 ml/L BAP, 750 µL/L bialaphos, and 1.3 ml/L Timentin stocks, stored at 4 °C.
 5. Solid barley pollen culture (KBP4PT) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, standard quality), 1 ml/L BAP, 12 ml/L L-glutamine, 750 µL/L bialaphos, and 1.3 ml/L Timentin stocks; appropriate amounts for a final volume of 0.5 L KBP4PT made up to an intermediate volume of 250 ml (double concentrated), adjusted to pH 5.9, heated to about 40 °C, and then mixed (1:1) with 250 ml Phytigel stock (melted by heating).
 6. Regeneration (K4NBT) medium: 50 ml/L K4N macro, 1 ml/L K Micro, 0.75 ml/L NaFeEDTA, 1 ml/L B5 organics, 4 ml/L L-glutamine, 100 ml/L maltose (1 M, standard quality), 1 ml/L BAP, 0.49 ml/L CuSO₄, 100 µL/L bialaphos, and 1.3 ml/L Timentin stocks; appropriate amounts for a final volume of 0.5 L K4NBT made up to an intermediate volume of 250 ml (doubled concentrated), adjusted to pH 5.9, heated to about 40 °C, and then mixed (1:1) with 250 ml Phytigel stock (melted by heating).

7.2.3 Materials for the Isolation of Embryogenic Pollen

1. Refrigerated centrifuge equipped with swing-out baskets
2. Waring blender (heat sterilizable)
3. Sterile Petri dishes with lid (3.5 cm in diameter)
4. Sterile screw-cap polypropylene centrifuge tubes (round bottom 12 ml and skirted conical 50 ml tubes)
5. Magenta boxes (autoclaved)
6. Nylon mesh (100 µm grid, autoclaved)
7. Hemocytometer (type Rosenthal)
8. Filter paper disks (7 cm diameter, ash-free, autoclaved)
9. Sterile filter-stopped tips (1 ml) for standard pipettes

7.2.4 *Materials for Agrobacterium-Mediated Transformation*

1. Temperature-controlled incubators equipped with a rotary shaker
2. Spectrophotometer
3. Erlenmeyer flasks (100 ml) with chicane
4. Sterile screw-cap polypropylene cryotubes (1.5 ml)
5. Sterile screw-cap polycarbonate round-bottomed tubes (12 ml)

7.2.5 *Materials for the Analysis of Transgenic Plants*

7.2.5.1 **Ploidy Determination and Colchicine-Induced Whole Genome Duplication**

1. Flow cytometer, e.g., Ploidy Analyser I (Partec, Münster, Germany)
2. Nylon-mesh filters, e.g., CellTrics (30 µm mesh diameter, Partec)
3. Staining buffer, e.g., CyStain UV Ploidy Staining Solution, Partec)
4. Wire brush
5. Sterile screw-cap polypropylene centrifuge conical tubes (50 ml)
6. 0.1 % w/v colchicine, supplied with 3 drops of Tween 20 per 100 ml

7.2.5.2 **Molecular Analyses**

DNA Isolation, PCR, and DNA Gel Blot Analysis

1. Liquid nitrogen
2. Safe-lock tubes (1.5 and 2 ml)
3. Phenol (equilibrated, stabilized)/chloroform/isoamyl alcohol 25:24:1 (AppliChem, Darmstadt, Germany)
4. Isopropyl alcohol (≥99.8 %)
5. Ethanol (70 %)
6. Taq DNA polymerase with respective PCR buffer
7. MgCl₂ (25 mM)
8. Primers for amplification of TALEN constructs, target, and selectable marker genes:
 - (a) *FokI*-R (forward, FokI-F1 ATCGAGATCGCCCGGAACAGCACC; reverse: FokI-R1 ATCATCTCGCCGCCGATCAGGAGC)
 - (b) Discrimination of the left and right TALEN units (forward: ubi-F1 TTCCGCAGACGGGATCGATCTAGG; reverse: TALEN-R2 TGGCGGCTTGGCGCGTGACAG)
 - (c) Target gene *gfp* (forward: GH-GFP-R2 TACGGCAAGCTGACCCTGAA; reverse: GH-GFP-F1 GGTCACGAAGTCCAGCAGGA)

(d) Selectable marker gene *Bar* (forward: GH-Bar-F1
GGTCTGCACCATCGTCAACC; reverse: GH-Bar-R1
TACCGGCAGGCTGAAGTCCA)

9. QIAquick PCR purification kit (Qiagen, Hilden, Germany)
10. Cloning pGEM-T vector kit (Promega, Madison, WI)
11. Agarose gel (0.8 %), freshly prepared
12. Gel electrophoresis system (Bio-Rad, Munich, Germany)
13. Positively charged nylon membrane (Hybond N+; Roche, Mannheim, Germany)
14. DIG probe synthesis kit (Roche, Mannheim, Germany)
15. DIG system for filter hybridization (Roche, Mannheim, Germany)

RNA Isolation and Reverse Transcriptase Reaction

1. TRIzol reagent (Invitrogen, Life Technologies, Darmstadt, Germany)
2. DNase I treatment kit (DNA-free kit, Ambion, Life Technologies, Darmstadt, Germany)
3. iScript Select cDNA synthesis kit (Bio-Rad, Munich, Germany)
4. Oligo (dT)₂₀ primers (Bio-Rad, Munich, Germany)

7.3 Methods

7.3.1 Vector Construction and Bacterial Strains

1. Design the gene-specific TALEN sequence pair using software tool, *TAL Effector Nucleotide Targeter 2.0* (<https://tale-nt.cac.cornell.edu/>).
2. Evaluate the potential off-target cleavage sites in the barley genome (The International Barley Genome Sequencing Consortium, 2012) using the kmasker tool (<http://webblast.ipk-gatersleben.de/kmasker/>) with standard parameters.
3. Customized TALEN units can be purchased from Invitrogen (Paris, France) and delivered in standard vectors.
4. Clone the left and right TALEN units under the control of maize *ubiquitin 1* promoter (Christensen and Quail 1996) and the *A. tumefaciens nopaline synthase* terminator using *SpeI/HindIII* cloning sites into pUbi-ABM destination vector (DNA Cloning Service, Hamburg, Germany).
5. Synthetic sequences encoding the Simian Virus S40 (SV40) Nuclear Localization Signal (NLS) and NLS-HA (SV40 NLS with an added hemagglutinin tag) are digested with *AscI/Eco47III* and ligated such that the NLS becomes attached to the N-terminus of pUbi-TALEN-Left and NLS-HA to the N-terminus of pUbi-TALEN-Right.

6. Each TALEN expression cassette is then introduced between the *Sfi*I cloning sites of the binary vector p7d35S, which harbors *Bar*, a gene for bialaphos resistance, driven by the cauliflower mosaic virus doubled enhanced 35S (CaMV d35S) promoter.
7. Binary vectors carrying left and right TALEN expression units are then separately introduced via electroporation into *A. tumefaciens* strain LBA4404/pSB1, which carries the disarmed Ti plasmid pAL4404 and the hypervirulence-conferring vector pSB1. Finally, two strains of *A. tumefaciens* LBA4404/pSB1 are obtained, one carrying binary vector with left and the other binary vector with right TALEN expression units.

7.3.2 Growth of Donor Plants

The quality of the isolated pollen cultures is strongly dependent on the conditions of the donor plants. Therefore, it is of high importance to keep the optimized conditions as described:

1. Germinate cv. “Igri” grains in a tray filled with germination substrate in a chamber providing a 12 h photoperiod ($136 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) with temperature regime of 14/12 °C for light/dark phases for 2 weeks.
2. Vernalize the seedlings for 8 weeks at 4 °C under an 8 h photoperiod.
3. After transfer to 18 cm diameter pots, fertilize the plants by providing 15 g Osmocote, and then return to the conditions described in step #1 under Sect. 7.3.2.
4. At the tiller elongation stage, transfer the pots to a glasshouse maintained at 18/16 °C with a minimum of 16 h photoperiod ($170 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density).

7.3.3 Isolation of Immature Pollen

7.3.3.1 Spike Pretreatment

Harvest spikes within the boots when the awns have just emerged from the sheath of the flag leaf, surface-sterilize boots by spraying with 70 % ethanol, remove the flag leaf sheath under aseptic conditions, and place the dissected spikes on a 10 cm diameter Petri dish (five spikes per dish) containing two moistened, 7 cm filter paper disks. After sealing, the plates are held at 4 °C for 4–5 weeks in the dark.

7.3.3.2 Isolation, Purification, and Pre-cultivation of Immature Pollen

All materials coming into contact with the explant need to be sterile and precooled to 4 °C, and all solutions should be kept on ice during the pollen isolation and

purification procedures. Liquid transfer is most effectively carried out using a battery-operated pipette with filter-stopped 5 or 10 ml pipette tips.

1. Ten to fifteen pretreated spikes are chopped into ca. 1 cm fragments and macerated in a blender in the presence of 20 ml 0.4 M mannitol. Usually, two bursts of 15 s each are performed in a Waring blender set on “low” speed.
2. The macerate is filtered through a 100 μ m mesh, which is placed on top of a Magenta box. The blender is flushed with 10 ml of 0.4 M mannitol, which is also passed through the mesh.
3. The debris remaining on the mesh is squeezed gently with tweezers, then returned to the blender for re-maceration (twice at 10 s) in another 10 ml of 0.4 M mannitol, and the macerate passed through the mesh, which is followed by flushing the blender again with 10 ml of 0.4 M mannitol. The latter is also passed through the mesh.
4. The material filtered through the mesh and collected in the Magenta box is transferred to the 50 ml centrifuge tube, and the Magenta box flushed with 5 ml 0.4 M mannitol, which is also added to the same tube. The suspension is centrifuged ($100 \times g$, 10 min, 4 °C).
5. The pellet is resuspended in 3 ml 0.55 M maltose in a round-bottomed 12 ml tube. The centrifuge tube is flushed with 2 ml 0.4 M mannitol, which is poured carefully over the top of the 0.55 M maltose suspension, thereby forming two distinct liquid layers.
6. The tube containing these two layers is subjected to density gradient centrifugation in swing-out baskets ($100 \times g$, 10 min, 4 °C) with the centrifuge set to give slow acceleration and deceleration so that the two layers do not become mixed. After the centrifugation step, the immature pollen is accumulated in the interphase. The interphase is withdrawn by pipetting, transferred to a fresh 50 ml tube to which 10 ml 0.4 M mannitol is added, and resuspended with gentle shaking. 100 μ L aliquot of the material is placed into a hemocytometer cell to estimate the population density. Meanwhile, the remaining microspores are pelleted by centrifugation ($100 \times g$, 10 min, 4 °C). Before the supernatant is withdrawn, the tube is left at stand for ca. 5 min to allow still-floating pollen to settle down.
7. Aliquots of 1 ml pollen suspension are transferred into 3.5 cm Petri dishes. The dishes are sealed with Parafilm or Nescofilm and held in the dark at 25 °C for 7–8 days prior to cocultivation with *A. tumefaciens*.

7.3.4 Agrobacterium-Mediated Gene Transfer to Embryogenic Pollen

7.3.4.1 Preparation of *A. tumefaciens* Stocks

1. As TALENs function as pairs, the left and right TALEN units are subcloned in separate binary vectors, from which two respective clones of *A. tumefaciens*

- strain LBA4404/pSB1 are produced. After electroporation with the selected binary vector, *Agrobacterium* is streaked out onto solidified CPY medium supplemented with 0.5 ml/L of tetracycline stock and 2.5 ml/L of spectinomycin stock and incubated at 28 °C for 48 h (see **Note 2**). Single colonies are sampled using a sterile toothpick and grown overnight with shaking at 180 rpm (28 °C) in a tube containing 3 ml liquid CPY supplemented with the respective antibiotics.
2. Plasmid DNA is isolated and the integrity of the binary vector is confirmed by restriction digest of plasmid DNA.
 3. A fresh culture is initiated by adding 20 µL overnight culture to 5 ml fresh CPY medium containing antibiotics and incubating for a further 24 h as described above until an OD₅₅₀ of approximately 2 has been reached.
 4. Positive clones are grown in 10 ml liquid CPY containing antibiotics for another 24 h. The resulting culture is split into two 12 ml round-bottomed tubes and centrifuged (3.220 × g, 12 min, at room temperature).
 5. The pellets are vortexed each in 2.5 ml fresh CPY without antibiotics, and the suspensions are unified in one of the tubes. The cell density is adjusted by the addition of an appropriate volume of medium.
 6. The cells are then mixed in an equal volume of 15 % w/v autoclaved glycerol, and 0.5 ml aliquots are transferred into 1.5 ml cryotubes, left for 1 h at room temperature, and then stored at –80 °C.

7.3.4.2 Cocultivation of Embryogenic Pollen Cultures and *A. tumefaciens*

1. A 0.5 ml glycerol stock of a strain LBA4404/pSB1 carrying an appropriate binary vector is added to 10 ml CPY medium containing 2.5 µL spectinomycin stock and incubated at 28 °C for 24 h with shaking (180 rpm).
2. The liquid cultures of each bacterial strain are split into two 12 ml round-bottomed tubes, which are centrifuged (3220 × g, 12 min, room temperature). The pellets are vortexed each in 2.5 ml CK medium and then transferred to a 100 ml Erlenmeyer flask, which is held at 28 °C for 1–3 h with shaking (100 rpm).
3. The KBP medium used to pre-cultivate the pollen is withdrawn through a 1 ml pipette tip. This procedure is done with particular care to avoid taking up any embryogenic pollen. Then, 1 ml of CK medium is added. It is important to minimize the time between the removal of the KBP medium and the addition of the CK medium, because microspores are rapidly damaged if they are dried.
4. One out of every ten dishes containing embryogenic pollen is left untouched to later provide a supply of feeder cells to support embryogenic development of the pollen cocultured with *A. tumefaciens*.
5. The *A. tumefaciens* cultures are diluted tenfold in water and the measured OD₅₅₀ used to determine the concentration of cfu present.
6. Each 1 ml of embryogenic pollen culture is challenged with 2.5×10^7 cfu. Immediately prior to the inoculation, the two *Agrobacterium* clones (one

carrying the left and the other the right TALEN unit) are mixed in a 1:1 ratio to obtain a final cell density of 2.5×10^7 per ml.

7.3.5 *Regeneration of Transgenics*

1. After 48 h of cocultivation, the CK medium is withdrawn as described above; the pollen is rinsed in 0.4 ml ASt medium, taking care to remove the bacterial mucilage from the wall and bottom of the dish with a pipette tip (*see Note 3*). The medium is then replaced with a fresh aliquot of 1.1 ml ASt, to which 100 μ L of non-cocultivated embryogenic pollen is added as a feeder. The sealed dishes are held in the dark at 25 °C for 1 week with gentle shaking (75 rpm).
2. The ASt medium is replaced by 1.1 ml S3 medium (taking up any embryogenic pollen should be avoided); no washing step is required. The sealed dishes are held in the dark at 25 °C for 1 week with shaking (65 rpm), after which the used S3 medium is withdrawn and refreshed by a new aliquot of the same medium and the dishes incubated for another week.
3. Two weeks after cocultivation, the formed microcalli are transferred to an ash-free filter disk placed over solid KBP4PT medium in a 10 cm Petri dish. After sealing, these dishes are held in the dark at 25 °C for 2 weeks.
4. Calli that reached more than 1 mm in diameter can be transferred into a 10 cm Petri plate containing K4NBT medium, while those which have not yet reached this size can be kept for another week on KBP4PT before being transferred to regeneration medium. The sealed K4NBT plates are held in the dark at 25 °C for 1 week and then transferred into the light.
5. After 3 weeks, the calli and any emerging regenerants are transferred to tissue culture boxes containing K4NBT and subcultured twice at 3 week intervals.
6. Regenerated plants are transferred to 6 cm diameter pots filled with soil substrate (Klasmann Petuniensubstrat) and placed in a tray covered by a transparent plastic hood to maintain a high-humidity environment. The tray is held in a chamber providing a 12 h photoperiod ($136 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) and a temperature regime of 14/12 °C for light/dark phases, respectively.
7. After 1 week, the hood is removed and the tray left uncovered for a further week. The plantlets are then vernalized for 8 weeks at 4 °C under an 8 h photoperiod.
8. After vernalization, the plants are grown under the conditions described for the donor plants.

7.3.6 Analysis of Putative Transgenic Plants

7.3.6.1 Ploidy Determination and Colchicine-Induced Whole Genome Duplication

1. During the vernalization period, flow cytometry of leaf tissue is used to determine the ploidy level.
2. For the isolation of nuclei, a leaf sample is supplied with ice-cold staining buffer (CyStain UV Ploidy Staining Solution, Partec, Münster, Germany), and the leaf tissue is disintegrated gently with a wire brush.
3. The resulting suspension is then filtered through a nylon-mesh filter (CellTrics, 30 µm mesh diameter, Partec).
4. The filtered cell suspensions of the regenerants and of controls from seed-derived wild type plantlets are analyzed by a Ploidy Analyser PA I (Partec) following the manufacturer's instructions.
5. Haploids identified are exposed to a colchicine treatment after the vernalization period as follows: the soil is washed from the roots, and the tillers and roots are trimmed to a length of about 5 cm and 3 cm, respectively. Each plant is placed in a 50 ml polypropylene conical tube containing 0.1 % w/v colchicine solution. The level of the liquid is adjusted to reach the base of the shoots. The tubes are capped and then held in the light for 6 h at 21 °C.
6. The plants are removed from the colchicine, and the roots carefully rinsed in water before being transferred into a 9 cm diameter pot filled with Petuniensubstrat. The pots are placed in a tray with a plastic hood as described above.
7. More than 80 % of plantlets survive the colchicine treatment, and out of the survivors over 90 % produce grain, which indicates that diploidization of germ line cells has taken place (*see Note 4*).

7.3.6.2 Molecular Analyses

DNA Isolation, PCR, and DNA Gel Blot Analysis

1. A 200–400 mg sample of fresh leaf tissue is snap-frozen in liquid nitrogen.
2. DNA is isolated using the method of Palotta et al. (2000) (*see Note 5*).
3. PCR assays of chosen genes are performed using 50–100 ng genomic DNA as template. In this particular example, four different PCR reactions for the following templates are performed: *FokI*-R nuclease domain, left and right TALEN units giving amplicons of different sizes allowing for differentiation, selectable marker gene (*Bar*), and target *gfp* gene. All PCRs are performed in reaction volume of 20 µl with the exception of *gfp* PCR assay, where reaction volume was set to 40 µl.
4. The *gfp* amplicons are subjected to purification and sequencing. Purification is performed using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany)

according to the manufacturer's instructions. Sequencing chromatograms are analyzed and aligned to the wild type sequence to see if there are differences (indels/mutations) in the targeted region.

5. The PCR products of the transgenic individuals showing chromatograms with multiple peaks after the TALEN target site, which might indicate that multiple mutated *gfp* alleles are present among the amplicons, are cloned into pGEM-T. After blue-white selection, plasmid DNA is isolated from the positive clones and sequenced.
6. In order to determine the DNA copy number of the integrated transgene, DNA gel blot is used. For that purpose, 25 µg aliquot of genomic DNA is digested overnight with suitable restriction enzymes (*see Note 6*). In case of the *gfp*-specific TALEN constructs, either *HindIII* or *SspI* is used. Digested DNA is electrophoresed through a 0.8 % agarose gel and transferred onto a positively charged nylon membrane.
7. The nylon membrane is then hybridized with a DIG-labeled probe (*gfp*, *FokI-R*, or *Bar*) generated from a PCR using a DIG-probe synthesis kit and the relevant primer pairs. Signal is then visualized using a DIG System for Filter Hybridization according to the manufacturer's instructions.

RNA Isolation and Reverse Transcriptase Reaction

In order to check if the integrated DNA copies are also transcribed, RNA is isolated from positive transgenic plants and reverse transcription is performed using *FokI-R* primers (*see Note 7*).

1. Leaf material is snap-frozen, ground by means of a Retsch mill, and 100 g powdered leaf material is used for total RNA isolation using TRIzol following the manufacturer's protocol.
2. The isolated total RNA is treated with DNase I, and then the cDNA first strand is synthesized using an iScript Select cDNA synthesis kit in the presence of oligo (dT)₂₀ primers. The resulting cDNA is used as template for subsequent PCRs based on the *FokI-R* domain specific primer pair (*see Note 8*).

7.4 Notes

1. A separate stock for each pH value (pH 5.0, pH 5.5, and pH 5.9) is required.
2. The disarmed Ti-plasmid pAL4404 present in LBA4404 and also the binary vectors used harbor the same spectinomycin resistance (*aadA*) gene. Consequently, a higher than standard concentration of spectinomycin is required (250 mg/L) to ensure the maintenance of the binary vectors. Tetracycline is used here in addition to select for *Agrobacterium* cells that carry the pSB1 plasmid which renders this LBA4404 derivative hypervirulent.

3. The bacterial mucilage after removal from the wall and bottom of the dish requires to be left inside the dish, as it contains much of the embryogenic pollen.
4. As haploid plants are sterile, only the plants that underwent genome doubling set grains.
5. For molecular analyses (DNA and RNA isolation, PCR) of the transgenics, it is recommended to use filter tips because false positives are often detected due to cross contamination of samples, particularly because of the frequent use of standard marker and/or selection genes.
6. The restriction enzyme(s) used to digest the genomic DNA for gel blot analyses should cut the DNA within the inserted T-DNA but should not cleave within the gene that is used for the hybridization. In addition, the enzyme should frequently cut the genomic DNA of the investigated species, as fragments larger than 10 kb can be difficult to detect.
7. While working with RNA, it is extremely important to keep all equipment and materials RNase-free. Use freshly autoclaved equipment and DEPC-treated solutions.
8. DNase I treatment, cDNA first-strand synthesis, and follow-up PCR are performed according to manufacturer's instructions provided with the kit.

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Chapter 8

Doubled Haploidy as a Tool for Chimaera Dissolution of TALEN-Induced Mutations in Barley

Maia Gurushidze, Hannes Trautwein, Petra Hoffmeister, Ingrid Otto,
Andrea Müller, and Jochen Kumlehn

Abstract Site-specific genome engineering is a breakthrough technology that facilitates the functional validation of genes and offers versatile novel opportunities of crop improvement. In the approach described in this chapter, the two units of transcription activator-like effector nucleases (TALENs) required for site-directed cleavage activity are separately used to produce homozygous transgenic lines, each carrying only one unit of the TALEN pair. Crossings of these plants result in pairwise combination of both TALEN units, which causes their activation during early zygotic embryogenesis in the hybrid caryopses and facilitates site-directed mutagenesis with unprecedented efficiency in a Triticeae species. As revealed by sequencing of target-specific PCR amplicons, the individual primary mutants are not only heterozygous for the target gene but also chimaeric typically harbouring multiple mutant alleles. We demonstrate the highly efficient production of homozygous mutant lines from the chimaeric primary mutants using haploid microspores as embryogenic founder cells for the generation of doubled haploid plants.

Keywords Customizable endonucleases • Genome engineering • Haploid technology • Pollen embryogenesis • Site-directed mutagenesis

M. Gurushidze • H. Trautwein • P. Hoffmeister • I. Otto • A. Müller • J. Kumlehn (✉)
Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research
(IPK), Corrensstrasse 3, 06466 Seeland/OT, Gatersleben, Germany
e-mail: kumlehn@ipk-gatersleben.de

8.1 Introduction

8.1.1 *Generation of Primary Mutants by Cross-Combination of Parental Lines Carrying Complementary Single TALEN Units*

Transcription activator-like effector nucleases (TALENs) represent a universal tool for site-directed mutagenesis as they are capable of inducing double-strand breaks (DSBs) at user-defined positions in the genome. The processing of a resulting DSB by the host cell's DNA repair machinery can lead to nucleotide deletions or insertions (indels) at the breakage site. TALENs are fusion proteins between a customizable, target-specific DNA binding domain (Moscou and Bogdanove 2009) and the nucleolytic domain of FokI endonuclease, with the former directing the nuclease to the genomic site of choice, while the latter cleaves the double-strand DNA (Christian et al. 2010). However, FokI exerts its activity only in a dimeric format, which is achieved by introducing a pair of TALEN units binding in appropriate distance and orientation to the two strands of the DNA (Bitinaite et al. 1998). Conventionally, the expression cassettes of left and right TALEN units are cloned into a single T-DNA used for plant transformation. As an alternative, two separate T-DNAs can be used in co-transformation approaches.

In barley, immature embryos or embryogenic pollen cultures have been used as recipient material for *Agrobacterium*-mediated transfer of TALEN-coding expression units, and both methods have resulted in moderate efficiency of site-specific mutagenesis (Wendt et al. 2013; Gurushidze et al. 2014). However, somatic mutations occurring in the primary transformants are not necessarily inherited to the next generation (Wendt et al. 2014). The advantage of the approach described in this chapter is that it induces mutations in cells whose genetic make-up is particularly likely to be transmitted to the generative tissues including the female and male gametophytes (i.e. embryo sac and pollen, respectively) of a given plant. To this end, the two TALEN units required for site-directed cleavage activity at the model *gfp* gene were separately used to produce homozygous transgenics, each carrying and expressing only one of these units. Pairs of resultant transgenics that express complementary TALEN units at homozygous state were used for reciprocal crosses. An additional advantage of the approach is that it allows validating the expression of the individual TALEN units in the plants before crossing. The combination of both complementary TALEN units within a hybrid plant results in TALEN activation during early zygotic embryogenesis, which is associated with extremely high efficiency of targeted mutagenesis in cells that genetically contribute to the germ line towards the next generation. As shown by sequencing of the cloned target-specific PCR amplicons, multiple mutant *gfp* alleles were present in each of the analysed seedlings, indicating unprecedented efficiency in site-directed mutagenesis in a Triticeae species. Thus, in the presented approach, there are only two successful transformation events necessary to generate the two lines carrying complementary single TALEN units. Once they are established in homozygous

state, no further transformation is necessary to generate mutants. A huge number of independent mutants can be generated by crossing these pre-validated single TALEN unit lines, which is a particularly valuable option, especially in plants that are not readily transformable.

8.1.2 Chimaerism Upon TALEN-Induced Targeted Mutagenesis

As mentioned above, every analysed individual of the hybrid offspring contained multiple mutant *gfp* sequences, i.e. produced TALEN mutants are chimaeric regarding the induced mutations. Virtually, the TALEN pair expressed in each cell could initiate independent mutation events resulting in extremely high diversity of the induced sequence modifications within one individual. Indeed, by sequencing up to 65 individual clones of the PCR amplicons derived from the target gene (*gfp*), as many as 61 different mutations were detected per hybrid seedling. Although chimaerism is a quite often occurring phenomenon in targeted mutagenesis, the proportion of heritable mutations strongly depends on the method. While none of the TALEN-induced mutations proved heritable among the seedlings of primary barley-mutant plants produced *via* immature embryo transformation (Wendt et al. 2014), multiple mutations were inherited within doubled haploid siblings using the method presented in this chapter (*see Note 1*).

8.1.3 Haploid Technology

Haploid technology is one of the most powerful biotechnological tools in plant breeding and functional genomic studies. The advantage of haploid plants is that homozygosity can be readily achieved at all loci *via* whole-genome duplication in just one generational step. Nowadays, haploid technology is also exploited to produce instantly homozygous mutants and transgenics (Kumlehn et al. 2006; Gurushidze et al. 2014).

Pollen embryogenesis is a phenomenon that allows generating haploid and doubled haploid plants from immature pollen. In this approach, each doubled haploid individual originates from a unique gametophytic cell, and accordingly, the resulting population of doubled haploid plants represents a collection of meiotically independently recombined but genetically fixed individuals. In this chapter, we describe the utilization of pollen embryogenesis for chimaera dissolution of TALEN-induced mutations in barley.

8.2 Materials

8.2.1 Growth of Parental Lines and Primary Mutant Plants

1. The parental lines used in crossing experiments carrying stably incorporated either left or right TALEN units represent winter-type cultivar 'Igri'. Parental lines were produced as described in the Chap. 7.
2. Grains of the parental lines were germinated on Substrate 2 (Klasmann, Germany), which is a 3:1:2 mixture of garden mulch, sand and white and black peats, respectively.
3. The soil substrate used after vernalization is a mixture of compost, Klasmann Substrate 2 and sand mixed at a ratio of 2:2:1. Osmocote (19 % N, 6 % P and 12 % K) was used as a fertilizer.
4. Petuniensubstrat (Klasmann, Germany) was used for the acclimation of in vitro regenerants to grow on soil substrate.

8.2.2 Stock Solutions and Culture Media

8.2.2.1 Solutions for Isolation, Purification and Induction of Embryogenic Development of Immature Pollen

Carbohydrates

Mannitol (0.4 M, 72.9 g/L): autoclaved and stored at 4 °C.

Maltose·H₂O (1 M, Sigma grade I, 360.3 g/L): filter sterilized and stored at room temperature.

Maltose·H₂O (1 M, standard quality, 360.3 g/L): filter sterilized and stored at room temperature.

Maltose·H₂O (0.55 M, standard quality, 198.2 g/L): filter sterilized and stored at 4 °C.

Mineral salts

NaFeEDTA (75 mM, 27.5 g/L): filter sterilized and stored at 4 °C

CaCl₂ (1 M, 147 g/L CaCl₂·2H₂O): filter sterilized and stored at 4 °C

CuSO₄·5H₂O (10 mM, 2.5 g/L): filter sterilized and stored at 4 °C

NH₄Cl (1 M, 53.5 g/L): filter sterilized and stored at 4 °C

K macro minerals (20×): 1.6 g/L NH₄NO₃, 40.4 g/L KNO₃, 6.8 g/L KH₂PO₄, 8.8 g/L CaCl₂·2H₂O and 4.9 g/L MgSO₄·7H₂O; filter sterilized and stored at 4 °C

K4 N macro minerals (20×): 6.4 g/L NH₄NO₃, 72.8 g/L KNO₃, 6.8 g/L KH₂PO₄, 8.8 g/L CaCl₂·2H₂O and 4.9 g/L MgSO₄·7H₂O; filter sterilized and stored at 4 °C

K micro minerals (1000×): 8.4 g/L MnSO₄·H₂O, 3.1 g/L H₃BO₃, 7.2 g/L ZnSO₄·7H₂O, 120 mg/L Na₂MoO₄·2H₂O, 25 mg/L CuSO₄·5H₂O, 24 mg/L CoCl₂·6H₂O and 170 mg/L KI; filter sterilized and stored at 4 °C

Organic supplements

KM organics (100×, Sigma K-3129): 2 mg/L *p*-aminobenzoic acid, 200 mg/L L-ascorbic acid, 1 mg/L D-biotin, 100 mg/L D-calcium pantothenate, 2 mg/L cyanocobalamin, 40 mg/L folic acid, 10 g/L myo-inositol, 100 mg/L nicotinamide, 100 mg/L pyridoxine-HCl, 1 mg/L retinol, 20 mg/L riboflavin and 100 mg/L thiamine-HCl; stored at -20°C .

Gamborg B5 organics (1000×): 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine-HCl and 10 mg/L thiamine-HCl; filter sterilized and stored at -20°C .

L-Glutamine (37 mg/ml): dissolved by adding a few drops of 0.1 M KOH and heating in a water bath, filter sterilized and stored at -20°C .

6-Benzylaminopurine (BAP, 1 mM): 225 mg/L dissolved in 30 ml hot H₂O and 3–5 drops 1 M NaOH, made up to the final volume, filter sterilized and stored at 4°C .

Morpholinoethanesulfonic acid monohydrate (MES, 1 M, 213.2 g/L, pH = 5.0, pH = 5.5 and pH = 5.9): dissolved in 40 ml H₂O, the pH adjusted with KOH (few pellets), stored at room temperature overnight, and the pH readjusted with either KOH or HCl, made up to the final volume, filter-sterilized and stored at 4°C .

Gelling agent

Phytigel (2×, 6 g/L): suspended in cold water of double-distilled quality (at best 1.5 g Phytigel per 250 ml unit), autoclaved and stored at room temperature.

8.2.2.2 Nutrient Media for Embryogenic Pollen Culture and Plant Regeneration

1. Starvation medium barley (SMB): 1 ml/L CaCl₂, 1 ml/L NH₄Cl, 2 ml/L MES pH 5.5 and 400 ml/L mannitol stocks; filter-sterilized and stored at 4°C .
2. Barley pollen culture (KBP) medium: 50 ml/L K macro, 1 ml/L K micro, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 12 ml/L L-glutamine, 4 ml/L BAP and 250 ml/L maltose (1 M, Sigma Grade I) stocks; pH adjusted to 5.9 and stored at 4°C .
3. Solid barley pollen culture (KBP4P) medium: for a final volume of 0.5 L KBP4P, appropriate amounts of 50 ml/L K macro minerals, 1 ml/L K micro minerals, 1 ml/L NaFeEDTA, 10 ml/L KM organics, 250 ml/L maltose (1 M, standard quality), 1 ml/L BAP and 12 ml/L L-glutamine stocks were mixed to an intermediate volume of 250 ml (doubled concentrated), adjusted to pH 5.9, heated to about 40°C and then mixed (1:1) with 250 ml Phytigel stock (melted by heating).
4. Regeneration (K4NB) medium: for a final volume of 0.5 L K4NB, appropriate amounts of 50 ml/L K4N macro minerals, 1 ml/L K micro minerals, 0.75 ml/L NaFeEDTA, 1 ml/L B5 organics, 4 ml/L L-glutamine, 100 ml/L maltose (1 M, standard quality), 1 ml/L BAP and 0.49 ml/L CuSO₄ stocks were mixed to an

intermediate volume of 250 ml (double concentrated), adjusted to pH 5.9, heated to about 40 °C and then mixed (1:1) with 250 ml Phytigel stock (melted by heating).

8.2.3 Materials for the Isolation of Embryogenic Pollen Culture and Plant Regeneration

1. Refrigerated centrifuge equipped with swing-out baskets.
2. Waring blender (heat sterilizable).
3. Sterile Petri dishes with lid (3.5 cm in diameter).
4. Sterile Petri dishes with lid (10 cm in diameter).
5. Divided Petri dishes with two sections and lid (10 cm in diameter).
6. Sterile screw cap polypropylene centrifuge tubes (round bottom 12 ml and skirted conical 50 ml tubes).
7. Magenta boxes (autoclaved).
8. Nylon mesh (100 µm grid, autoclaved).
9. Haemocytometer (type Rosenthal).
10. Filter paper discs (7 cm diameter, ash-free, autoclaved).
11. Sterile filter-stopped tips (1 ml) for standard pipettes.

8.2.4 Materials for the Molecular Analyses of Pollen-Derived Plants

1. Liquid nitrogen.
1. Safe-lock tubes (1.5 and 2 ml).
2. Phenol (equilibrated, stabilized) to chloroform to isoamylalcohol (25:24:1) (AppliChem, Darmstadt, Germany).
3. Isopropyl alcohol (≥99.8 %).
4. Ethanol (70 %).
5. Taq DNA polymerase with respective PCR buffer.
6. MgCl₂ (25 mM).
7. Primers for amplification of target gene *gfp* (forward: GH-GFP-R2 TACGGCAAGCTGACCCTGAA; reverse: GH-GFP-F1 GGTCACGAACTCCAGCAGGA).
8. QIAquick PCR purification kit (QIAGEN, Hilden, Germany).
9. Cloning pGEM-T vector kit (Promega, Madison, WI).
10. Agarose gel (0.8 %); freshly prepared.
11. Gel electrophoresis system (Bio-Rad, Munich, Germany).

8.2.5 *Materials for Ploidy Determination and Colchicine-Induced Whole-Genome Duplication*

1. Flow cytometer, e.g. Ploidy Analyser I (Partec, Münster, Germany).
2. Nylon mesh filters, e.g. CellTrics (30 μm mesh diameter, Partec).
3. Staining buffer, e.g. CyStain UV Ploidy staining solution (Partec).
4. Wire brush.
5. Sterile screw cap polypropylene centrifuge conical tubes (50 ml).
6. 0.1 % w/v colchicine, supplied with three drops of Tween per 100 ml.

8.3 Methods

8.3.1 *Growth of Primary Mutant Plants*

1. T1 grains of homozygous transgenic plants of cv. 'Igri' expressing single TALEN units were germinated (*see Note 2*) on germination substrate in a chamber providing 12 h photoperiod ($136 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) with temperature regime of 14/12 °C for light/dark phases for 2 weeks.
2. Vernalization of the seedlings was performed at 4 °C under an 8 h photoperiod for 8 weeks.
3. The seedlings were then transferred to 18 cm diameter pots, fertilized by providing 15 g Osmocote and grown under controlled conditions as described in **Step 1** of this section.

8.3.2 *Crossing of Pairs of Complementary Single TALEN Plants and Analysis of Hybrid Plants*

Left and right TALEN unit-carrying homozygous barley lines were grown under the conditions described above (Sect. 8.3.1). Crossing partners were chosen in such a way that the resulting hybrids possess both TALEN units. After reciprocal crossing of the selected TALEN carrying lines, the hybrid plants were analysed as follows: the target sequence (*gfp*) was amplified and cloned into pGEM-T vector. The DNA was isolated from various numbers of positive clones and sequenced to gain preliminary information about mutation frequencies.

8.3.3 *Spike Preparation*

Spikes were harvested when the awns had just emerged from the sheath of the flag leaf, boots were surface-sterilized by spraying with 70 % ethanol, and the flag leaf sheath was removed under aseptic conditions. Dissected spikes were placed in one section of a divided Petri dish (10 cm in diameter), whereas the other section was supplied with sterile water to provide saturated humidity in the dish's airspace. The dishes were sealed and placed at 4 °C for 2 weeks in the fridge.

8.3.4 *Isolation, Purification and Inductive Treatment of Immature Pollen*

1. Ten to fifteen pretreated spikes were chopped into ca. 1 cm pieces and macerated in a blender in the presence of 20 ml 0.4 M mannitol. Two bursts of 15 s each were performed in a Waring blender set on 'low' speed (*see Note 3*).
2. The macerate was filtered through a 100 µm mesh into a Magenta box. The blender was rinsed with 10 ml of 0.4 M mannitol, which was also passed through the mesh.
3. The debris remaining on the mesh was squeezed gently with tweezers transferred back to the blender and re-macerated twice for 10 s in another 10 ml of 0.4 M mannitol. The macerate was again filtered through the mesh, and the blender was rinsed again with 10 ml of 0.4 M mannitol. The latter was then also passed through the mesh into a Magenta box.
4. The material collected in the Magenta box was transferred to the 50 ml centrifuge tube, and the Magenta box flushed with 5 ml 0.4 M mannitol, which was also added to the same tube. The suspension was centrifuged ($100 \times g$, 10 min, 4 °C).
5. The supernatant was discarded, and the pellet was re-suspended in 3 ml 0.55 M maltose in a round-bottomed 12 ml tube. The centrifuge tube was flushed with 2 ml 0.4 M mannitol, which was poured carefully over the top of the 0.55 M maltose suspension, so that two distinct liquid layers were formed.
6. The tube containing these two layers was subjected to density gradient centrifugation in swing-out baskets ($100 \times g$, 10 min, 4 °C) (*see Note 4*). After the centrifugation step, the immature pollen accumulated in the interphase. The interphase was carefully withdrawn by pipetting, transferred to a fresh 50 ml tube to which 10 ml 0.4 M mannitol was added and resuspended with gentle shaking. 100 µL aliquot of the material was placed into a haemocytometer cell to estimate the population density. Meanwhile the remaining microspores were pelleted by centrifugation ($100 \times g$, 10 min, 4 °C). After centrifugation, the tube was left in the centrifuge for ca. 5 min before the supernatant was withdrawn to allow settling down of the floating pollen.

7. The pollen was resuspended in SMB with a density of 200,000 microspores/ml; aliquots of 1 ml pollen suspension were transferred into 3.5 cm Petri dishes. The dishes were sealed and held in the dark at 25 °C for 48 h to induce embryogenic competence.

8.3.5 *Regeneration of Pollen-Derived Plants*

1. After the inductive treatment, the SMB was withdrawn through a 1 ml pipette tip (see Note 5). The medium was then replaced with 1 ml KBP medium, and the sealed plates were further held at 25 °C in the dark for another 2 weeks.
2. Then, the formed embryogenic calli were transferred to an ash-free filter disc placed over solid KBP4P medium in a 10 cm Petri dish. After sealing, these dishes were held in the dark at 25 °C for 2 weeks.
3. Calli that reached more than 1 mm in diameter can be transferred into a 10 cm Petri plate containing K4NB medium, while those which have not yet reached this size can be kept for another week on KBP4P before being transferred to regeneration medium. The sealed K4NB plates were held in the dark at 25 °C for 1 week and then transferred into the light.
4. After 3 weeks, the calli and any emerging regenerants were transferred to tissue culture boxes containing K4NB and subcultured twice at 3-week intervals.
5. Regenerated plants with roots were transferred to 6 cm diameter pots filled with soil substrate (Klasmann Petuniensubstrat) and placed in a tray covered by a transparent plastic hood to maintain a high-humidity environment. The tray was held in a chamber providing a 12 h photoperiod ($136 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) and a temperature regime of 14/12 °C for light/dark phases, respectively.
6. After 2 weeks, the hood was removed and the tray left uncovered for a further week. The plantlets were then vernalized for 8 weeks at 4 °C under an 8 h photoperiod.
7. After vernalization, the plants were grown under the conditions described for the donor plants.

8.3.6 *Analysis of Pollen-Derived Plants*

8.3.6.1 Ploidy Determination

During the vernalization period, flow cytometry of leaf tissue was used to determine the ploidy level as follows:

1. For the isolation of nuclei, a leaf sample was supplied with ice-cold staining buffer (CyStain UV Ploidy staining solution, Partec, Münster, Germany), and the leaf tissue was disintegrated gently with a wire brush.

2. The resulting suspension was then filtered through a nylon mesh filter (CellTrics, 30 µm mesh diameter, Partec).
3. The filtered cell suspensions of the regenerants and of controls from caryopsis-derived wild-type seedlings were analysed by a Ploidy Analyser PA I (Partec) following the manufacturer's instructions.

8.3.6.2 DNA Isolation, PCR and Sequencing

1. A 200–400 mg sample of fresh leaf tissue was snap-frozen in liquid nitrogen.
2. DNA was isolated using the method of Pallotta et al. (2000) (see Note 6).
3. The target *gfp* gene was amplified by PCR reaction using 50–100 ng genomic DNA as template. PCRs were performed in reaction volume of 40 µl.
4. The *gfp* amplicons were subjected to purification and sequencing. Purification was performed using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.
5. The PCR products of the transgenic individuals showing chromatograms with multiple peaks in the TALEN target site, which indicated the presence of multiple *gfp* alleles among the amplicons, were cloned into pGEM-T vector. After blue-white selection, plasmid DNA was isolated from individual positive clones and sequenced.
6. Sequencing chromatograms were analysed and aligned to the wild-type sequence to see if there were differences (indels/mutations) in the targeted region.

8.3.6.3 Mutant Comparison

The target *gfp* gene of haploids and doubled haploids, derived from hybrid plants' pollen cultures, was amplified and sequenced. *gfp* was amplified and individual clones sequenced also from the pollen donor hybrid plants (see Note 7). The number and types of the mutations carried by doubled haploid mutants were recorded and compared with the mutations present within the parental (pollen donor) chimaeric hybrid plants. For example, 58 clones were sequenced for the pollen donor plant #39/4, and all the 58 sequences proved to be mutated (Fig. 8.1). From this donor, 10 doubled haploids were then produced. Whereas in three of those, it was not possible to amplify *gfp* (see Note 8), amplification and sequencing were successful in the remaining seven DH plants, five of which carried mutant sequences, and all of these five mutations proved to be unique (Fig. 8.2) (see Note 9). As expected, doubled haploid mutants possessed their mutated allele in homozygous state.

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GFP-WT      CACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-1   CACCCCTGGTGAACCGCATCGAGCTGAAGG----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-2   CACCCCTGGTGAACCGCATCGAGCTGAAGG----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-3   CACCCCTGGTGAACCGCATCGAGCTGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-4   CACCCCTGGTGAACCGCATCGAGCTGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-5   CACCCCTGGTGAACCGCATCGAGCTGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-6   CACCCCTGGTGAACCGCATCGAGCTGAAG-----CITCAAGGAGGACGGCAACATCCTGGGGCACAGCTGGAGT
39/4_c1-7   CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-8   CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-9   CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-10  CACCCCTGG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-11  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----CACAAAGCTGGAGT
39/4_c1-12  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-13  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-14  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-15  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-16  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-17  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-18  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-19  CACCCCTGGTGAACCGCATCGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-20  CACCCCTGGTGAACCGCATCGA-----CITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-21  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-22  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-23  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-24  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-25  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-26  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-27  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-28  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-29  CACCCCTGGTGAACCGCATCGAGCTGAAG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-30  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-31  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-32  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-33  CACCCCTGG-----CGCACCATCTTCTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-34  CACCCCTGG-----CGCACCATCTTCTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-35  CACCCCTGG-----CGCACCATCTTCTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-36  CACCCCTGGTGAACCGCATCGA-----CGTGTGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-37  CACCCCTGGTGAACCGCATCGAGCTGAAG-----CGTGTGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-38  CACCCCTGGTGAACCGCATCGAGCTGAAGG---ACITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-39  CACCCCTGGTGAACCGCATCGAGCTGAAGG---ACITCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-40  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----ACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-41  CACCCCTGGTGAACCGCATCG-----GCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-42  CACCCCTGGTGAACCGCATCG-----AGCTGAAGGGCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-43  CACCCCTGGTGAACCGCATCGAGCTGAAGGGC--GACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-44  CACCCCTGGTGAACCGCATCGAGCTGAAGGGC--GACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-45  CACCCCTGGTGAACCG-----GAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-46  CACCCCTGGTGAACCGCATCGAGCTGAAGGGC--AACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-47  CACCCCTGG-----GGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-48  C-----TTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-49  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----ACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-50  CACCCCTGGTGAACCGCATCGAGCTG-----CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-51  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-52  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-53  CACCCCTGGTGAACCGCATCGAGCTGAAGG-----AACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-54  CACCCCTGGTGAACCGCATCGAGCTGAAGG---TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT
39/4_c1-55  CCGCCTGCAAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGGATTTCTATAGTGTACACTAA
39/4_c1-56  CCGCCTGCAAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGGATTTCTATAGTGTACACTAA
39/4_c1-57  CACCCCTGGTGAAGTTTGAGGCGACACCTGGTGAACCGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAA
39/4_c1-58  CACCCCTGGTGAACCGCATCAAGTCCGCCATGCCGAAGCTACGTCAGGAGCGCCACCATCTTCTTCAAGGAGGACGG
    
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Fig. 8.1 Alignment of the various cloned *gfp* mutant sequences of the hybrid plant #39/4. The underlined sequences represent TALEN-binding sites in wild-type *gfp*. Deletions are indicated with *dashes*. Note that long insertions in addition to deletions are present in the last four sequences

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GFP-WT      CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   WT
39/4_DH-1   CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   WT
39/4_DH-2   CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   WT
39/4_DH-3   CTGGTGAACCGCATCGAGCTGAAGG-----AGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -15
39/4_DH-4   CTGGTGAA-----CTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -24
39/4_DH-5   CTGGTGAACCGCATCGA-----CTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -15
39/4_DH-6   CTGGTGAACCGCATCGAGCTGAAGG-----CTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGTA   -109
39/4_DH-7   CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAAGCTGGAGT   +1

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Fig. 8.2 *gfp* alleles found in the pollen-derived homozygous doubled haploids produced from the hybrid plant #39/4 are aligned with the wild type *gfp* sequence

8.4 Notes

- Five distinct mutations were inherited among 10 doubled haploid siblings in the example presented in this chapter.
- Cultivar ‘Igrı’ lines expressing either the left or right TALEN units were produced by transformation of immature pollen cultures using the method described in Chap. 7. For more details about the TALEN vectors, see Gurushidze et al. (2014).
- All materials coming into contact with the explant need to be sterile and pre-cooled to 4 °C, and all solutions should be kept on ice during the pollen isolation and purification procedure. Liquid transfer is most effectively carried out using a battery-operated pipette with filter-stopped 5 or 10 ml tips.
- At this step, it is critical that the two layers do not become mixed. For that reason, the centrifugation step is performed with the acceleration and deceleration forces of the centrifuge being reduced to 30 %.
- The exchange of SMB to KBP medium is to be done with particular care to avoid taking up embryogenic pollen. To this end, the pipette should be slightly pressed with its tip to the bottom of the dish, so as to control the uptake of medium, while the cultivated pollen is collecting around the tip without being taken up.
- For molecular analyses (DNA isolation, PCR) of the transgenics, it is recommended to use filter tips because false positives are often detected due to cross-contamination of samples, particularly because of the frequent use of standard marker and/or selection genes.
- Ten to 65 clones were analysed per hybrid plant. Mutant alleles represented 62.5 to 100 % of the individually cloned sequences.
- Large deletions including at least one primer-binding site might be the most plausible reason for failed amplification.
- Among the five different mutations found in pollen-derived 10 doubled haploid siblings, 2 were already detected among the 58 mutations of their hybrid mother plant (39/4), while 3 mutations were ‘new’, i.e. not present among the 58 previously sequenced clones.

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Part III
Phenotypic Screening

Chapter 9

Field Evaluation of Mutagenized Rice

Material

Sydney D. Johnson, Dennis R. Taylor, Thomas H. Tai,
Joanna Jankowicz-Cieslak, Bradley J. Till, and Alpha B. Jalloh

Abstract Since the discoveries of the beneficial effects of some mutations on plants, scientists have made use of both physical and chemical mutagens to develop new varieties of crops and ornamentals. More than 3200 varieties have been produced through mutation induction, and these have contributed towards improving food security in many countries.

Proper field techniques can greatly enhance the chances of identifying desirable phenotypes. The protocol described here deals mainly with field techniques successfully employed and developed in the evaluation of rice mutants for over 8 years in the uplands and the hydromorphic lowlands at the Rokupr Agricultural Research Centre (RARC) of the Sierra Leone Agricultural Research Institute (SLARI) between 2005 and 2012 to evaluate gamma and X-ray-treated rice seeds.

Keywords Mutation breeding • Field observation • Bulk selection • Single panicle selection • Observational yield trials • Replicated yield trials • Generation of mutant population

9.1 Introduction

Since the discoveries by Muller and Stadler, plant scientists have made use of the effects of induced mutations to develop new varieties of crops and ornamentals using both physical and chemical mutagens (Muller 1927; Stadler 1928). More than

S.D. Johnson (✉) • D.R. Taylor • A.B. Jalloh
Rokupr Agricultural Research Centre (RARC), Sierra Leone Agricultural Research Institute (SLARI), PMB, 1313 Freetown, Sierra Leone
e-mail: dysorosyd4953@yahoo.com

T.H. Tai
Crops Pathology and Genetics Research Unit, USDA-ARS, Davis, CA 95616, USA
Department of Plant Sciences, University of California, Davis, CA 95616, USA

J. Jankowicz-Cieslak • B.J. Till
Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria

3200 new varieties have been produced through mutation induction, and these have contributed towards improving food security in many countries (MVD 2016; Jankowicz-Cieslak and Till 2015). Breeding was, and is still, a game of numbers where phenotypes play a central role, whether routine hybridization is involved or reverse genetics such as Targeting Induced Local Lesions IN Genomes (TILLING) is contemplated (Kurowska et al. 2011). The end result of a high-throughput approach or simple hybridization is to produce desirable phenotypes that meet the demands of end users.

When employed properly, field techniques can greatly enhance the chances of identifying desirable phenotypes and therefore should be well planned before starting. The use of radiation techniques to produce mutants is increasingly becoming important in the development of high yielding, disease tolerant crop varieties as well as ornamentals (Ahloowalia et al. 2004).

Whilst the literature on mutation breeding dealing directly with a methodology for field evaluation of mutants is scarce (El-Degwy 2013), field evaluation of mutants is routinely conducted in many research institutes around the world to identify mutants with desirable traits for incorporation into existing varieties to improve yields and confer resistance or tolerance to new threats of diseases or pests in crop varieties as well as flowers and ornamentals. Evaluation of mutant phenotypes is also increasingly employed in reverse-genetic approaches such as TILLING (Kurowska et al. 2011).

The protocol presented in this chapter deals mainly with field techniques successfully used for the evaluation of rice mutants over a 5-year period in the uplands and the hydromorphic lowlands at RARC, one of seven SLARI centres in Sierra Leone. Field evaluations are especially important when screening mutant populations. This is because the frequency of induced mutations, whilst orders of magnitude higher than natural mutations, is still relatively low. The most densely mutagenized populations have been produced with chemical mutagens. For example, a mutation density of $\sim 1/280$ kb in rice has been reported (Till et al. 2007; Tsai et al. 2011). Physical mutagens such as gamma irradiation have been shown to produce large genomic indels with only a few such lesions reported per line (Yuan et al. 2014; Henry et al. 2015). This means that the chance of mutating a gene that would alter the desired trait is quite low, and thousands of plants need to be routinely screened for a chance of success. Field evaluations should accommodate large numbers and ensure that rare phenotypic variations can be easily spotted. Once phenotypes are detected, efforts can be made to develop molecular markers, thus reducing the workload for producing new varieties.

Prior to field evaluation, care should be taken for the development of a suitable mutant population (Fig. 9.1). Homogeneous seeds are selected and tested for viability and treated with the appropriate mutagen. Mutagenesis procedures and important considerations are described in Chaps. 2, 3, 4 and 6 of this book. Importantly, the first generation after irradiation of seed (called the M_1) is chimeric and not suitable for phenotyping. In rice and other sexually propagated species, meiotic propagation removes chimeras (*see* Chap. 1). Phenotypic selection can therefore begin in the second (M_2) generation. When M_1 plants are grown, a choice

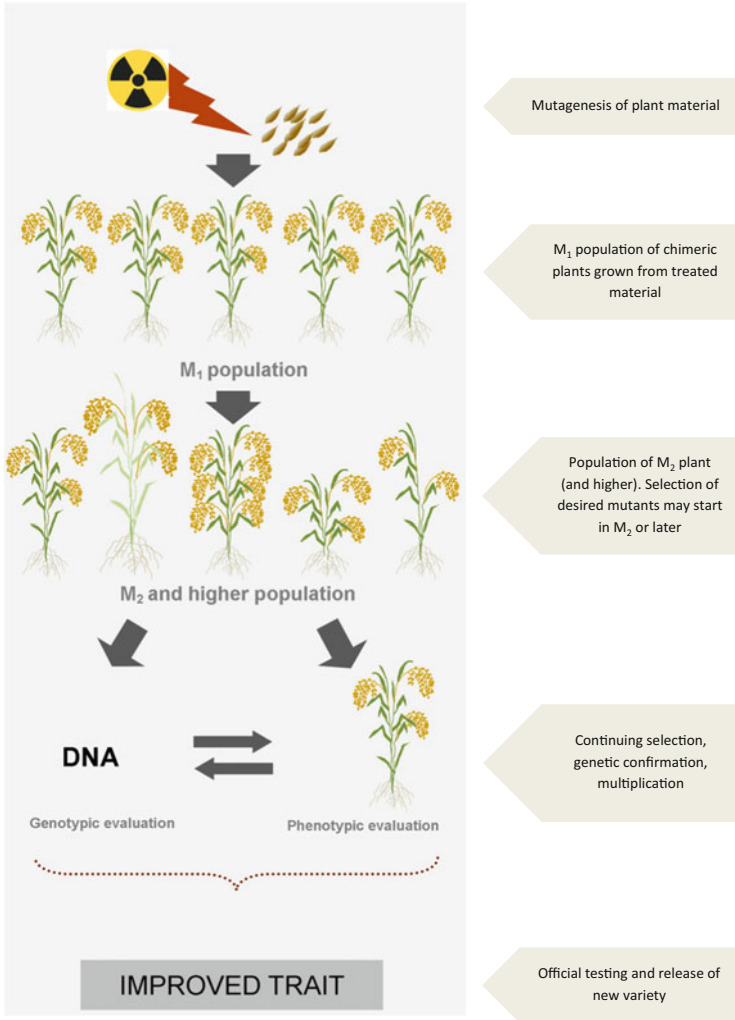


Fig. 9.1 An overview of the production and screening of mutant crop populations. Mutations are induced in seed or other plant materials. The first generation after mutagenesis is termed the M₁. When multicellular tissues like seed are mutagenized, the resulting plants are chimeric with different cells harbouring different mutations. Chimeric material is not suitable for screening. Plants are propagated and screening methods can be applied to the first non-chimeric generation (the M₂) in seed mutagenized material. Phenotypic and/or genotypic selections are applied to identify plants with desired improved characteristics. Further propagation and validation of traits are usually performed to make pure-breeding material. This material is tested prior to release as a new variety

must be made regarding organization of the mutant population. The two main options are between a bulk or pedigree population structure. Bulk approaches, as the name implies, involve collecting all seeds from the M_1 together and sowing M_2 progeny together. Here it is impossible to trace back an observed trait to a specific founding mutant plant. The advantage is that it requires little work in organizing and tracking seed. The disadvantage is that it is difficult to estimate mutation densities, and efforts must be made to determine if two plants showing similar phenotype are allelic. Pedigree approaches track the lineage as early as the M_1 . Information is collected and each generation and the genetic relationship of each seed can be tracked. This approach is especially important in TILLING as described in Chap. 18. The disadvantage is that it is very labour intensive. In addition, a computerized system and database for seed organization and tracking is advised when handling large populations. The advantage is that genetic relationship of plants with similar phenotypes is known, and precise estimations on mutation density can be calculated. The choice of strategy depends on the research objective, and the two need not be mutually exclusive as lines can be developed in later generations through single plant selections from bulk populations. The protocol below describes this combined approach.

9.2 Materials

9.2.1 Plot Design

1. Mutagenized rice seeds (*see Note 1*).

9.2.2 Field Preparation and Planting

1. Heavy equipment for land preparation.

9.2.3 Rice Culture

1. Equipment for weeding.
2. Herbicides.
3. Fertilizers.

9.2.4 Field Observations and Trait Evaluation

1. Equipment for evaluating traits of interest.
2. Data collection equipment (e.g. laptop or tablet computer, tape recorder).
3. Field equipment (land preparation and planting).
4. Field maintenance supplies (e.g. fertilizers, herbicides).
5. Equipment for evaluations (e.g. data recorders, specific instruments for phenotyping particular traits if needed).
6. Equipment and supplies for harvesting and processing seeds (e.g. threshers, drying equipment, storage supplies like envelopes).

9.3 Methods

9.3.1 Plot Design

1. Conduct bulk irradiation of seeds at desired dosages or obtain mutagenized seeds from a service lab (*see* **Notes 1** and **2**).
2. Establish plot layout. Choose the number of varieties and treatment dosages to be evaluated based on available field space and labour (*see* **Note 3** and **Fig. 9.2**).

9.3.2 Field Preparation and Planting

1. Prepare field for planting using standard practices for your region (*see* **Note 4**).
2. Sow seeds in rows with and intra-row spacing of 20 cm between seeds (single seed per hill) and an interrow spacing of 20 cm (*see* **Fig. 9.1** and **Note 6**).

9.3.3 Rice Culture

1. Field management. As with other field crop species, the two major management issues during rice culture are weed management and fertilizer application (*see* **Note 5**). Additionally, disease and pest management can be of significant concern.

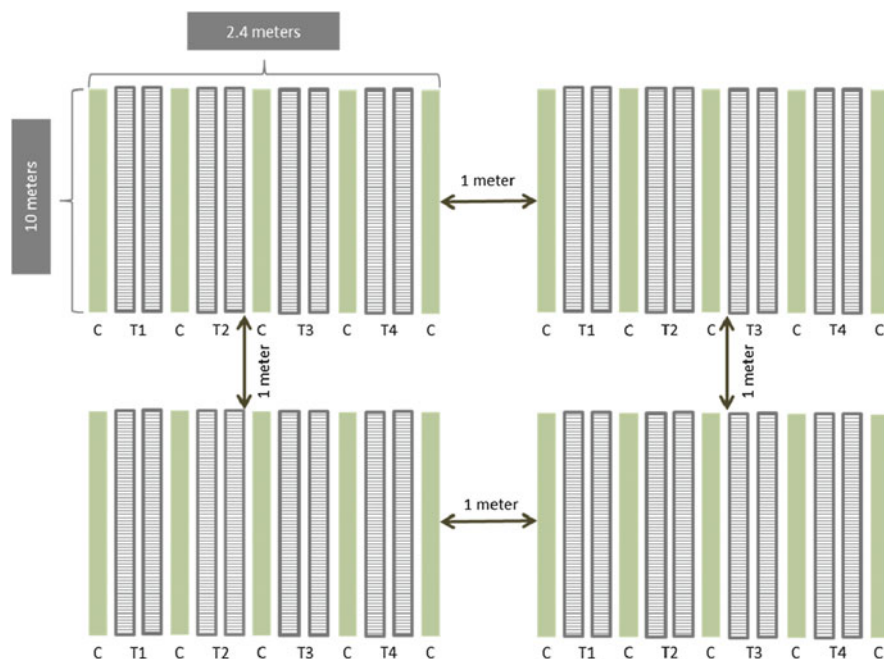


Fig. 9.2 Planting scheme of mutant populations. An individual genotype is evaluated in a single plot. Four plots are diagrammed in this figure. Plants treated with different dosages of mutagen are evaluated in the same plot (labelled T1, T2, T3 and T4 for the different treatments). Treated materials are flanked by control plants of the same genotype that have not been irradiated. Interspersing nonirradiated material helps control for GxE interactions when phenotypically selecting mutants (e.g. variations in field drainage). Plots are 10 m long with seed spacing of 20 cm within and between rows. Each plot is separated by 1 m. Care should be taken when organizing field experiments so that there is sufficient time and labour to evaluate all the planted materials. Phenotypic evaluation should not begin before the second (M_2) generation after mutagenesis

9.3.4 Field Observations and Trait Evaluation

1. Conduct regular field observations. All events observed in the field environment that are likely to affect the outcome of the activities should be recorded (*see Note 6*).
2. Evaluate traits of interest. Field evaluation of mutants is conducted to identify those with desirable/superior traits for varietal release or incorporation in other breeding procedures (*see Note 7*). Procedures for collection of trait data are well established for rice (e.g. Standard Evaluation System for Rice 1996). Standard practices (including the type of traits and the methods employed for evaluation) for the growing region should be employed. It is preferable for at least two people to be involved in data collection (*see Note 8*). Initial analysis of data collected can be restricted to the use of simple averages and standard errors (*see Note 9*).

3. Make selections of individuals (*see Note 10*). Seeds should be harvested, cleaned, dried to a moisture content of approximately 14 % and stored in plastic or paper bags in a cool, dry area. Additional seed treatments (e.g. for insect pests) may be needed.
4. Repeat field evaluation process to determine stability of selected mutants and to assess other agronomic traits. These observational and replicated yield trials are performed using standard recommended designs (*see Note 10*).

9.4 Notes

1. Mutagenized rice seed may be generated by employing caesium-137 or cobalt-60 gamma irradiators to induce mutations. A homogeneous seed source should be used for mutagenesis to prevent contamination from other varieties or landraces which could be mistaken for induced mutants. Seeds can be irradiated and stored for months before sowing. Cold storage of seed is preferable. However, when yield trials are being performed (*see Note 11*), seed should be stored at ambient temperatures to mimic conditions used by farmers.
2. The Plant Breeding and Genetics Laboratory of the FAO/IAEA provides irradiation mutagenesis services to member states. For groups who are able to perform irradiation mutagenesis locally, it should be noted that the dosage rate of the irradiator may affect the spectrum and density of induced mutations. Seed irradiation and dosage optimization should be performed according to published protocols (Mba et al. 2010). Special care should be taken when conducting dosage optimization using X-ray irradiators as the physical variations of different machines (e.g. fixed versus orbital rotation) may have an impact on actual dosage delivered to the seeds. Dosage depends on the responses of species and variety types. It is recommended that a preliminary varietal sensitivity test be performed to identify the appropriate dose range. At the Sierra Leone Agricultural Research Institute, initial varietal sensitivity of the local *O. glaberrima*, the institute's released *O. sativa* and NERICA rice varieties showed that favourable responses could be obtained from 100 to 400 Gy using both gamma rays using cobalt-60 and X-ray sources. Dosages above 500 proved fatal. The LD50 was between 350 and 440 Gy.
3. It is good practice to have a reasonably large population to facilitate the identification of beneficial mutants. It is however necessary to limit the total number of treatments to handle. For example, starting with ten varieties, the number of irradiation treatments including the controls (i.e. M_0) should not exceed five, resulting in 50 different treatment combinations. Assuming 100 seeds are irradiated per treatment, this will provide a working population (M_0 and M_1) of 650 seeds per variety and 6500 seeds for ten varieties. Using the

recommended intra-row spacing of 20 cm and an interrow spacing of 20 cm, the total amount of space required for one variety with five irradiation treatments including the control is 2.4×10 m plus 1 m separation between different mutagenic sources as shown in Fig. 9.2. Each variety should be sown in separate rows according to the level of irradiation commencing with control (nonirradiated seeds) followed by irradiated seeds. The principle of randomization is adopted in assigning treatments. Since each treatment consists of variety by levels of irradiation, varieties are initially randomized using a randomization table, or other method of randomization, followed by randomizing irradiation levels within varieties, essentially a split plot design. Intra- and interrow spacing 20 cm is considered adequate as segregation is expected after irradiation treatment.

4. Standard methods for preparing fields for planting of rice *via* direct seeding should be employed. This may involve the use of pre-emergent herbicides and pre-plant fertilizers which typically consist of major nutrients nitrogen (N), phosphorus (P) and potassium (K). It may be necessary to supplement with various mineral nutrients (e.g. zinc, iron). The methods, quantities, types of fertilizers and when to apply depend on the soil native fertility, the recommended rates and the cropping history. Whether the investigation is located on the uplands or irrigated lowlands, split application of N, P and K is usually recommended. Mini bunds are normally erected around plots to avoid fertilizer drift from plot to plot.
5. Depending on the available resources and local cultural practices, weed management may be conducted using application of herbicides during early stages of growth as well as manual weeding. Potential sensitivity of mutants to herbicides is a consideration. Midseason (i.e. mid-tillering stage of development) application of nitrogen may be desirable. When applying fertilizers, it is important not to go beyond what is optimally required. The need for application of fertilizers is justified as the genotypes should not suffer from nutrient stress. Excessive fertilization is also not recommended as the mutants should be exposed to as normal a condition as what operates in other field evaluations. Undue environmental effects that could mask the expression of mutant phenotypes should be limited to the extent possible. It is therefore important that the experimenter applies what normally operates in the region. Mutants should not be given any special treatment from the normal population.
6. After irradiation, it is best to sow one seed per hill except where the number of mutants is large as in M_2 and M_3 , in which case two seeds could be placed per hill. If both seeds emerge, one would have to be transplanted and labelled. It is recommended to store mutants and controls in a refrigerator or a cold room especially in warm and humid regions to avoid climatic/weather conditions on seed viability. This is necessary to distinguish the effect of mutagenesis from local storage conditions. Monitoring of field conditions (e.g. air and water temperature) which may affect development of the plants should be conducted on a regular basis. If available, data loggers that are able to record thousands of measurements of temperature and relative humidity are ideal although manual

measurements or data from nearby weather stations are alternatives for obtaining information.

7. In the evaluation process, mutants which are sufficiently different from the controls are identified. Labelling is expected, but it should not be done at the expense of objectivity. Rather than have all the details, labels should be clear but coded, and when a mutant is spotted with desirable traits, its real identity can be verified later. This is necessary to reduce bias in selection. Observers should be open-minded and not be restricted towards the objectives of the investigation as mutants with traits other than those set out to look for could be spotted and noted on the field by tagging. The Standard Evaluation System for Rice (1996) is useful in making observations on agronomic traits, crop damage (such as diseases, insects, rodents and birds), physiological stresses including acidity and drought, morphological characters and grain quality. Some important selection criteria should include tiller number, panicle number, panicle size, grain size and quality, number of filled spikelets per panicle, adaptation to local conditions and acceptable plant height and physiological maturation period.
8. It is preferable to have more than one observer to assess the plots at different stages of plant growth. Regular visits are essential with the growth phases of the crop providing a guide as to the number of visits. Three visits during each growing period are normally adequate: emergence, tiller production, reproductive and ripening stages. Where there are clear disagreements between observers, the team can visit the field together to arrive at some objective conclusion. Field activities are normally fraught with many predators ranging mostly from mammals, insects and birds. Effort should be made to erect barriers to restrain pests such as rodents and birds. Rodents usually attack the crop throughout the growing period, birds usually at the seeding and reproductive stages. A minimum of two people should be involved in data collection. Always write down as much details on the field before leaving as it is quite easy to forget details that were not recorded. Data obtained on the field should be transferred to a computer either from field notebooks or the use of data loggers. The raw data should be transferred; no transformation should be made on the field. It is always a good practice to have a spare copy of the raw data kept separately just in case of natural disasters like fire. It may be necessary to save seeds from each treatment for possible laboratory investigations, for example, Near-Infrared Reflectance Spectroscopy (NIRS) or physico-chemical analysis.
9. Analyses of data collected should be restricted to using simple averages and standard errors. This can be done without losing information on selected mutants as the analysis does not interfere with the selections made. Data on individual mutants selected are computed and compared with the controls (M_0) and other mutants not selected to obtain trends in plant characters amongst nonselected mutants, selected mutants and controls. Such information is useful and could provide benchmarks for future investigations.
10. Whilst selection could commence at the M_2 stage, mutant plants with interesting traits should be harvested separately. Single panicle selection is the preferred method of harvesting. Other plants are harvested according to treatment.

At the M_3 , M_4 and M_5 stages, panicles of selected mutants are also harvested, labelled and stored separately (Fig. 9.3). The rest of the materials are bulked by treatment. A number of options exist for selected mutants between M_2 and M_5 . Some of the selections could be fixed to produce double haploids (Maluszynski et al. 2003) in tissue culture, whilst others could be utilized in reverse-genetics activities such as TILLING (Till et al. 2006); the rest of the selected mutants, when fixed, could be incorporated in yield trials.

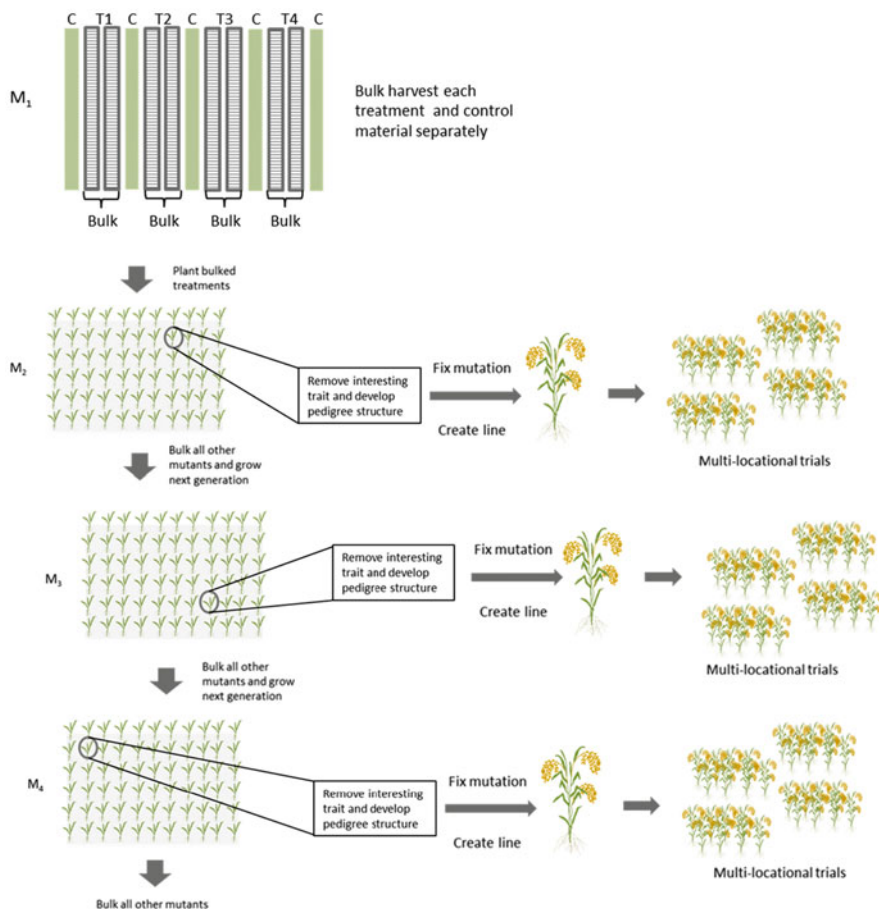


Fig. 9.3 Plot design, bulk screening and mutant selection of rice. Different treatments should be planted in rows (grey bars) flanked by non-mutagenized controls (green bars). Seed from a single treatment/genotype are bulked and phenotypic evaluation begins in the M_2 generation (middle panel). Plants with interesting phenotypes are selected and individually harvested. A pedigree approach is followed with these plants whereby mutations are fixed so that traits are pure-breeding and lines are created. This material is then evaluated in multi-location trials prior to varietal release. The rest of the seed from the plot (similar treatment/genotype) is bulked and the next generation planted and the selection process repeated (bottom panel). This is continued for several generations or until no further interesting plants with novel characteristics are recovered

11. At the M_5 generation, mutants are generally stable enough for observational yield trials (OYT) and replicated yield trials (RYT) using any of the recommended standard designs. Many field designs are available for replicated yield trials, ranging from completely randomized design, randomized complete block, partially incomplete blocks, split plots to fan designs to select from for trial establishment. Nonirradiated controls should be included in the evaluation of observational and replicated trials to determine superiority of mutants over parents. Selection of the appropriate or relevant design depends on the objective of the evaluation, and assistance could be sought from a biometrician or consulting recognized books on statistics and experimental designs (e.g. Cochran and Cox 1968; Cox 1958; Gomez and Gomez 1984).
12. Irradiation could be successfully used to produce beneficial changes in plant traits. This method is a lot safer, and seeds can be easily handled in the field with little or no risk to the environment or individuals involved in handling them. This method could be combined with in vitro techniques to reduce the period necessary for traits to be fixed. From experience, it is necessary for at least 4–6 growth cycles before one could be fairly certain that any traits observed on the phenotypes could be fixed.
13. It would be advisable in future for the number of varieties to be limited as the number of plant materials increased exponentially after the M_2 stage. Also with a larger number of varieties, the amount of data that could be recorded would be limited.

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Chapter 10

Root Phenotyping Pipeline for Cereal Plants

Michal Slota, Mirosław Maluszynski, and Iwona Szarejko

Abstract The proposed system for the phenotypic analysis of root traits that is presented here enables the precise description of the root growth kinetics of cereal plants. The designed pipeline is composed of a drip irrigation system to supplement plants with a medium, a high-resolution root system scanning facility and a method for comprehensive image analysis. The system enables low-effort, accurate and highly repeatable analysis of features of the root system of cereal seedlings and young plants until the early tillering stage. This system employs an automatic drip irrigation line, which is controlled remotely by a programmable logic controller (PLC). The PLC adapter used facilitates the automated control of all system modules, thus allowing the rate of the medium flow to be adjusted for the supplementation of plants. The system employs measuring sensors for the continuous monitoring of the parameters of the culture medium. This continuous sensing of medium parameters can be applicable for mineral nutrition studies and abiotic stress response testing. The installed drip lines are injected into transparent acrylic tubes (500 mm high, 32/30 mm in outer and inner diameter, with a circular opening in the bottom of 3 mm in diameter) that are filled with glass beads. The acrylic tubes are placed in opaque cover tubes that permit the non-destructive observation of the growth of the root system. Enhanced imaging quality contributes to an increase in the precision of the results that are obtained in the course of the analysis of root parameters using specialised root scanners coupled with the WinRHIZO system. This novel phenotyping pipeline permits noninvasive observation of root system growth adjusted for the subsequent root image acquisition with a reduced background noise. The method combines automated control of plant growth conditions with good imaging quality and high replicability of growth parameters.

Keywords Root system • Root system architecture • Hydroponics • Root phenotyping

M. Slota • M. Maluszynski • I. Szarejko (✉)

Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Jagiellonska 28, 40-032 Katowice, Poland

e-mail: iwona.szarejko@us.edu.pl

10.1 Introduction

10.1.1 *Issue of Root Phenotyping*

The analysis of root system growth and development remains a challenge in the area of plant phenotyping. Classical root phenotyping approaches in the field are extremely laborious and require a considerable effort to properly characterise the root system architecture under natural conditions (Böhm 1979). Root systems of field-grown plants form extensive networks in the soil, which can hinder their extraction and proper observation (Zhu et al. 2011). Excavation techniques, including soil cores and soil profiles, must be applied to study root systems under such conditions (Prasanna et al. 2013). By contrast, root system studies carried out under greenhouse or growth chamber conditions offer a more homogeneous experimental setting due to the ability to better control the environmental variables. Nonetheless, greenhouse-based assays of root phenotyping still remain time-consuming and labour-intensive when a soil substrate is applied for plant growth. Soil-based root phenotyping experiments are extremely limited by the difficulty of observing root growth and development without the disruption of the surrounding soil core (Menon et al. 2007). Moreover, the rooting volume of plants growing in pots of varying sizes significantly influences the calculated root system volume (Poorter et al. 2012). Therefore, multiple experimental setups for the in vitro screening of plant root traits have been developed. To facilitate the visualization of root growth, plants can be grown in hydroponic cultures, on surfaces of agar or paper or in clear gel media in transparent containers (Zhu et al. 2011). Most of these methods are restricted to relatively young plants, which may not directly correlate with the architecture of the fully developed root system (Zhu et al. 2011). The application of in vitro assays in plant phenotyping can also lack the capability of imitating the complex natural conditions and can frequently cause stress itself. Root screening assays that involve gel media tend to expose roots to light and may cause hypoxia (Zhu et al. 2011). On the other hand, much progress has recently been made in the investigations of the roots of small plants on agar plates, which can easily be adopted for the purpose of automated large-scale growth screening (Nagel et al. 2009; Yazdanbakhsh and Fisahn 2009).

10.1.2 *Root Phenotyping of Cereal Plants*

Cereals have a fibrous root system that is composed of embryonic roots and adventitious (crown) roots that emerge postembryonically (Smith and De Smet 2012). Isolated embryonic roots are formed in the early stage of plant development, which are initially the only roots that allow water uptake from the soil. Embryonic roots penetrate the soil deeply and have numerous lateral roots. During late development, plants produce a smaller number of thicker and less branched adventitious

roots that grow beneath the soil but that do not penetrate the deeper layers (Coudert et al. 2010). A specific spatial and temporal pattern of the root growth of monocots poses a challenge for high- or moderate-throughput root phenotyping. The complex root systems of monocotyledonous plants require special efforts in order to maintain the equilibrium between the analysis throughput and imaging resolution. As the throughput of a screening method increases, it causes a reduction in the accuracy and precision of the measurements (Cobb et al. 2013). The process of high-throughput phenotyping depends on a combination of specialised techniques for plant growth, imaging and image analysis (Clark et al. 2013). Many screening techniques have been applied for rapid and reproducible measurements under controlled conditions, including aeroponics, hydroponics, wax-petrolatum layer, soil-filled chambers or pipes (Prasanna et al. 2013). It is necessary to consider the degree to which a root phenotype will be expressed in the target experimental conditions. Manual techniques remain reliable for small experiments that involve simple measurements with fewer replicates (Clark et al. 2013). However, there is a growing interest in exploring new techniques in order to capture and extract phenotypes from a larger number of plants with greater precision and reduced subjectivity (French et al. 2009). It is essential to adjust a plant growth system to the purpose of the image capture. The key to maintaining precision in a phenotyping experiment is to employ a stable instrument setup that can effectively ensure imaging quality (Cobb et al. 2013). Image acquisition that employs digital cameras or scanners for the purpose of imaging is straightforward for non-soil grown plants. By contrast, plants grown in a soil substrate must either be separated from the soil or imaged using minirhizotron tubes for two-dimensional (2D) image capture (Zhu et al. 2011). Several root screening approaches involve the application of three-dimensional (3D) imaging techniques. The most promising technique for noninvasive 3D imaging in soil is X-ray computed tomography (CT) and magnetic resonance imaging (MRI) (Metzner et al. 2015). There are also other applicable approaches that include selective plane illumination microscopy (SPIM) and optical projection tomography (OPT) that are adapted for plants grown in transparent media (Downie et al. 2012). Regardless of the method that is used for image acquisition, it is essential to properly analyse the data obtained in a reproducible manner. Large image datasets require the application of novel software solutions in order to process and extract meaningful estimates of phenotypic variation. Most image analysis tools employ predefined processing and analysis methods in semi-automatic and automatic procedures in order to quantify multiple phenotypes from single images or groups of images (Cobb et al. 2013). Numerous commercial and open-licence software packages have been developed to solve specific tasks (Lobet et al. 2013).

10.1.3 Proposed Root Phenotyping Pipeline

In spite of all of the progress that has been made in the field of plant root phenotyping, there is still a gap between the complex, multifunctional commercial platforms which have been developed for high throughput and precision screening and the simpler root phenotyping protocols which can be adjusted for specific plant species and screening assay. The described protocol offers the possibility to develop the moderate-throughput, reliable and cost-effective method that would facilitate the precise characterisation of the root system architecture in cereals. The system employs an automatic drip irrigation line which transports a medium solution to individual plants. Thus, the designed system offers a possibility to carry out mineral nutrition studies by altering the composition of aqueous medium. The watering pumps which supplement the plants with a medium can be placed in the separate tanks containing differed medium variants. The plant growth system provides randomised conditions maintained in an automated manner. A built-in programmable logic controller (PLC) computer provides a controllable power-line access for the connection of all of the system accessories (water pumps, air pump, heating device) as well as collects data from external sensors (temperature sensor, pH/redox or other ion-selective electrodes). This setup facilitates the monitoring of culture medium parameters (pH, temperature, redox potential) and the generation of different time-lapse graphs of the changes in parameters. Due to the application of the glass beads as a substrate which provides the mechanical impedance for root growth, the parameters such as a total root length and a density of lateral roots have higher values compared to plants grown under conventional hydroponic conditions. The imaging of root systems can be performed in a destructive (with a use of the flatbed scanners coupled with the WinRHIZO software) as well as a non-destructive manner (using a digital camera for the image acquisition). The measurements of the root system which are acquired using the proposed system were demonstrated to be reproducible within the biological repetitions of the tested barley genotypes in each replication of the experiment. The robustness of the system maintenance has been repeatedly tested and optimised (*see* **Notes 1 and 2**, and Table 10.3). The novel protocol for the root phenotyping of cereal plants presented here consists of a complete pipeline for maintaining plant growth combined with image acquisition and data analysis methods (Fig. 10.1).

10.2 Materials

10.2.1 Design of a Plant Growth System

Note: The description of the functioning of the system refers to the numbering of its components in Figs. 10.2a and b.

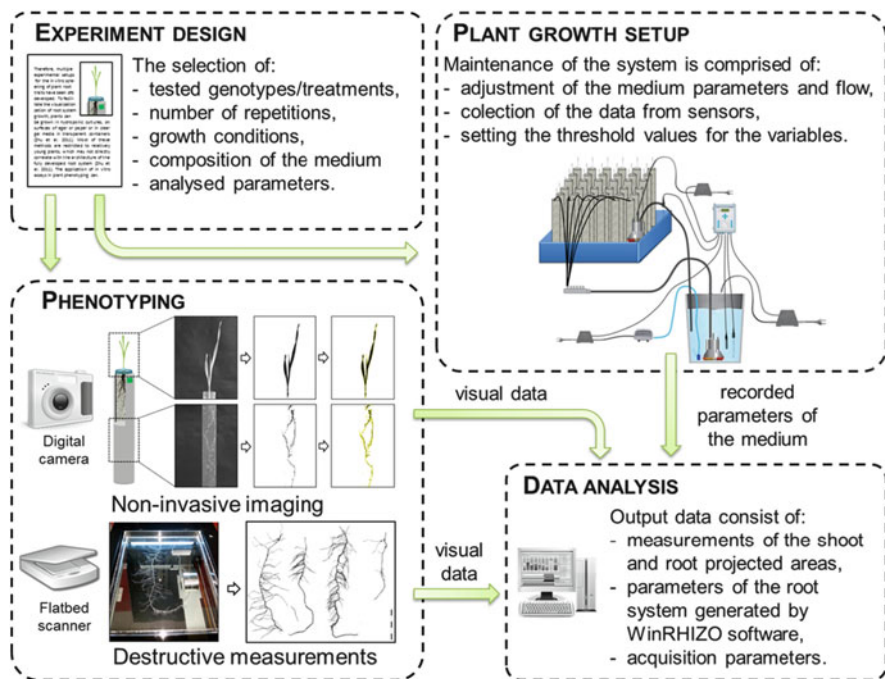


Fig. 10.1 The overview of the pipeline for the preparation of phenotyping experiments. The pipeline consists of the following stages: experiment design, maintaining the growth setup, plant phenotyping and the data acquisition and analysis

1. The system uses an automatic drip irrigation line that is controlled remotely by the programmable logic controller (PLC).
2. The PLC adapter coupled with a PLC computer (Fig. 10.2; 13) enables the automated control of all of the system modules: water pumps, air pumps and heating devices. It offers a power-line access for water and air pump transformers (Fig. 10.2; 1 and 2).
3. Medium flow is maintained by adjusting the afferent (Fig. 10.2; 3) and efferent pumps (Fig. 10.2; 11). The PLC adapter allows the independent programming of the afferent and efferent pumps, which can operate in a continuous or discontinuous module with preset time intervals.
4. The afferent pumps are placed in opaque canisters (Fig. 10.2; 4) with a water level indicator for medium storage. When the canister is filled with the medium, a pH electrode and a thermal sensor are injected to each canister.
5. Aerating pipes (Fig. 10.2; 6) are inserted into the medium canisters for the proper aeration of the medium solution by the air pumps (Fig. 10.2; 5).
6. The system is equipped with measuring sensors for the continuous monitoring of the parameters of the culture medium: temperature, pH, redox state, and concentration of specific ions.

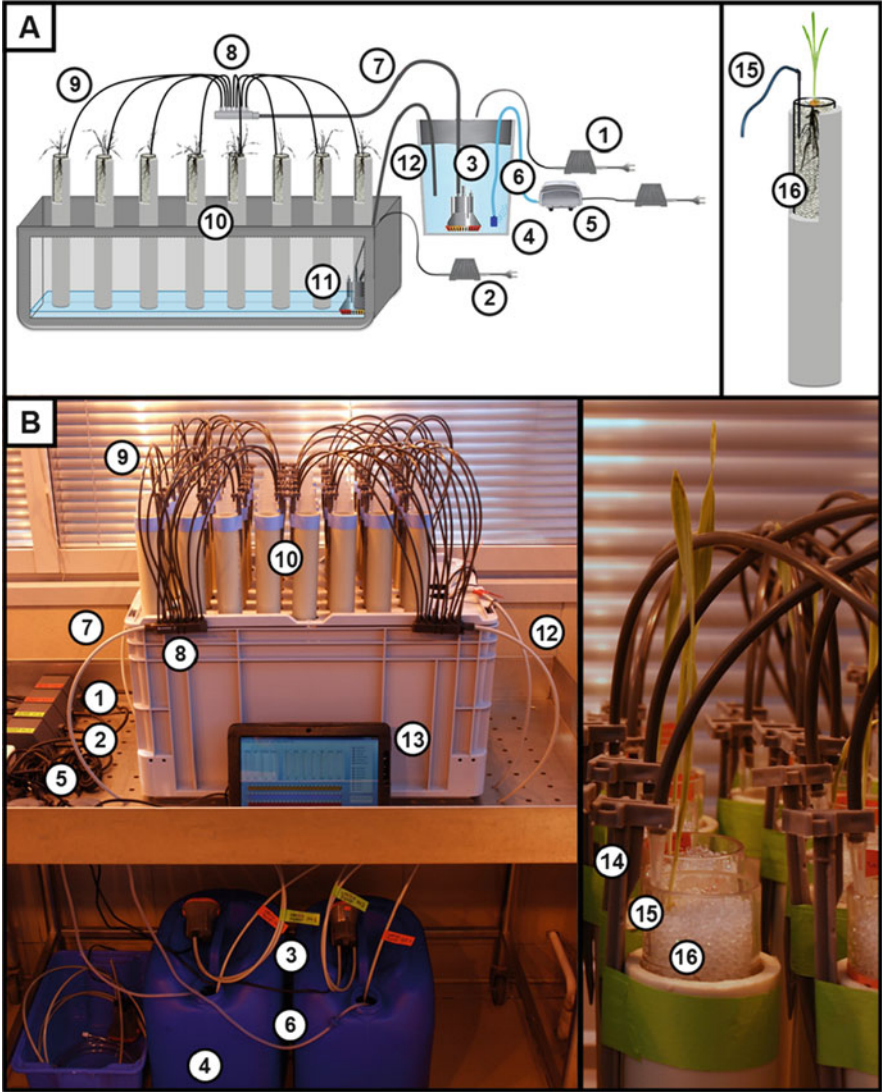


Fig. 10.2 Overview of the plant growth system. Schematic layout (a) and photographs (b) of the watering system that consists of a transformer for an afferent pump (1), a transformer for an efferent pump (2), an afferent water pump (3) placed in the canister with the culture medium (4), air pumps (5), an aerating pipe (6), a supply pipe (7), a distributor with 12 outlets (8), distribution pipes (9), acrylic tubes filled with a substrate (10), an efferent pump placed on the bottom of a stackable box (11), drain tubing (12), a tablet connected to a PLC adapter (13), a pipe peg (14), a distribution pipe with a plastic tip (15), an acrylic tube filled with glass beads (16)

7. PLC-controlled experimental conditions can be monitored, and all of the medium flow parameters can be modified by setting the desired threshold values. The solution that is applied allows the water flow efficiency to be adapted for the current capacity of the experiment that is being conducted.
8. Supply pipes (Fig. 10.2; 7) transfer the medium to water distributors (Fig. 10.2; 8) connected to drip lines (Fig. 10.2; 9). Each drip pipe capped with a plastic tip (Fig. 10.2; 15) is attached to the pipe pegs (Fig. 10.2; 14) and inserted into the acrylic tubes which are filled with glass beads (Fig. 10.2; 16).
9. The transparent tubes are placed into opaque cover tubes to protect the roots from the light. Transparent, 3-mm soda-lime glass beads (Sigma, Cat. No. Z265926-1EA) allow for the noninvasive observation of root system growth. This substrate also facilitates the root system cleaning, which improves the final image quality.
10. The acrylic tubes, which are equipped with a bottom drainage opening to ensure the proper draining of the medium, are placed together in a stackable box. The excess medium is drained off from the bottom of the box into the medium canister by efferent pumps through the drain tubing (Fig. 10.2; 11).

10.2.2 Root Scanning Setup

Note: The root scanning setup employs an STD4800 scanner (Regent Instrument) and an Epson Perfection V700 photo scanner equipped with the WinRHIZO System. For additional guidelines, please refer to the operating instructions for the STD4800 scanner and the WinRHIZO technical manuals. The description of the system's setup uses the numbering of its components in Figs. 10.3a and b.

1. The scanner (Fig. 10.3; 1) should be installed on a vibration-free table to avoid any image noise. The scanner has an additional transmissive lighting system—a transparency unit (TPU). The reflective white plastic in front of the scanner cover must be removed to scan with the TPU light (Fig. 10.3; 2).
2. The scanner should be connected to a PC with a USB2 cable (Fig. 10.3; 3). All of the necessary drivers and analysis software are provided with the system.
3. A positioning system (Fig. 10.3; 4) is to be used to adapt the scanner for scanning plant roots. The positioning system is made of semiopaque plastic blocks to accommodate different scan area sizes.
4. Translucent waterproof trays (Fig. 10.3; 5) that match the positioned scan areas are supplied with the system for scanning immersed objects. An appropriate tray should be placed within the positioning system.
5. When applying the trays, spacers should be installed to raise them to the height of the TPU cover.

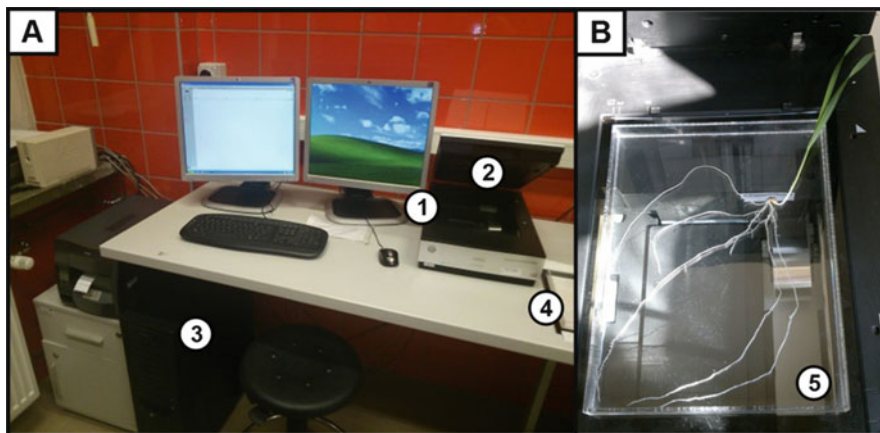


Fig. 10.3 Imaging setup for root analysis. The PC (a) is connected to the scanner (b) with a TPU lighting system. The scanner (1) with the cover removed (2) is connected to the PLC computer (3). The WinRHIZO system accessories consist of a scanner positioning system (4) and a translucent waterproof tray (5)

10.3 Methods

10.3.1 Preparation of Culture Media

1. Use the following ingredients to prepare the culture medium (the catalogue numbers that are used refer to the Sigma–Aldrich product line):
 - Ammonium phosphate monobasic ($\text{NH}_4\text{H}_2\text{PO}_4$), Cat. No. 216003
 - Potassium nitrate (KNO_3), Cat. No. P8291
 - Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), Cat. No. 31218
 - Magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), Cat. No. 63140
 - Boric acid (H_3BO_3), Cat. No. 202878
 - Manganese chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), Cat. No. 221279
 - Zinc sulphate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), Cat. No. 204986
 - Molybdc acid ($\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$), Cat. No. 232084
 - Copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Cat. No. 209198
 - Ferric sodium ethylenediaminetetraacetate (NaFeEDTA), Cat. No. E6760

All of the chemical compounds used should be chemically pure for the purpose of analysis (AR signature—analytical reagent).

2. Use specified quantities of each compound for the preparation of the medium stocks (macro, micro and Fe) (Table 10.1).
3. Dissolve all compounds in deionised water and autoclave the stocks at 121 °C, 1.1 bar for 45 min.
4. Store all medium stocks at 4 °C.

Table 10.1 Hoagland medium composition (Hoagland and Arnon, 1950)

Group	Compound	Stock volume	Quantity [g]
Macro	NH ₄ H ₂ PO ₄	250 ml	28.76
	KNO ₃		25.28
	Ca(NO ₃) ₃ ·4H ₂ O		59.04
	MgSO ₄ ·7H ₂ O		61.62
Micro	H ₃ BO ₃	1000 ml	2.86
	MnCl ₂ ·4H ₂ O		1.81
	ZnSO ₄ ·7H ₂ O		0.22
	H ₂ MoO ₄ ·H ₂ O		0.02
	CuSO ₄ ·5H ₂ O		0.08
Fe	NaFeEDTA	100 ml	1

- Adjust the pH of the medium at 5.9–6.1 using 1 N NaOH (Sigma, Cat. No. 221465).
- Prepare the medium in advance in an opaque canister with a tap and a water level indicator and store them at 4 °C.

10.3.2 Controlling the System and Monitoring the Medium Parameters

Note: The automatic drip irrigation system is controlled by a programmable logic controller (PLC) that provides a convenient power-line access for the connection of all of the system's peripherals. The PLC adapter is also equipped for the continuous sensing of parameters of the culture medium. For additional guidelines, please refer to the operating instructions for the applied PLC adapter.

- The afferent pumps, efferent pumps and air pumps should be connected to the power-line adapter. The adapter has six 230 V outputs for connecting the programmable devices. Use submersible water pumps (4×) with a maximal operating pressure of 1 bar (14 psi) and a maximal flow of 250 l/h with three outlets. Use air pumps (2×) with a minimal oxygen flow of 320 l/h. For additional guidelines on assembling the pumps, please refer to the technical manual from the pumps supplier.
- The PLC adapter (Fig. 10.4a) can be programmed using in-system programming (ISP) and in-application programming (IAP). For setting the switch on/off times of the defined outputs, program the PLC adapter directly or use the PLC software for PCs (Fig. 10.4b).
- Define the switch on/off times of the outputs using the software that is provided. Select the *Tools menu* and browse the *Output parameters*.
- Define the length of the switch on time and switch off time interval for the device.

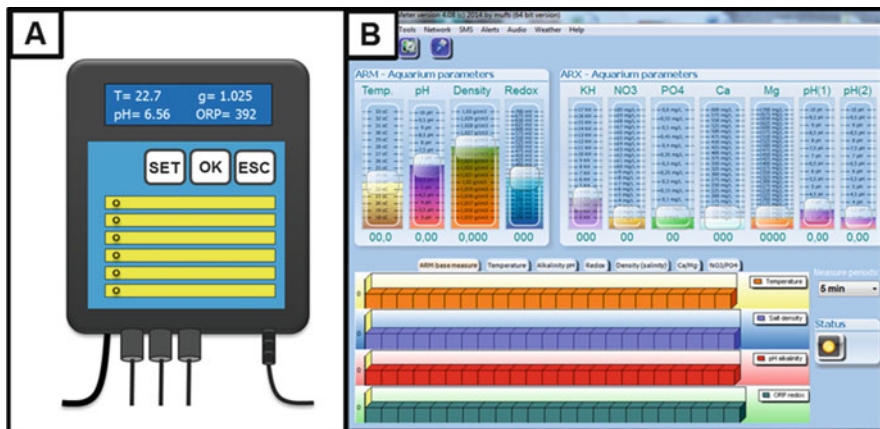


Fig. 10.4 Programmable logic controller (PLC) for the watering system control (a) and dedicated software for the continuous acquisition of the parameters of the culture medium (b)

5. Connect the PLC adapter with the PLC computer using a USB cable.
6. Import the selected parameters to the PLC device by choosing the *Tools menu/Output parameters/Send to device*.
7. Connect two pH electrodes (pH range 5–9) to the PLC device.
8. Calibrate the pH electrodes separately using the PLC adapter menu. Press the OK button for 2 s. Press SET to choose the desirable calibrating parameter (pH4, pH9, temperature) for each electrode. Insert a calibrating electrode into appropriate buffer (pH4, pH9). Wait for about 1 min to ensure that you get a stable measurement and press the OK button to save the result. The calibrating process can be terminated by pressing the ESC button.
9. Calibrate the temperature sensor using the same option as described in point 8 using a conventional thermometer measurement as a reference.
10. The measurement data from the pH electrodes and temperature sensors can be exported in the .csv format. To import the data choose the *Tools menu/Parameters settings/Load from device*.

10.3.3 Experiment Preparation and Maintenance

1. Autoclave the appropriate amount of soda-lime glass beads, 48 acrylic tubes and any necessary laboratory glassware. The autoclave program should be applied as follows: 121 °C, 1.1 bar, 45 min.
2. Sterilise grains in a 5 % solution of sodium hypochlorite (Sigma, Cat. No. 71696) for 15 min. Remove the sodium hypochlorite solution and wash grains with sterile water 3 times for 5 min.

3. Plant the grains in sterile square plastic 120 × 120 mm Petri plates (Gosselin, Cat. No. BP124-05) filled to mid level with sterile moist vermiculite or filter paper. Incubate the grains at 4 °C for 24 h and then transfer the plates to an incubator at 24–25 °C for the next 48 h.
4. Fill 48 acrylic tubes with soda-lime glass beads leaving a space of approximately 5 cm at the top.
5. Transplant the germinated seedlings into the acrylic tubes and replenish them with an additional portion of soda-lime glass beads leaving a space of approximately 1 cm at the top.
6. Place the distributor pipes that are capped with plastic tips that are attached to the pipe pegs near the edges of the tube and cover the top opening of it with a piece of cotton wool.
7. Fill the medium container with the appropriate amount of the medium.
8. Check all of the tubing connections and plug the water and air pump transformers into the power line that is connected to the PLC computer.
9. Start the pumps and set the water and air pumps programs using the PLC software.
10. Place the system into a growth chamber under controlled conditions: temperature 22/20 °C during the day/night, photoperiod 16/8 h and illumination of 320 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Supplement the plants with the medium for the entire duration of the experiment (14 days).
11. Replace the medium in the medium canister every 4 days.

10.3.4 *Medium Exchange*

1. Use an additional submersible water pump with a maximal operating pressure of 1 bar (14 psi) and a maximal flow of 250 l/h.
2. Before beginning the medium exchange, make sure that the drip irrigation system is paused using the PLC adapter.
3. Attach a single supply pipe to one of the three water connections. Close the remaining water connections by screwing on the end caps.
4. Place the other end of the supply pipe that is connected to the water pump onto the water draining system.
5. Submerge the pump inside the medium canister.
6. Connect the medium discharge pump to the transformer and plug it directly into the power supply. Keep the pump turned on until the medium canister is completely emptied.
7. Attach a rubber pipe to the tap of the medium supply canister. Place the end of the pipe into the empty canister and turn the tap on. The medium supply container should be placed above the medium exchange containers for it to work effectively.
8. Leave the tap turned on until the medium canister is refilled.
9. Repeat **steps 5–7** for the second medium canister.

10. After exchanging the medium in both canisters, turn on the drip irrigation system again.

10.3.5 Experiment Termination and Root System Cleaning

1. After the experiment is completed, stop the drip irrigation line using the PLC adapter.
2. Carefully disconnect the drip pipes and remove the acrylic tubes from the cover tubes.
3. Remove the piece of cotton wool that is covering the tube opening.
4. Carefully remove the plant after moistening the substrate and pouring the water through bottom drainage opening with a syringe (100 ml) or a beaker.
5. Rinse the plant root system with tap water above the sieve to remove the remaining glass beads. The extracted seedlings should be kept wet. Keep the plants in tap water in properly marked 50 ml conical centrifuge tubes until the roots are scanned using the WinRHIZO system.
6. The time period preceding the root system analysis should be kept to a minimum.

10.3.6 Root System Analysis Using WinRHIZO System

Note: For additional extensive guidelines, please refer to the WinRHIZO technical manual.

1. Before the beginning the analysis, make sure that the STD4800 scanner is connected to the PC with a USB2 cable and that all of the necessary drivers and analysis software are installed.
2. Cut the roots of a single plant using a sharp pair of scissors in order to separate all of the embryonic roots.
3. Place the roots (1–2 embryonic roots at a time) on a waterproof tray (Regent Instruments). The roots should be entirely immersed in water. If necessary refill the tray with tap water.
4. Position the roots in order to avoid any overlapping lateral roots and ensure that they are distributed randomly.
5. Before starting the WinRHIZO software, the personal protection key should be connected to the USB port.
6. Start the WinRHIZO software and select the EPSON Perfection V700/V750 TWAIN as the source.
7. After opening the WinRHIZO program, select the image acquisition parameters by entering the menu *Image Acquisition Parameters*. Choose the option ‘*With tray*’ and select the appropriate scan area.

system [cm], root system surface [cm²], root system volume [cm³], root diameter [mm] and the number of tips. All measurements which can be conducted using the WinRHIZO software and non-destructive techniques for root and shoot imaging in time series are summarised in Table 10.2.

10.4 Notes

1. The subsequent stages of the preparation of an experiment indicating required time are presented in Table 10.3 (the robustness of the system relates to the experiment carried on 48 plants).

Table 10.2 List of the parameters that can be measured using the system

Organ	Measurements	Units	Destructive	
Shoot	Projected shoot area	cm ²	No	
		cm ²	No	
	Shoot height	cm	No	
Root	Root system depth	cm	No	
	Projected root area	cm ²	No	
	Total length of the root system	} WinRHIZO	cm	Yes
	Projected root surface		cm ²	Yes
	Average root diameter		mm	Yes
	Root surface area		cm ²	Yes
	Root volume		cm ³	Yes
	Number of root tips		–	Yes
	Number of root forks		–	Yes

Table 10.3 Stages of the experiment in terms of their time-consuming

Stages of the experiment		Duration
Experiment preparation	Grain surface sterilisation	30 min
	Preparation of tubes and substrate	2 h
	Medium preparation	2 h
Plant growth	Grain incubation [4 °C]	24 h
	Grain incubation [24 °C]	72 h
	12 days of the growth of plants (+ noninvasive imaging)	45 min/day
Data acquisition and analysis	Experiment termination	2 h
	Roots scanning (48 plants)	6 h
	WinRHIZO analysis	4 h
	Data analysis (WinRHIZO)	2 h
	Data analysis (projected shoot and root areas)	3 h

2. WinRHIZO software can generate two types of files—Images and Data. The commands for each of these can be found in the *Image* and *Data* menus, respectively. Image files can be saved in the .tiff format, which can be read by most image-based software packages. Analysis data are saved in the ASCII text format and have the .txt extension.

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Chapter 11

Breeding New Aromatic Rice with High Iron Using Gamma Radiation and Hybridization

Phuong Tan Tran and Cua Quang Ho

Abstract The goal of many breeding programmes is the combination of several improved traits to produce a cultivar that meets demands of farmer and consumer. For example, breeding aromatic rice varieties having both high quality and yield is an objective in Vietnam to satisfy domestic consumers and increase value in the export market. Genetic variation is the starting point for any breeding programme. In some cases sufficient variation exists and traditional hybridizations and introgressions are suitable for cultivar development. In other cases, new variation, such as that created through mutagenesis, is required for the development of new traits. Thus, a combinatorial approach using both hybridization and induced mutations can be considered when the goal is a new cultivar expressing several improved traits. We have taken this approach in rice breeding to generate lines with improved aroma and high iron content. Here we provide a protocol for mutation induction, hybridization and phenotypic analysis for the improvement of aroma and iron content in rice using a combined mutation and hybridization approach. Example data from this work is shown. This approach can be easily adapted for other traits of interest.

Keywords Aroma • Badh2.1 • Bioavailable iron • Iron content • High quality • Hybridization • Pedigree selection

11.1 Introduction

There is an increasing demand for the production of high-grain-quality aromatic rice to meet the domestic demand and also for export. Though there are many aspects which impact quality, key features that influence the market value of rice are aroma, kernel shape, cooking quality and taste. Aromatic rice is unique and prized in many countries. Local aromatic rice varieties in Vietnam have high value in the domestic market. There are famous varieties such as Tao Huang, Nang Thom in the south and the Tam group (Tam Xoan, Tam Thom) in the north. These

P.T. Tran (✉) • C.Q. Ho
Soc Trang Department of Agriculture and Rural Development, Soc Trang, Vietnam
e-mail: trantanphuong2005@gmail.com

varieties have disadvantageous traits such as photoperiod sensitivity; long growth duration (160–180 days); high stature (150–185 cm); thin, long, drooping leaves; and sparseness between grains. The consequence of these negative traits is low productivity calculated in the range of 2–3 tons/ha/crop. The advantage of these varieties is their better adaptation to the poor land and changing climatic conditions. Both genetics and environment affect traits such as aroma. For example, basmati rice loses the aroma when grown outside the Punjab in Pakistan and India. It is thought that Punjab climate and/or soil is important for producing a strong aroma (Efferson 1985). Khao Dawk Mali 105, the most important aromatic rice cultivar in Thailand, is reported to have the strongest aroma and best quality when grown in the Tung Kula Rong Hai region in northwest Thailand (Yoshihashi et al. 2004). Ideally, high-yielding, aromatic and photoperiod-insensitive varieties can be bred to serve increasing demands of customers in domestic and the world markets and to increase incomes for farmers.

The success of any breeding programme relies on genetic variation in the form of altered alleles that control or contribute to the traits of interest. There are vast genetic resources available for rice (e.g. <http://irri.org/about-us/our-organization/genetic-resources-center>). Traditional breeding approaches seek to generate new combinations of alleles that result in an improved variety. Yet, some traits (alleles) may not be available in existing germplasm or available only in genotypes that are recalcitrant due to linkage drag with negative traits. New alleles can be created at a high frequency using mutagenesis (*see* Chap. 1). Thus mutagenesis can be considered to support breeding objectives. In some cases mutagenesis of elite cultivars can be performed and improved varieties can be directly released (*see* Chap. 9). This is quite common and about 62 % of all officially registered mutant varieties are produced in this fashion (*see* Chap. 1 and MVD 2016). Hybridization of mutant alleles with “natural” alleles already present in germplasm is another approach. We provide an example of hybridization of mutant and natural alleles to produce high aroma and high bioavailable iron accessions in this chapter. The general approach for directed hybridizations is shown in Fig. 11.1.

In addition to requisite genetic variation, appropriate screening techniques are needed to produce improved varieties. For rice aroma, many researchers have examined the trait by a sensory test. Buttery et al. (1983) identified the characteristic aroma compound in steam volatile oils of cooked aromatic rice as 2-acetyl-1-pyrroline (2AP). Yoshihashi (2002), by a method using isotope-labelled analogues of 2AP, reported that the concentrations of 2AP are presented in milled and brown rice, rice bran, husk and seedlings. 2AP was not detected in root and it does not form during cooking or postharvest processing. Solid phase microextraction (SPME) has emerged as a rapid and efficient tool for the extraction and quantification of the aroma compounds (Stashenko and Martínez 2007). It is a rapid, simple, versatile and solvent-free technique and has integrated sampling, extraction, concentration and sample introduction of volatile compounds into gas chromatography (GC) in a single step resulting in high sample throughput (Soria et al. 2009; Picó et al. 2007).

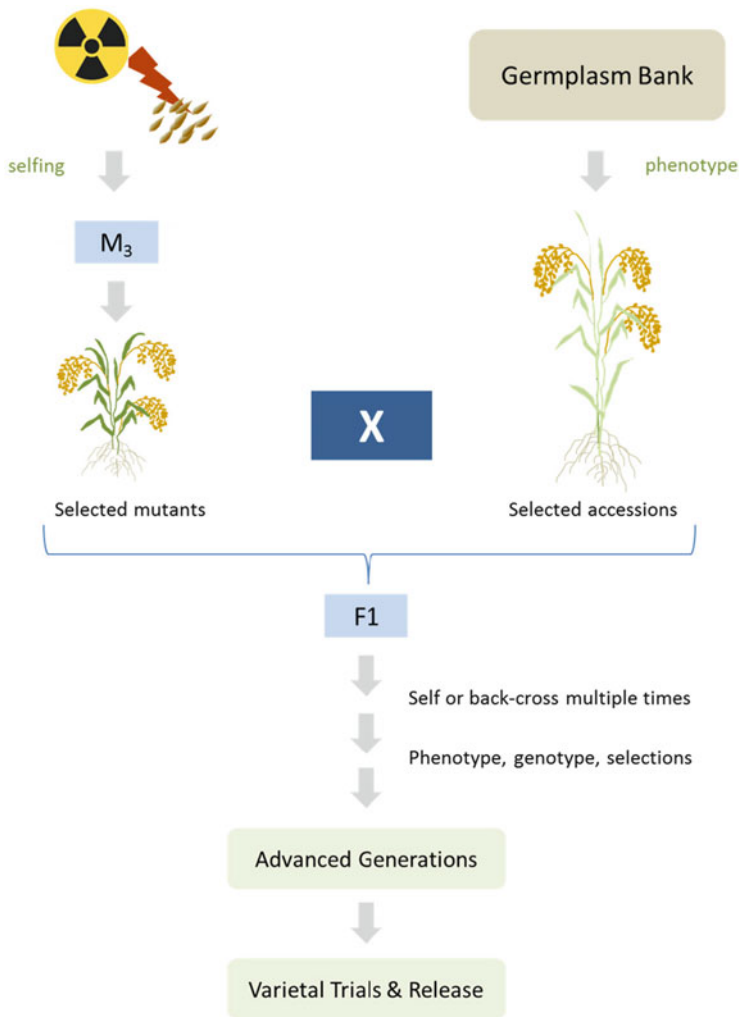


Fig. 11.1 Development of new rice cultivars through hybridization of mutant and natural alleles. Mutant populations are developed by treating seed with gamma irradiation (*top left*). Plants are self-fertilized and screened for improved traits. Phenotypic analysis is also carried out on accessions from a germplasm bank. Selected accessions are then hybridized with selected mutants. Since most traits will be recessive, the resulting F1 is further crossed. Different crossing strategies can be used such as selfing or backcrossing to the selected accession to introgress mutant alleles. Crossing and phenotypic analysis continues until traits are stabilized. At this time plants can be subjected to requisite varietal trials followed by eventual release. Figure courtesy of Dr Joanna Jankowicz-Cieslak and Dr Bradley J. Till of the FAO/IAEA Joint Programme

Micronutrient malnutrition, the result of diets poor in vitamins and minerals, affects more than half of the world’s population. Women and children are especially susceptible to deficiencies in micronutrients, particularly vitamin A, iron and

zinc. As a result they are at risk of disease, premature death, lower cognitive capacity and poor quality of life. Nutritionally improved staple food provides an inexpensive, cost-effective, sustainable, long-term means of delivering micronutrients to the poor. Brown rice is a more nutritious food in comparison with white rice owing to the fact that the bran layer contains high amounts of vitamin B1, plus other nutrients and micronutrients. Other important components, γ -tocotrienol and γ -oryzanol in the bran covering of brown rice, have the effect of reducing cholesterol in the blood, an important factor causing cardiovascular disease (Chen and Cheng 2006). Brown rice is also suitable for people who follow the “macrobiotic diet” which not only brings much nutrients but also has a sweet taste created by the contribution of many enzymes on sugar and proteins inside grains. Researches on iron content in brown rice samples showed that iron content changes depending on varieties, IR64 (12.58–12.88 mg/kg), Jasmine 85 (12.84–18.50 mg/kg) and OMCS2000 (11.77–14.78 mg/kg), and about 2/3 of iron content is lost through milling (Tran et al. 2004). However, it is difficult to stabilize the iron content and aromatic level because iron and zinc contents vary in different regions and growing seasons (Liang et al. 2006). Based on research and evaluation of iron-enriched rice domestically and internationally, and responding to the national strategy on nutrition, we have conducted cross-breeding for iron-enriched aromatic rice varieties by using mutant materials, local aromatic varieties and improved aromatic varieties to contribute to nutrition security for households.

11.2 Materials

1. Rice seed from accessions with desired traits (*see Note 1* and Table 11.1 for examples).
2. Rice mutant lines (*see Note 2*).
3. KOH (potassium hydroxide).
4. A cobalt-60 facility (*see Note 3*).
5. Scale for measuring seed weight.
6. Ruler.
7. Hygrometer.
8. Test tubes (1.3 × 10 cm).
9. Dehuller (e.g. Satake).
10. Atomic absorbance spectrophotometer.
11. Taq polymerase.
12. MgCl₂.
13. dNTPs.
14. Gene-specific primers.

Table 11.1 Rice accessions with variation in aroma

SDK ^b	Name of Vietnamese local rice variety	Aroma ^a	SDK ^b	Name of Vietnamese local rice variety	Aroma ^a
Vietnamese local aromatic rice varieties					
	Nang Thom Cho Dao	+	5121	Tam Con	+
233	Tam Tuc Tay Bac	+	5122	Tam Nghia Lac	+
268	Tam Den Ha Dong	+	5124	Tam Hai Giang	+
274	Tam Ap Be Ninh Binh	+	5126	Tam Ap Be	+
314	Tam Xoan Bac Ninh	+	6212	Tam Co Rut	+
316	Tam Nghe Hat Do	+	6216	Tam Thom	+
5117	Tam Xuan Dai	+	6240	Tam Cao Cay	+
5119	Tam Xuan Hong	+	6250	Tam Tieu	+
5120	Tam Nghia Hong	+	2376	Du Nghen	+
219	Tam Tron Hai Duong	-		Tam Thom Hai Hau TT1	+
Vietnamese improved aromatic varieties					
	Soc Trang 3 (ST3)	+		Soc Trang 13 (ST13)	+
	Soc Trang 5 (ST5)	+		Soc Trang 14 (ST14)	+
	Soc Trang 6 (ST6)	+		Soc Trang 15 (ST15)	+
	Soc Trang 10 (ST10)	+		Soc Trang 17 (ST17)	+
	Soc Trang 12 (ST12)	+		Soc Trang 18 (ST18)	+
	Huong Com	+			
	Hoa Sua	+			
Rice control					
	Jasmine 85	+			
	Khao Dawk Mali 105	+		VND95-20	-

^a(+) aroma, (-) non-aroma^bSDK: seed bank number

11.3 Methods

11.3.1 Preparing a Mutant Population

1. Irradiate seed (*see Note 4*).
2. Sow seed in a field nursery.
3. Transplant mutant plants to an experimental field after 25 days nursery growth.
4. Propagate material until the M₃ generation (*see Note 5*).
5. Begin phenotypic evaluation in the M₃ generation (*see Note 6*).
6. Select interesting plants for further characterization and hybridization (*see Note 7*).
7. Continue phenotypic evaluations (*see Note 7*).

11.3.2 *Phenotypic Analysis of Aroma*

1. Harvest seed when 90 % in panicles are ripe. Note that M₁ plants from seed mutagenized material is not suitable for phenotypic analysis as it is chimeric.
2. Air-dry until humidity reaches 14 %.
3. Dehull seed from individual plants.
4. Combine 40 seeds and 5 ml 1.7 % KOH in a test tube.
5. Cover tube and let stand for 15 min at room temperature.
6. Evaluate aroma by smell (*see Note 8*).

11.3.3 *Genotypic Analysis of Aroma*

1. Extract genomic DNA from selected material (*see Note 9*).
2. Design primers for a PCR-based marker assay (*see Note 10*).
3. Combine 0.25 µl Taq DNA polymerase, 1 µl of genomic DNA, 2.5 µl of 10× buffer, 3 mM MgCl₂, 4 µl of dNTPs and 2.0 µl of each primer in a total volume of 25 µl.
4. Incubate samples: 94 °C for 2 min followed by 35 cycles of 5 s at 95 °C, 5 s at 58 °C and 5 s at 72 °C, concluding with a final extension of 72 °C for 5 min.
5. Analyse PCR products using a 2 % agarose gel containing 0.5× TBE (*see Note 11*).

11.3.4 *Chromatographic Analysis of Aroma Compounds*

1. Place 3.5 g of milled rice with 500 µl of water in a 10 ml vial (*see Note 12*).
2. Equilibrate samples at 80 °C for 5 min.
3. Introduce a Supelco[®] VB/Carboxen/PDMS (divinylbenzene/Carboxen/polydimethylsiloxane) fibre in the headspace surrounding the rice at the same temperature for 15 min for solid phase microextraction (SPME) of aroma compounds.
4. Analyse extracts using a Hewlet Packard 5890 Série II gas chromatograph using a non-polar DB-5 (J&W Scientific) capillary column (length 60m, 0.32 mm, film thickness 0.25 µm).
5. Use helium as a carrier gas at a flow rate of 1.9 ml min⁻¹ at 25 °C.
6. Perform injection in splitless mode first (5 min for SPME), then in split mode to the end of the cycle (38.5 min for SPME).
7. Warm the column at 40 °C for 5 min, then apply the following temperature programmes for SPME: from 40 to 115 °C at a rate of 3 °C/min, then from 115 to 220 °C at 30 °C/min, and finally maintain at 220 °C for 5 min.
8. Maintain the detector port at 250 °C.

9. Calculate 2AP from the area ratio between 2AP's peak and the internal standard (*see* Sect. 11.5 for example data).

11.3.5 Combination of Traits Through Hybridization

1. Select materials for crossing (*see* Note 13).
2. Perform crosses to generate F₁ material.
3. Perform phenotypic evaluation of F₁ material (*see* Note 14).
4. Self-fertilize material to produce a segregating F₂ population.
5. Begin phenotypic and genotypic evaluations and selections to choose material for further propagation.
6. Continue propagation for several cycles (e.g. F₁₁) to ensure that traits are fixed and pure bred (*see* Fig. 11.1 and Sect. 11.5.1 crossing schemes to combine and fix traits in rice).

11.4 Notes

1. It is good practice to choose, when available, multiple accessions having similar traits. This material will be used in hybridizations with mutant lines. Having a diverse set of starting material diversifies the alleles for introgression and should increase the chances of success. In the example, 20 Vietnamese local aromatic rice varieties that were tall, bold grain shape and short-day length photoperiod sensitive were chosen. They have had hard texture when cooked and the average yield is approximately 3–4 tons/ha. Nine aromatic ST rice varieties and Hoa Sua, Huong Com have growth duration of 105–115 days, improved phenotype with 5–6 ton/ha yield and high-quality grain length 7.5–7.8 mm, slender grain, non-chalkiness of endosperm and amylose content of 17–19 %.
2. Seed for irradiation should be homogeneous and crossable with the other accessions chosen. In the example provided in this protocol, Zazu and Huyet Rong, a wild-type, tall and photoperiod-insensitive variety, was selected. The average yield is approximately 3–4 tons/ha. This is a local aromatic variety specific to Vietnam, having hard texture when cooked, aroma, long grains, dark-red bran layer and purple-yellow husk.
3. Other sources of gamma irradiation such as caesium and other types of mutagenesis such as X-ray irradiation and also chemical mutagenesis can be used. It is important that the mutagenic treatment is optimized.
4. The procedure to obtain the mutants may be summarized as follows: Seeds of Zazu and TT1 were incubated in a water bath at 33 °C for 48 h to induce germination to obtain high-frequency gene mutation. They were then irradiated by gamma-rays from a ⁶⁰Co facility at two doses: 12 krad (120 Gy) and 15 krad

- (150 Gy). After 24 h, the seeds were sown in a field nursery to obtain the first generation (M_1). Phenotypic selections began at the M_3 generation selected by staff Le Xuan Tham and Nguyen Thi Thu Hien.
5. A bulking procedure can be used until interesting phenotypes are identified. At this point a pedigree approach should be taken whereby mutations are fixed. Individuals are propagated through self-fertilization, and phenotypes are evaluated and selected at each generation to develop pure-bred lines. In our work we carried mutant lines to M_{10} and hybridized material to F_{11} . Further description of creating pure-bred material from initially bulked material is described in Chap. 9.
 6. In addition to specific traits of interest, it is useful to collect data on general traits. Data on plant height (cm), number of effective tillers/plant, panicle length (cm), number of filled grains/panicle, 1000 seed weight (gr), days to maturity and grain yield (ton/ha) were recorded in our example. After harvesting, the seeds of each genotype were dehulled for evaluation of the grain quality, viz. grain size (grain length), grain shape (grain length-breadth ratio) and also aroma. In addition, in our work analysis for bioavailable iron was conducted by atomic absorbance spectrophotometer (AAS) method and the related methods at Da Lat Nuclear Research Institute. See Sect. 11.5 for example data.
 7. It is useful to phenotypically characterize accessions prior to using in hybridizations to ensure plants actually show the desired traits. For example, in our work we chose to perform analysis for bioavailable iron conducted by atomic absorbance spectrophotometer (AAS) method and the related methods at Da Lat Nuclear Research Institute. See Sect. 11.5 for example data. Analysis of mutant plants can continue until desired mutant traits are found. During the early stages of propagation (e.g. M_3), there is a chance that other mutations (alleles) are co-segregating and epistatic interactions may limit the ability to identify plants with interesting traits. As propagation proceeds, alleles should segregate away from each other and phenotypes stabilize. At some point the chance of finding new traits is low. We typically do not carry observations past the 11th generation.
 8. The detection of fragrance can be carried out *via* sensory or chemical methods, although each has their disadvantages. Chemical methods involving smelling leaf tissue or grains after heating in water or reacting with solutions of KOH (Sood and Sidiq 1978) can cause damage to the nasal passages. Sensory methods therefore have their limitations when processing large numbers of samples, but it gives results in a shorter time and at lower costs for the rice breeder. Aroma evaluation by smell is subjective. It is ideal to select a group of people for the evaluation and average the scores. For example, there were five people in our testing team, they smelled at well-aired places and classified the aroma by four groups: strongly aromatic (score 7), moderately aromatic (score 5), lightly aromatic (score 3) and non-aromatic (score 1). The score of one sample is the average score of three repeated times of smelling, each time is 10 min apart. It is useful to include control material known to have good aroma

and control material known to have poor aroma when performing subjective sensory methods.

9. Methods for low-cost extraction of genomic DNA are described in Chap. 14.
10. Molecular markers may have been developed for traits arising from natural alleles that have spread in populations through evolution and breeding. In the case of rice aroma, the accumulation of 2AP in aromatic rice is explained by the loss of function mutations in the *badh2* gene (Bradbury et al. 2005; Chen et al. 2008). At least ten non-functional alleles of the *badh2* gene have now been identified (Shi et al. 2008; Sakthivel et al. 2009; Kovach et al. 2009). We used this data to develop four primer sets to evaluate aroma markers in varieties from Vietnam using a simple PCR assay to evaluate differences in amplicon mobility (*see* Sect. 11.5). It is important to note that this approach is inefficient and difficult when evaluating new alleles created by mutation because newly induced mutations did not previously exist in the population and could be in any gene or regulatory region. To create markers for mutant alleles, the best approach is traditional mapping/cloning or through the aid of genome sequencing (*see* Chap. 1).
11. Suitable primer combinations show clearly different band mobilities. Higher-resolution gels such as polyacrylamide can be used when agarose is insufficient.
12. For samples analysed by SPME-GC, collidine was added as an internal standard.
13. It is best to choose well-characterized materials for hybridization.
14. This is important when using mutant material to confirm if alleles are dominant or recessive. If desired combinations of traits are observed in the F_1 , one can consider doubled haploid approaches to instantly fix alleles. Doubled haploidy (DH) can also be applied in the F_2 or later generations. Chapter 16 of this book provides a protocol on validation of putatively DH plants.

11.5 Example Data

11.5.1 *Breeding New Aromatic Rice in High Bioavailable Iron by Using Gamma Radiation and Crossing*

11.5.1.1 *Results of Aroma Testing by Sensory from M_3 to M_{10} Generations*

We selected dark-red bran layer rice grains and tested aroma in rice. Results of line choice and aroma testing by sensory from M_3 to M_{10} generations (Table 11.2) showed that the rate of strong aroma increased from 8.22 to 12 % and aromatic lines of total aroma tested lines increased correlatively from 30.14 to 60 %, and the mean aroma score of populations increased from 3.55 to 4.68. From M_3 we got 5311 M_3 plants (Table 11.2). From this, 111 M_3 plants were chosen for testing aroma.

Non-aromatic lines fluctuated from 35.14 % in the M₃ generation to 8.61 % in M₅ generation; therefore, average aroma score of the population continuously increased after each generation. From seventh to tenth generations, there were no non-aromatic lines and five strong aromatic lines were selected in M₁₀. These strong aromatic lines were planted consecutively and without replications to evaluate several agronomic criteria such as length of flag leaf, length of dynamic leaf, panicle length, number of tillers/hill and panicles/hill, hypothetical yield and actual yield. External characteristics such as grain length, grain width, grain shape and zero-score chalkiness of endosperm indicated that zero-score chalkiness of endosperm of these mutant rice lines fluctuates from 65.2 to 78.1 % and long and slender grains (ratio of length to width was 3.2–3.4), so these lines have nice grain shape and good quality and value. They have low gelatinization temperature, medium gel consistency and amylose content between 17.3 and 21.1 %; therefore, they are classified as soft rice which is still soft over 24 h after cooking. Moreover, they have long-lasting aroma and strong aroma. Comparing evaluated characteristics including the growth duration time (105 days) and bioavailable iron content, we chose two new aromatic rice varieties named as Red 06 (from 12 krad irradiation dosage) and Red 156 (from 15 krad irradiation dosage). Bioavailable iron content of Red 06 and Red 156 are 44.4 ppm and 35 ppm, while bioavailable iron content of Zazu is 19.5 ppm.

11.5.1.2 Hybridizations to Create High Aroma and Iron Rice

The availability of the rice mutant resource is already helping researchers in their quest to gain insights into the biology of this commercially important crop. These efforts are critical to understand gene function and breeding. Some promising mutant rice lines are discovered, but some characters need to be modified. Therefore we elaborated a strategy in which we use these promising mutant rice lines for crossing with other rice varieties (e.g. Fig 11.2).

Results of lines chosen and aroma testing (Table 11.3) showed that the rate of slightly aromatic and non-aromatic lines was high at early generations, and it gradually decreased until traits were fixed in selected lines.

Nine strongly aromatic lines which were selected were also taken for analysis for bioavailable zinc and bioavailable iron contents by AAS method (Table 11.4). The results showed that zinc content of the nine lines is the same as that of existing inbred varieties.

11.5.1.3 Further Hybridizations to Combine Traits

In the scope of this research, we selected four different parental lines for breeding. Parental lines selected for breeding were evaluated for their agronomic and quality traits. The results are shown in Tables 11.5 and 11.6.

Table 11.2 The result of aromatic lines selected over segregating generations

G ^a	Lines used for aroma testing	Aromatic scale								Mean aroma of populations (scale)
		7	%	5	%	3	%	1	%	
Using gamma Co ⁶⁰ at 12 krad dosage										
M2	22	1	4.55	4	18.18	11	50.00	6	27.27	3.00
M3	73	6	8.22	22	30.14	31	42.47	14	19.18	3.55
M4	479	43	8.98	165	34.45	185	38.62	87	18.16	3.69
M5	246	25	10.12	99	40.08	91	36.84	32	12.96	3.95
M6	166	19	11.45	81	48.80	57	34.34	9	5.42	4.33
M7	71	8	11.27	34	47.89	29	40.85	0		4.41
M8	34	4	11.76	19	55.88	11	32.35	0		4.59
M9	26	3	11.54	16	61.54	7	26.92	0		4.69
M10	25	3	12.00	15	60.00	7	28.00	0		4.68
Using gamma Co ⁶⁰ at 15 krad dosage										
M2	27	2	7.41	5	18.52	5	18.52	15	55.56	2.56
M3	111	2	1.80	11	9.91	59	53.15	39	35.14	2.57
M4	432	8	1.85	55	12.73	254	58.80	115	26.62	2.80
M5	267	10	3.75	54	20.22	180	67.42	23	8.61	3.38
M6	379	12	3.17	102	26.91	265	69.92	0		3.66
M7	132	14	10.61	74	56.06	44	33.33	0		4.55
M8	173	17	9.83	105	60.69	51	28.48	0		4.61
M9	121	12	9.92	76	62.81	33	28.70	0		4.65
M10	52	5	9.62	33	63.46	14	26.92	0		4.65

^aG Mutant generation

According to quality characteristics shown in Table 11.5, mutant Tam Thom lines T1 and T2 have a very high rate of chalkiness of endosperm which is an obstacle in cross-breeding. However, T1 and T2 are not photosensitive to short-day light and have several other important characteristics such as maintenance of aroma of Tam Thom, closeness between grains, moderate susceptibility to leaf blast (caused by *Pyricularia oryzae*) and low amylose content. Mutant Tam Thom T1 line has brown–yellow colour of grain husk and aroma score of 4.1 which is nearly equal to the aroma score of Tam Thom Hai HauTT1. Tam Thom Hai HauTT1 is highly resistant to leaf blast, and Hoa Sua is an extra long and slender grain, very early maturing and semidwarf. These are the basic characteristics transferred into progenies to create specific characteristics of new rice varieties (Fig. 11.3).

In classical plant breeding, selection typically involves evaluating a breeding population for one or more traits at field trials. In pedigree breeding method, selection of desirable plants is made at early generations for traits of higher heritability. So, effective phenotypic screening will be less expensive for selection in large populations. These F₂ populations were developed, and stringent phenotypic selection based on phenotypic preference (like early maturity, panicle length, grain shape, aroma, etc.) was carried out on segregating populations to obtain

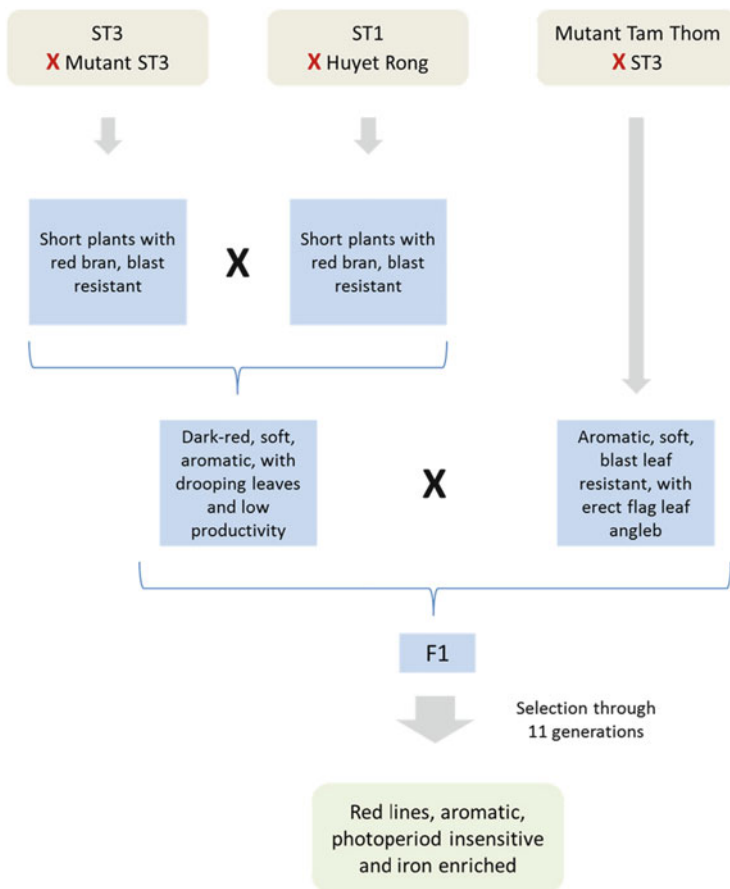


Fig. 11.2 Hybridization between five parents by cutting husks method. Crossing begins between ST3 and mutant ST3 (*top left*), ST1 and Huyet Rong (*middle*) and ST3 and Mutant Tam Thom (*right*). Resulting plants are phenotyped and plants with desired traits are selected (*blue boxes*). Hybridization of these materials is done and then is propagated through 11 generations to produce a line with the desired combination of traits (*bottom box*). Since the F_2 population contains heterozygotes, and heterozygote and dominant homozygote are not easily distinguishable, crossing is continued. Strongly aromatic lines continued to separate highly into four groups such as strongly aromatic, medium aromatic, slightly aromatic and non-aromatic at early generations, and the separation decreased through propagation until traits are pure bred. The rate of strongly aromatic and aromatic lines increased from 1.79 to 8.04 % of total aroma tested lines correlatively from F_2 generation to F_{11} generation, and the mean aroma score of populations increased from 2.35 to 4.69. To the F_{11} generation, we have selected 9 strongly aromatic and 75 aromatic lines. Figure courtesy of Dr Joanna Jankowicz-Cieslak and Dr Bradley J. Till of the FAO/IAEA Joint Programme

agronomically desirable plants and reduce the population size. Selection of desirable plants is also similar in the next segregating populations until pure breeding lines are produced.

Table 11.3 The result of aromatic lines selected over segregating generations

G ^a	Lines used for aroma testing	Aromatic scale								Mean aroma of populations (scale)
		7	%	5	%	3	%	1	%	
<i>Separating generations of five parents (RED ST)</i>										
ST3/Mutant ST3//ST1/Huyet Rong/// Mutant Tam Thom T5/ST3										
F2	3120	56	1.79	312	10.00	1320	42.31	1432	45.90	2.35
F3	4651	74	1.59	501	10.77	1812	38.96	2064	44.38	2.26
F4	4551	79	1.74	513	11.27	2013	44.23	1946	42.76	2.44
F5	3656	65	1.78	451	12.34	1751	47.89	1389	37.99	2.56
F6	3541	79	2.23	564	15.93	2015	56.90	883	24.94	2.91
F7	3621	110	3.04	662	18.28	2068	57.11	781	21.57	3.06
F8	2893	211	7.29	711	24.58	1720	59.45	251	8.68	3.61
F9	2113	189	8.94	611	28.92	1142	54.05	171	8.09	3.77
F10	1723	162	9.40	752	43.64	743	43.12	66	3.83	4.17
F11	112	9	8.04	75	66.96	29	25.89	0	0	4.69

^aG mutant generation**Table 11.4** The evaluation results of bioavailable zinc and bioavailable iron contents of the nine lines plus control

No.	Name of lines	Bioavailable zinc content (ppm)	Bioavailable iron content (ppm)
1	R110-755	21.2 ± 2.1	14.6 ± 1.0
2	R34RD-840	25.6 ± 2.2	24.8 ± 1.9
3	R35RD-869	21.1 ± 1.7	19.8 ± 1.2
4	R75-797	20.8 ± 2.0	21.8 ± 1.1
5	R76-696	21.0 ± 1.8	65.1 ± 2.3
6	R75-747	21.0 ± 2.1	15.7 ± 1.2
7	R51-723	24.5 ± 1.9	25.8 ± 1.4
8	R857-821	29.8 ± 2.5	44.3 ± 2.2
9	R8-786	23.7 ± 1.6	22.2 ± 1.2
10	Jasmine 85		12.8 ± 0.9

Table 11.5 Agronomic characteristics of rice materials

Name of parents	Growth duration (days)	Plant height (cm)	Brown grain length (mm)	Reaction to brown plant hoppers (scale)	Reaction to leaf blast (scale)
Mutant T1	95	115 ± 5.7	7.0 ± 0.2	7	5
Mutant T2	99	101 ± 3.2	7.1 ± 0.2	7	5
Tam Thom Hai HauTT1	118	145 ± 6.2	5.6 ± 0.2	3	2
Hoa Sua	90	100 ± 1.0	7.8 ± 0.1	9	6

Table 11.6 Quality characteristic of parents

Name of parents	Weight of 1000 grains (gramme)	Zero-scale chalkiness of endosperm (%)	Gelatinization temperature (scale)	Amylose content (%)	Gel consistency (mm)	Aroma (scale)
Mutant T1	19.6 ± 0.3	14.6 ± 4.0	6.6 ± 0.5	18.4 ± 1.4	71.5 ± 1.9	4.1 ± 0.04
Mutant T2	19.7 ± 0.3	22.4 ± 2.4	6.4 ± 0.5	19.5 ± 0.9	61.2 ± 2.1	3.4 ± 0.04
Tam Thom Hai Hau TT1	19.1 ± 0.3	97.6 ± 1.1	5.6 ± 0.5	16.9 ± 0.4	77.7 ± 1.7	4.4 ± 0.04
Hoa Sua	24.1 ± 0.3	99.8 ± 0.8	5.7 ± 0.2	12.1 ± 0.4	60.2 ± 3.5	2.7 ± 0.81

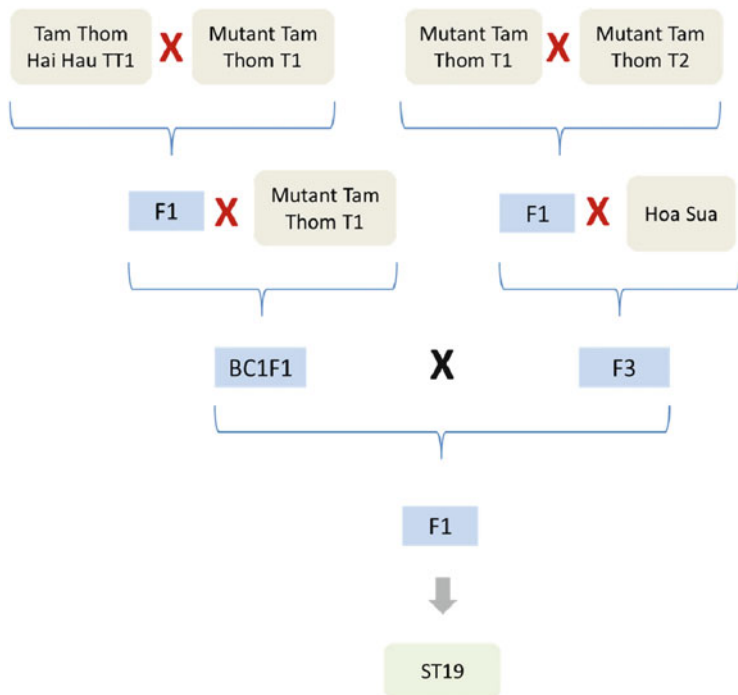


Fig. 11.3 Crosses carried out to produce the variety ST19 using varieties and advanced mutant lines. Successive rounds of back- and outcrosses were carried out to create a line with combined characteristics. Figure courtesy of Dr Joanna Jankowicz-Cieslak and Dr Bradley J. Till of the FAO/IAEA Joint Programme

Table 11.7 The result of aromatic lines selected over segregating generations

G ^a	Lines used for aroma testing	Aromatic scale								Mean aroma of populations (scale)
		7	%	5	%	3	%	1	%	
<i>Separating generations of four parents (ST19)</i>										
	TT1/2*Mutant Tam Thom T1	Hoa Sua/Mutant Tam Thom T2				Mutant Tam Thom T1				
F2	2527	56	2.22	817	32.33	1326	52.47	328	12.98	3.48
F3	427	17	3.98	151	35.36	208	48.71	51	11.94	3.63
F4	268	18	6.72	122	45.52	101	37.69	27	10.07	3.98
F5	333	30	9.01	168	50.45	130	39.04	5	1.50	4.34
F6	493	53	10.75	264	53.55	176	35.70	0		4.50
F7	277	30	10.83	148	53.43	99	35.74	0		4.50

^aG segregating generation

In segregating populations of three combinations, we separated aroma into four groups such as strongly aromatic (score 7), moderately aromatic (score 5), lightly aromatic (score 3) and non-aromatic (score 1) (Table 11.7). Resulting lines from the hybridizations were further characterized for traits including amylose, 1000 grain weight and iron (Table 11.8).

11.5.1.4 2-Acetyl-1-pyrroline Analysis in Aromatic Rice

The high demand for fragrant rice cultivars in markets worldwide has driven the development of methods for quantifying 2-acetyl-1-pyrroline (2AP) and distinguishing fragrant and nonfragrant cultivars. The concentration of 2AP is controlled by a recessive gene for fragrance (*mgr*) mapped on rice chromosome 8. Methods for the determination of the volatile compounds in rice have schemes for collection, concentration, separation and quantification. The method of preference for the pre-concentration of flavour compounds is solid phase microextraction (SPME).

We determined the concentration of 2AP in 62 samples of rice grains (brown rice). The concentration of 2AP by SPME-GC analysis varied among seasons and ecological cultivated sites (Table 11.9).

11.5.1.5 Molecular Screening for Aroma in Segregating Rice Lines

To facilitate the selection of plants with improved aroma coming from natural alleles, we used a molecular marker strategy to test for the *badh2* gene. Variations in this gene are thought to have major effects on rice aroma (Bradbury et al. 2005b; Chen et al. 2008). An example gel showing size polymorphism variation between aromatic and non-aromatic rice is shown in Fig. 11.4.

Table 11.8 Characteristics of high bioavailable iron rice cultivars and aromatic rice cultivars

No.	Characters	Red 06	Red 11	Red 156	Aromatic rice 12	RED ST	ST16	ST19	ST20
1	Growth duration time (days)	105	105	108	110	108	112	95-105	115
2	Flag leaf length (cm)	30.4	26.0	38.1	30.9	31.0	38.1	25.48	32.1
3	Plant height (cm)	121.5	91.1	127.5	111.1	112.5	90.5	91.26	102
4	Length of panicles (cm)	28.6	26.1	28.1	29.5	27.5	26.3	26.18	25.7
5	Tillers/hill	16.5	18.5	18.4	22.2	13.7	10.3	10.79	16.2
6	Panicles/hill	11.8	14.5	15.2	14.2	9.8	8	7.76	8.5
7	Weight of 1000 grains (gramme)	24.6	23.3	25.8	27.9	27.8	25.5	23.01	25.3
8	Grains/panicle	135.6	115.0	112.2	117.5	110.0	133	151	131
9	Total filled grains/panicle	103.5	108.1	81.5	103.0	94.9	121	101.83	88.8
10	Length of kernel (mm)	6.7	6.6	6.7	7.8	7.5	8.6	7.65	8.2
11	Width of kernel (mm)	2.0	1.8	2.1	1.8	2.0	1.8	1.72	1.7
12	Zero-score chalkiness of endosperm (%)	78.1	95.0	75.2	95.2	88.0	100	100	100
13	Gelatinization temperature (scale)	6.0	6.0	6.0	7.0	7.0	7.0	7	6.8
14	Gel consistency (mm)	65.6	63.5	54.4	65.4	75.0	67	55.73	64.7
15	Amylose content (%)	18.5	15.6	17.4	19.1	13.12	14.1	10.6	12.4
16	Aroma	Strong	Strong	Light	Strong	Strong	Strong	Strong	Strong
17	Yield (ton/ha)	4.7	5.6	5.1	6.7	4.5	5.1	5.2	5.7
19	Bioavailable iron content (ppm)	44.4	55.1	35		65.1		10.8	
20	Brown rice colour	Red	Red	Red	White	Red	White	White	White

Table 11.9 The change of aroma content in aromatic rice among growing seasons

Cultivated name	2AP content (ppb)		Sensory test by smelling (scale)	
	Dry season	Wet season	Dry season	Wet season
ST3	6.03	1.77	5.3	1.9
ST10	6.80	2.40	4.5	2.5
ST12	8.73	2.86	5.1	2.5
ST16	5.97	2.84	5.8	3.4
ST17	6.83	2.63	4.2	3.3
ST18	4.47	2.07	4.9	3.7
ST19	4.90	2.57	5.0	2.6
ST20	8.83	1.58	4.3	2.9
Jasmine 85	6.10	2.52	4.7	2.9

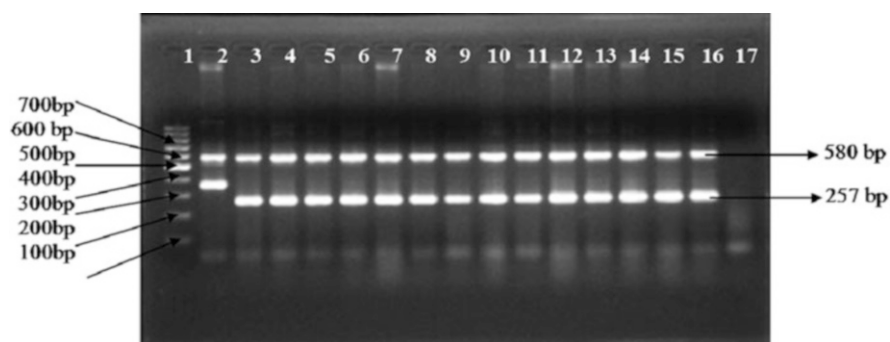


Fig. 11.4 PCR analysis of new aromatic rice varieties for the presence of aroma gene. Lanes 1, 100 bp ladder; 2, VD95-20 is non-aromatic; 3, ST10 is aromatic; 4, Red ST is aromatic; 5, 6, 7, Red 06 from 12 krad; 8, 9, 10, Red 156 from 15 krad; 11, 12, 13, Red 11 from mutant Zazu/Red ST; 14, 15, 16, aromatic rice 12 from mutant Zazu/ST10 and 17, water

11.6 Conclusion

Breeding aromatic rice varieties having high quality and yield in order to increase value and to serve increasing demand of customers domestically and for exportation is essential. Multiline crossing was carried out on mutant Tam Thom, and aroma improved rice varieties by using pedigree method. After a strict process of selection by qualitative and quantitative anticipated targets (life cycle, leaf and stem phenotypes, grain dimensions, aroma), we have selected aromatic rice varieties named ST16, ST19 and ST20. These varieties have growth duration of 95–115 days, improved phenotype (plant height of 102 cm, strongly tillering) with actual yield higher than 5 ton/ha and high-quality grain length >7.5 mm, slender grain, non-chalkiness of endosperm and low amylose content. Their grains are of high quality so they have high economic value.

Aroma in 20 Vietnamese local aromatic rice varieties (19 varieties of Tam group in the north of Vietnam and Nang Thom Cho Dao in the south of Vietnam) and Red

06, Red 156, Red 11, aromatic rice 12, Red ST, ST16, ST19 and ST20 are explained by the *badh2.1* allele. Aroma sensory and 2AP content in the dry season were higher than in the wet season. Rice aroma was segregating in before the establishment of pure-bred lines necessitating phenotypic and genotypic screening of material. Breeding would be further aided through establishing the interrelated scale between aroma scale and 2AP content. The strategy of mutant generation and selection and hybridization with accessions harbouring natural alleles allowed us to combine novel and existing traits to create new rice varieties. The methods can be adapted for other breeding objectives in rice and other seed-propagated crops.

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Chapter 12

Utilising NIRS for Qualitative and Non-destructive Identification of Seed Mutants in Large Populations

Johann Vollmann and Joanna Jankowicz-Cieslak

Abstract Phenotyping of large plant populations for genetic research or plant breeding is often time-consuming and expensive. Seed composition is a primary breeding objective as this determines quality for various markets, e.g. food, fodder and industrial processing. Near-infrared reflectance spectroscopy (NIRS) is a fast developing analytical tool for seed composition screening. For example, it is utilised in plant breeding programmes to predict compositional concentrations in various samples. NIRS can be used to detect variation between seed lots and between individual seeds and can be used to identify and isolate new phenotypes including mutants based on spectroscopic sample properties. Spectral data of seed samples may be subjected to principal component analysis to separate groups and individuals with distinct compositional properties. Spectroscopic outliers such as mutants with novel seed quality alleles may then be selected based on principal component scores. Outliers represent a small subset of the entire population, and these may be subject to more rigorous analyses (chemical, physiological and genetic). In determining their potential exploitation, NIRS is a high-throughput phenotyping platform that can be used to reduce large sample sizes, e.g. a mutant population to manageable numbers.

Keywords Near-infrared reflectance spectroscopy • Spectroscopic fingerprinting • Outlier identification • Mutant preselection • High-throughput phenotyping

J. Vollmann (✉)

Plant Breeding Division, Department of Crop Science, University of Natural Resources and Life Sciences Vienna (BOKU), Tulln an der Donau, Austria
e-mail: johann.vollmann@boku.ac.at

J. Jankowicz-Cieslak

Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, IAEA Laboratories Seibersdorf, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria

12.1 Introduction

Field, glasshouse and laboratory-based phenotyping of large and genetically diverse populations are often time-consuming and costly and are considered a major bottleneck in plant genetic improvement (Araus and Cairns 2014). Rapid developments in genotyping have generated massive data banks, but this has not been matched by phenotyping; this has led to the so-called phenotype gap which limits our ability to link genes to traits. Near-infrared reflectance spectroscopy (NIRS) is a fast analytical method based on reflection/absorption from functional chemical groups in the near-infrared region of light (about 800–2500 nm wavelength range). This is suitable to predict the concentration of major organic components in seed or other biological materials without requiring extensive sample preparation. Non-destructive NIRS methods enable rapid prescreening that may be performed on seed prior to field or glasshouse propagation. While most NIRS applications are implemented in agriculture, food and environmental industries, it is also becoming popular in pharmaceutical and medical fields due to improvements in instrumentation and in statistical analysis (Bosco 2010). Owing to the low per-sample cost and speed of measurement and analysis, NIRS has the potential to serve as a component of high-throughput phenotyping platforms in various crop improvement and quantitative genetics studies.

In plant breeding, NIRS has been utilised in two ways. Quantitative analysis involves the prediction of concentrations of identified components. In this approach, calibration standards need to be developed to assign measured spectra to specific components accurately (e.g. Sato et al. 2012; Xie et al. 2014). The second major approach is qualitative analysis. This involves the classification of samples according to their spectroscopic properties. Samples are clustered according to their similarity/dissimilarity (Munck 2007). This is based on “spectroscopic fingerprints” of each sample. Different statistical criteria can be applied to separate outliers from “normal” samples. This approach is ideally suited to projects involving large populations where outliers are expected at a rare frequency, such as with chemically or irradiated mutant plant populations. Low densities of induced mutations typically mean that many thousands of plants need to be screened to identify rare variants with the desired improved trait (Jankowicz-Cieslak et al. 2011). Moreover, in contrast to quantitative analysis for predicting concentrations of individual analytes, qualitative NIRS analysis does not require reference samples and reference chemistry analysis for the development of calibrations.

Apart from genotype identification and verification based on seed (Turza et al. 1998; Wu et al. 2008), NIRS can be used in the identification of transgenic food materials (Alishahi et al. 2010) or seeds, such as separating RoundupReady® from non-GMO soybeans (Esteve Agelet et al. 2012). NIRS can also be used in mutant identification, for example, barley endosperm mutants such as those expressing high lysine or high and low beta-glucan can be differentiated from normal barley genotypes based on their chemometric patterns (Jacobsen et al. 2005). In wheat, starch mutants carrying non-functional alleles in the amylose

synthesis gene resulting in the “waxy” phenotype can be detected by NIRS (Delwiche et al. 2011). A major step forward in NIRS is the analysis of single seeds, e.g. NIRS analysis can distinguish individual amylose-free seeds in both hexaploid and tetraploid wheat (Dowell et al. 2009). Thus, waxy wheat seeds could be selected in segregating populations, for purifying advanced breeding lines or for mutant identification and isolation. In maize, prediction of constituents such as starch, oil or protein concentration and seed weight is feasible on individual seeds through classical calibrations based on partial least square regression statistics, while oil, starch and protein mutant phenotypes can be identified from principal component analysis of spectral data, implemented using single seed glass tube NIR spectrometer. The design of the instrument enables high-throughput data collection and is of great interest for single seed-based selection, genetic screening and seed phenomics (Spielbauer et al. 2009).

Evidence from different crop species demonstrates the potential of utilising NIRS spectral data for classifying seed samples according to spectral similarity. A major concern in identifying mutants (traditionally mutagenised by seed treatments with chemical or physical mutagens) is their low frequency; usually thousands of individuals or lines must be evaluated to identify the rare novel phenotypes of interest. We have developed a high-throughput preselection method utilising qualitative NIRS analysis of rice seed in which rare and novel phenotypes can be identified. Since the outliers are from the same population and share a common and highly homogenous genetic background, any change is easily picked up and is a potential mutant that may be validated by further analyses. A practical, user-friendly method for NIRS-based screening of mutant seed populations is given below for spectroscopic outlier detection.

12.2 Materials

12.2.1 *Equipment and Hardware*

1. NIRS equipment, e.g. Bruker Matrix-I FT-NIR machine (*see Note 1*)
2. Mill, equipped with grid nets for uniform grinding, e.g. CT 1093 Cyclotec Sample Mill (FOSS, Sweden)
3. Funnel
4. Spoon
5. Brush
6. Small scale vacuum cleaner
7. Sample glass cuvettes compatible with NIRS equipment

12.2.2 Software

1. OPUS 7.5 (Bruker, Ettlingen, Germany) or software compatible with NIRS equipment
2. Unscrambler[®] (Camo Software AS, Oslo, Norway)
3. Standard spreadsheet software for data handling

12.2.3 Plant Materials

1. Mutant populations (*see Note 3*).
2. Dry seeds with moisture content below 14 % (*see Notes 4 and 5*).

12.3 Methods

12.3.1 Seed Preparation

1. Dry mature seed *per* mutant population or line to have moisture content below 14 %, in a standard desiccator or a dry room under ambient conditions (*see Note 5*).
2. Grind seed to a fine powder (*see Note 6*).

12.3.2 NIRS Analysis

12.3.2.1 Destructive Approach

1. For each accession or a mutant line, grind 3–5 g of dry seeds (*see Note 7*).
2. Disassemble the mill and clean it with a vacuum cleaner and brush so that no particles of the previous sample remain (*see Note 8*).
3. Fill the sample containers with the milled sample according to manufacturer's instructions.
4. Scan sample in the NIRS instrument to collect and record spectral reflectance characteristics of the samples (*see Note 9*).
5. Repeat the measurement 2–4 times, turning the sample cup or mixing the sample each time (*see Note 10*).
6. Perform statistical analysis (*see Notes 11–15*, Figs. [12.1](#), [12.2](#) and [12.3](#)).

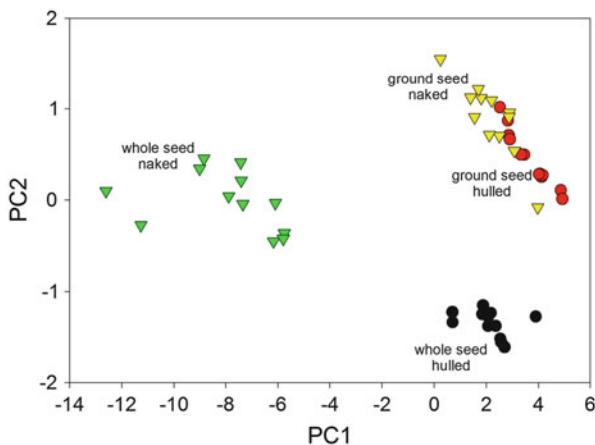


Fig. 12.1 Classification of a test set of 12 rice mutant lines based on principal component analysis of NIRS spectra as influenced by sample pretreatment such as dehulling or grinding of samples

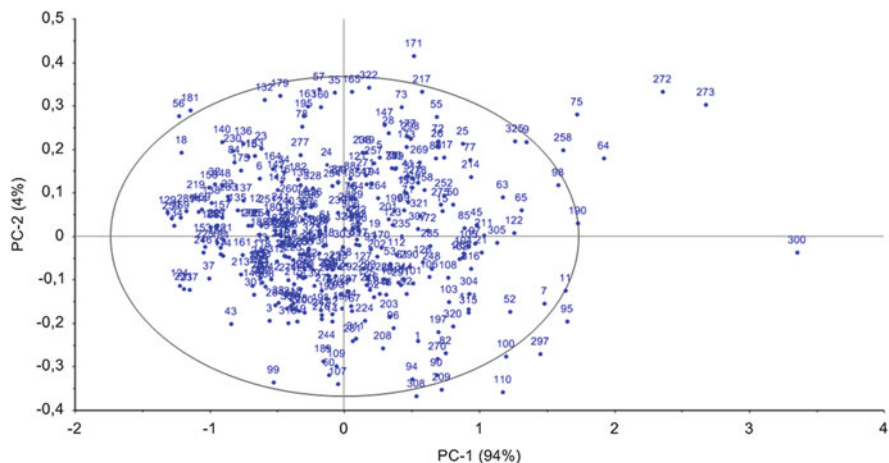
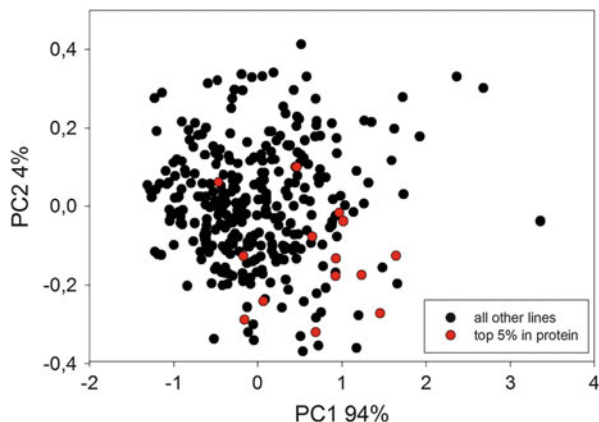


Fig. 12.2 Classification of a mutant population of 329 rice samples using PC analysis of spectroscopic data. Spectroscopic outliers are visualised as samples outside the boundaries of a Hotelling's T2 statistics ellipse

12.3.3.2 Non-destructive Approach

1. For each accession/mutant line prepare 3–5 g of dry seeds.
2. Fill sample containers with milled samples according to manufacturer's instructions.
3. Scan sample in the NIRS instrument to collect and record spectral reflectance characteristics of the samples (*see Note 9*).
4. Repeat the measurement 2–4 times, turning the sample cup or mixing the sample prior to each measurement. If the amount of sample material permits, use a new portion for each measurement.

Fig. 12.3 Spectroscopic classification of 329 rices mutant lines with the top 5 % of samples highest in seed protein content highlighted in red



5. Keep the seed for future analysis or for mutant multiplication.
6. Perform statistical analysis (*see* **Notes 11–15**, Fig. 12.1, Fig. 12.2 and Fig. 12.3).

12.3.3 Statistical Analysis

1. Download spectroscopic data from the NIRS computer.
2. Import sample spectra to Unscrambler or other statistical software suitable for multivariate data analysis.
3. Carry out principal component analysis for data reduction and clustering of samples (*see* **Notes 12–15**).
4. Save PCA scores calculated for each sample of the population in a spreadsheet software programme.
5. Use PCA scores to plot x - y graphs of the samples to visualise clustering (similar phenotypes) and outliers (unique phenotypes).

12.4 Notes

1. Various types of NIRS instruments with different technologies are available on the market at present. Newer models often have better performance so that more spectral information can be recovered from a sample. Among important properties are resolution, light throughput, wavenumber accuracy, repeatability and signal-to-noise. Beside the near-infrared region (typically 800–2500 nm), some instruments have an extended wavelength range (e.g. 400–2500 nm) including the range of the visible spectrum of light; this may be of interest in measuring colour differences which can be caused by variation in carotenoids, anthocyanins or other components of seed samples. NIRS instruments also differ in

sample presentation methods which have an influence on surface reflection, particle size and other effects; depending on sample presentation modes, seed samples might either need to be finely ground, might be scanned non-destructively or analysed as single intact seeds. Here we provide Bruker Matrix-I as an example of a modern FT-NIR instrument which is very well suited for combined single seed and bulk material analysis.

2. Proprietary software is required for most instruments in order to carry out basic instrument operations such as optical adjustments or collection of spectral data from samples. Programmes are normally available to assist in the development and validation of chemometric calibrations from sample sets with reference data, or they can be used to predict seed composition from external calibrations. These are commercially available for major crop species and include the most important grain components such as for instance starch, protein and pigments.
3. Mutant populations should be developed following established procedures (Lee et al. 2014). It is important to note that the first (M_1) generation after mutagenesis is chimeric, and most mutations will not be heritable, also the M_1 generation suffers from physiological disorders as a result of mutagenic treatments. Therefore, mutation screening should not be carried out until the M_2 or subsequent generations. Seed weight and number are important considerations when selecting the generation to screen. One should select a generation where there is at least 3–5 g of seed *per* line.
4. In quantitative NIRS analysis for predicting particular analytes using calibrations, reference samples need to be collected from different environments (e.g. locations, growing seasons) to cover environmental variation. In qualitative NIRS analysis for classifying samples, spectral variation caused by environmental effects might bias classification results. Therefore, samples to be classified have to be grown in the same environment (field location, greenhouse) to avoid environmental effects. Moreover, repeated controls have to be used to be able to monitor and estimate environmental variation effects. A dry seed with moisture content below 14 % is a suitable material for NIRS analysis. Samples differing in water content cannot be analysed, as very broad water peaks in certain regions of the NIRS spectrum mask useful spectral variation between samples.
5. Generally, seed of cereal crops can be stored at a moisture level of 14 % or below. In dry environments, this level is reached at full maturity; in moist or high-humidity environments, some postharvest drying may be necessary. Moisture level of 14 % or below is reached when single seeds cannot be indented with finger nails.
6. Depending on seed size and the sample presentation method of the NIRS instrument, grinding of seeds into a homogeneous powder or replicated measurements (e.g. mix by inverting the vial 2–4 times) of the sample might be needed for avoiding surface reflection and to obtain representative and reproducible spectral data. Larger amounts of sampling materials measured in higher number of replications might avoid the need for grinding thus enabling non-destructive measurements in nonhomogenous (i.e. large seeded) samples. However, non-destructive measurement vs. grinding or dehulling of samples has a strong effect on spectroscopic sample classification, as shown in Fig. 12.1.

7. The method described here is a destructive method that involves grinding the entire seed (embryo and endosperm) to make the measurement. In advanced lines, induced mutations should segregate in a Mendelian fashion. In order to recover mutations identified in NIRS, one should retain 20 or more seeds *per* line.
8. Cross-over contamination from previous measurements can lead to errors.
9. Depending on the NIRS instrument and the measuring mode used, scanning a sample takes between 30 s and 2 min.
10. For qualitative NIRS analysis to classify samples, general statistical software packages suitable for data reduction and multivariate analysis could be utilised. A machine-independent software package dedicated to spectral data analysis (spectra pretreatment and transformation, calibration development and validation, multivariate classification, etc.) is Unscrambler[®] (Camo Software AS, Oslo, Norway), which is widely used in multivariate analysis of spectroscopic data.
11. In spectroscopic data analysis for sample classification, specific data pretreatment methods such as multiplicative scatter correction or standard normal variate transformation are used to adjust for radiation-scattering effects. Various other data pretreatment methods such as first or second derivative functions might be applied as well for reducing data noise and enhancing sample signals on a trial-error basis, but data pretreatment might cause information loss thus reducing the discrimination power of spectral data.
12. Spectral data are subject to principal component analysis (PCA), and PCA scores for samples are calculated and further used in score plots to visualise classification results. Either full spectra or specific wavelength regions can be utilised to calculate PCA models. PCA classification models can be validated, and models can be selected based on the level of total variance explained. In mutant populations, spectroscopic outliers can subsequently be detected based on the distance to untreated control genotypes of the same genetic background. In Fig. 12.2, 329 rice samples derived from a mutant population are classified by PCA analysis of spectroscopic data; outliers are visualised as samples outside the boundaries of a Hotelling's T2 statistics ellipse which could be subject to further analysis.
13. In sample populations segregating for qualitative characters such as the waxy wheat trait, samples with known group membership (e.g. waxy vs. normal starch) could be used as anchors to classify unknown samples or for developing discriminant analysis functions based on the PCA scores.
14. If calibrations for quantitative determination of individual analytes are available, they might be combined with spectroscopic classifications to gain additional information about individual samples. In Fig. 12.3, the sample set of Fig. 12.2 (329 rices samples, mutant population) was subject to quantitative analysis of seed protein content: The majority of the 5 % of samples with highest protein content are appearing in the lower right section of the scatter plot as highlighted. Using quantitative calibrations, the concentrations of various other seed components of interest can be calculated and considered for further selection steps, as given in Table 12.1 for the highlighted samples of Fig. 12.3.

Table 12.1 Protein content and other parameters of the top 5 % in protein content of Fig. 12.3 rice mutant population as compared to the control mean

Line	Ash	Fat	Fibre	Protein	Moisture
281	4.58	5.19	3.50	13.45	9.96
52	4.20	5.78	4.89	13.52	8.78
27	4.20	5.74	4.07	13.65	9.24
189	4.00	5.92	4.90	13.78	9.78
97	4.52	5.79	5.94	13.80	9.33
315	4.08	5.73	4.71	14.12	8.29
241	4.90	5.52	4.69	14.24	9.68
11	4.55	6.45	6.18	14.41	7.78
297	4.61	5.39	4.34	14.60	9.92
21	4.31	5.88	5.73	14.63	9.43
304	3.82	5.97	4.59	14.76	10.29
248	4.35	5.96	4.68	15.30	10.13
90	4.75	5.96	3.73	16.34	9.15
17	4.80	6.10	5.45	16.55	9.45
Control mean	3.75	4.94	5.51	11.25	4.65

15. False detections: In addition to genotype, the composition of a seed is influenced by the environment in which it develops. Environmental effects could be at the scale of the plant environment or the micro-environment in which the seed develops. For example seeds that develop at the extremities of an inflorescence may not receive the same nutrient supplies as those that develop in central locations and have better links to the vascular system; seed composition may also be affected by pests and diseases. Such affected seeds may be detected as having abnormal NIR spectra. Measures can be taken to discard such “off-type” seeds, e.g. they are often small or deformed and removed by sieving. Or they can be included in the preselections and retested in subsequent generations.

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Chapter 13

Proteome Analyses of *Jatropha curcas*

Fatemeh Maghuly, Gorji Marzban, Ebrahim Razzazi-Fazeli,
and Margit Laimer

Abstract Plant proteomes are complex and therefore their analyses represent major technical challenges. In fact, proteome analyses depend on several crucial steps, such as the amount of proteins, extraction, separation, visualization, identification, quantification, and the interaction between proteins and other molecules in a plant tissue at a given time.

To date it is recognized, that there is no single method able to describe an entire plant proteome. Even though several alternatives exist, the most widely used methodology for proteome analysis includes two-dimensional gel electrophoresis (2-DE), mass spectrometry, and bioinformatics tools.

The *Jatropha* proteome represents a valuable field of study related to gene functions since many biochemical pathways of fatty acids and secondary metabolites in this species provide an alternative potential source to fossil oil useful for production of biodiesel. Therefore, a detailed proteome analysis of *J. curcas* using gel-based electrophoresis combined with identification by mass spectrometric analyses could help to understand its potentials as a source of biofuel.

Due to the low correlation between protein expression level and mRNA in plant tissues, it is not advisable to predict a final amount of protein from quantities of mRNA. However, it is generally accepted that protein analysis allows to identify so far unknown genes and to assign them a function.

This chapter provides a selection of methods, reproducible with the highest resolution and quality of results from sample preparation to proteins identification of *J. curcas*.

Keywords Sample preparation • Protein extraction • SDS-PAGE • 2-DE • Coomassie staining • Silver staining • Mass spectrometry • Protein sequence databases

F. Maghuly • G. Marzban • M. Laimer (✉)

Plant Biotechnology Unit (PBU), Department of Biotechnology (DBT), University of Natural Resources and Life Sciences (BOKU), VIBT, Muthgasse 18, 1190 Vienna, Austria
e-mail: margit.laimer@boku.ac.at

E. Razzazi-Fazeli

VetCore Facility, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

13.1 Introduction

Jatropha curcas has attracted a great deal of attention worldwide, regarding its potential as a new energy plant. The press cake from seeds, rich in protein (60–63 %), could be an ideal protein source with a high content of essential amino acids, even higher than soybean (45 %) (Maghuly and Laimer 2013). However, seeds of toxic *J. curcas* contain a range of toxins and anti-nutritional compounds which render the seedcake and oil unsuitable for use as animal feed and for human consumption (Maghuly and Laimer 2013). Accordingly, in an effort toward better understanding, engineering, and to determine gene function simultaneously, the detail proteomics analysis of *J. curcas* is necessary (Maghuly and Laimer 2013).

It is well known that every plant cell type of *J. curcas* expresses a unique transcriptome and proteome at different stages of differentiation depending on its developmental fate. Knowing when and where a gene product (RNA and/or protein) is expressed can provide important clues to its biological function (Bouchez and Höfte 1998). The facility with which a high-throughput approach can be used for the study of gene expression depends largely on the level of regulation (Bouchez and Höfte 1998). To understand the function of all genes in a given organism, it needs to know not only, which genes are expressed, when and where, but also what the protein end products are and under which conditions they accumulate in certain tissues (Mathesius et al. 2003). At the transcriptome level, however, results must consider that mRNA abundance and protein level are not always correlated (Gygi et al. 1999; Maghuly et al. 2009), that low-copy number mRNAs (potentially very important for regulation) are not detected as readily as abundant mRNAs, and that gene expression studies do not provide reliable information about protein levels, the subcellular localization of gene products or the posttranslational modifications occurring to a protein that may be essential for its function, transport, and activation (Maghuly et al. 2009; Rose et al. 2004). This information will complete the annotation of genes and contribute to the identification of their function (Zivy et al. 2007).

Proteomics, a combination of several methodological approaches used to study the global changes in proteins, provide an essential bridge between the transcriptome and the metabolome, because the levels of various metabolites are the result of direct enzymatic activity and of indirect cellular regulation over transcriptional or biochemical processes (Zivy et al. 2007; Cook et al. 2004; Gray and Heath 2005; Kaplan et al. 2004).

Therefore, comprehensive proteomic studies may provide new information on proteins that play an integral role in functional genomics and on the gene coding sequence and increase the understanding of protein function in specific cellular compartments. Furthermore, proteomics provide major advantages linked to the

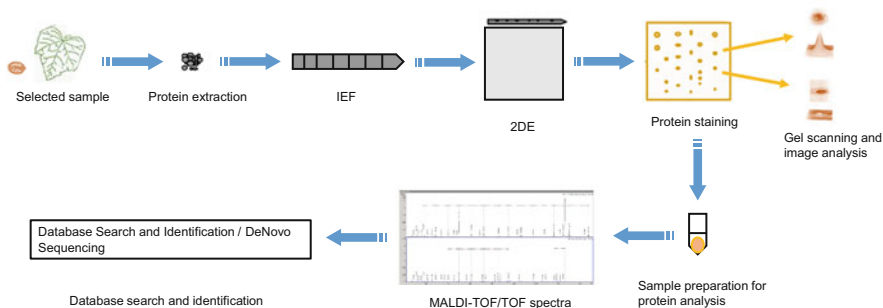


Fig. 13.1 Outline of the experimental approach to proteomic analyses of *Jatropha* tissues

high-throughput capacity due to the ability to perform simultaneously the analysis of hundreds of proteins from the same sample (Maghuly et al. 2011).

Analyses of large portions of the proteome are now possible through techniques such as two-dimensional gel electrophoresis (2-DE) or mass spectrometry (MS). These tools can provide a more direct assessment of biochemical processes by providing information about the main proteins involved in the enzymatic, regulatory, and structural functions encoded by genes and regulated by transcription factors. Although these proteomic techniques are powerful in being able to analyze the complex plant proteome, it constitutes considerable challenges, especially because of low protein concentration and the presence of inhibiting compounds in the cell (Flengsrud 2007). It is crucial to use an effective extraction protocol to avoid interferences and to increase the protein yield because it influences further protein separation and data generation. In addition, the choice of detection methods after protein separation is also important for data visualization.

In this chapter, two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), and bioinformatics tools suitable for *Jatropha curcas* proteomes are described. The proteins are extracted from the tissues under study, which are also relevant for other plants, and the protein mixtures are separated by isoelectric focusing (IEF) in a conventional Immobiline DryStrip gel (IPG strip). Entire strips are transferred into a set of gels, and proteins are visualized by Coomassie blue (Tu et al. 2005; Sinchaikul et al. 2005) and silver staining for gel image analysis (Sinchaikul et al. 2005) (Fig. 13.1).

The proteins spots are separated, excised from the gel, and digested with trypsin. The tryptic digests are subjected to MS/MS TOF to obtain data on the peptides sequences. MS/MS data are used to identify the proteins through search of protein sequence databases (Giorgianni and Beranova-Giorgianni 2005) for the analysis of *J. curcas* tissue.

13.2 Materials

Sample preparation for protein analysis is prone to various contaminations particularly human keratin from the skin and hair, sheep keratin from clothes, and proteinaceous contaminants from dust. Therefore, try to protect the samples against these influences in every preparation step, especially for protein identification. Important means to achieve this are

1. Wear a clean lab coat.
2. Wear powder-free nitrile gloves; latex gloves contain natural rubber and so significant amounts of keratin and other proteinaceous materials. Pipet tip boxes should not be filled with bare hands, but always in the laminar flow.
3. Work in dust-free/reduced environment: e.g., in a laminar flow.
4. Use clean laboratory equipment: the working area and any tools that will be in contact with the sample should be wiped with an ethanol soaked wipe.

13.2.1 *Sample Preparation and Protein Isolation*

1. 100 mg of *Jatropha curcas* tissue
2. Liquid nitrogen
3. 2 ml Eppendorf tubes
4. Retsch Mill (Retsch GmbH & Co. KG, Haan, Germany)
5. TRIZOL (Invitrogen, San Diego, CA)
6. Cold and room temperature (RT) Centrifuge
7. Chloroform
8. Isopropanol
9. 0.3 M guanidine hydrochloride
10. 95 and 100 % ethanol
11. 8 M urea

13.2.2 *Determination of the Protein Concentration*

1. Bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co Rockford IL)
2. Protein standard: bovine serum albumin (BSA)
3. Incubator 37 °C
4. Late reader: Tecan Infinite M200 reader for 96-Well plates (TECAN, Männedorf, Switzerland)

13.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Novex[®] 4–20 % Tris–Glycine Mini Gels
2. Running buffer (1×): 25 mM Tris–HCl, 192 mM glycine, 0.1 % (w/v) SDS (store at 4 °C)
3. SDS-PAGE sample loading buffer (2×): 50 mM Tris–HCl, pH 6.8, 0.1 M DTT, 10 % glycerol, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue (aliquot and store at –20 °C)
4. Protein marker (PeqLab, Erlangen, Germany)
5. Mini SDS-PAGE electrophoresis, Novex System (Invitrogen, San Diego, CA)
6. Thermos mixer (Eppendorf, Hamburg, Germany)

13.2.4 Separation of Protein by Two-Dimensional Gel Electrophoresis (2-DE)

13.2.4.1 First-Dimension Isoelectric Focusing (IEF)

1. Rehydration solution: 3 M Urea, 2 M Thiourea, 2 % CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1 % DTT (Dithiothreitol), 2 % Ampholyte, and 0.1 % bromophenol blue
2. 13 cm Immobiline pH 3–10 NL Dry Strip[™] (IPG) (GE Healthcare, Piscataway, NJ, USA)
3. Ettan IPGphor 3 IEF System and Ettan IPGphor 3 strip holders (GE Healthcare, Piscataway, NJ, USA)
4. Paraffin
5. Filter paper blocks (GE Healthcare, Piscataway, NJ, USA)
6. Milli-Q water (deionized water)

13.2.4.2 2-DE

1. Equilibration buffer: 50 mM Tris–HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % SDS, a trace of bromophenol blue
2. Solution A: Equilibration buffer plus 2 % DDT
3. Solution B: Equilibration buffer plus 2.5 % Iodoacetate
4. 12 % acrylamide gel: 34.4 ml, Milli-Q water, 24 ml 40 % Acrylamide-Mix, 20 ml 1.5 M Tris–HCl (pH 8.8), 0.8 ml 10 % SDS, 0.8 ml 10 % APS (Ammonium persulfate), 0.032 ml TEMED (N,N,N,N-Tetramethylethylenediamine) (add TEMED and APS to the gel solution prior to gel casting)
5. 1 % agarose: 1 g agarose in 100 ml of running buffer (1×) and 100 µl bromophenol blue (Heat them in a microwave oven until the agarose is completely dissolved. Allow the agarose to cool to 40–50 °C before using)

6. Running buffer (1×): 25 mM Tris–HCl, 192 mM glycine, 0.1 % (w/v) SDS (Store at 4 °C)
7. Isobutanol
8. Agarose
9. Bromophenol blue
10. Parafilm
11. Electrophoresis chamber device for 13 × 13 cm vertical gels (PeqLab, Erlangen, Germany)

13.2.4.3 Protein Staining

Silver Staining for Analytical Gels

1. Fixing solution: 40 % (v/v) ethanol, 10 % (v/v) acetic acid, 50 % (v/v) Milli-Q water
2. Glutaraldehyde solution: 1 % Glutaraldehyde and 0.5 M sodium acetate
3. Naphthalene disulfonic acid solution: 0.05 % w/v 2,7-naphthalene-disulfonic acid (250 mg), Milli-Q water (500 ml)
4. Ammoniacal silver nitrate solution: Silver nitrate solution (4 g in 20 ml water). Combine: ammonium hydroxide (5.7 ml, 29 % solution) and sodium hydroxide (1.0 ml, 10 N stock solution) (*see Note 1*)
5. Formaldehyde solution: 0.01 % w/v citric acid (50 mg); 0.1 % formaldehyde (1.35 ml, 37 % stock), water to 500 ml
6. Stop solution: 5 % Tris, 2 % acetic acid

Silver Staining Compatible with Mass Spectrometry

1. Use MALDI-MS compatible silver-staining kit (ProteoSilver™ Plus Silver Stain Kit, SIGMA).
2. Fixing solution: 40 % (v/v) ethanol, 10 % (v/v) acetic acid, 50 % (v/v) Milli-Q water.
3. 30 % ethanol solution (washing solution): 30 ml ethanol 100 and 70 ml water.
4. Sensitizer solution: 1 ml ProteoSilver™ Sensitizer and 99 ml water per 100 ml solution (prepare within 2 h before use).
5. Silver stain solution: 1 ml of ProteoSilver™ Silver Solution and 99 ml water per 100 ml solution (prepare within 2 h before use).
6. Developer solution: 5 ml of ProteoSilver™ Developer 1 and 0.1 ml ProteoSilver™ Developer 2 and 95 ml water per 100 ml solution (prepare immediately before use (<20 min)).
7. Stop solution: 15 ml of the ProteoSilver™ Stop Solution.

Coomassie Staining

1. Coomassie-staining solution: 0.1 % (w/v) Coomassie brilliant blue R250, 40 % (v/v) methanol, 10 % (v/v) acetic acid, 50 % Milli-Q water
2. Coomassie-destaining solution: 40 % (v/v) methanol, 10 % (v/v) acetic acid, 50 % Milli-Q water
3. 5 % acetic acid washing solution

Post-fluorescence Gel Staining (SYPRO[®] Ruby Protein Gel Stain)

1. SYPRO fix solution: 50 % Methanol, 7 % acetic acid (both should be pro-analysis)
2. SYPRO stain solution: SYPRO[®] Ruby protein gel stain (Molecular Probes, Paisley, UK) (1 L of solution provides sufficient material to stain 6–8 gels (13 × 13 cm))
3. SYPRO wash solution: 10 % Methanol, 7 % acetic acid
4. Polypropylene or polycarbonate containers (It should be very clean and rinsed with ethanol before use)
5. Trichloroacetic acid (for IEF gels only)
6. Milli-Q water
7. Orbital shaker

13.2.5 Gel Scanning and Image Analysis

1. Laser scanner Typhoon FLA95000 image scanner (GE Healthcare, Piscataway, NJ, USA)
2. ImageMaster 2D Platinum version 5.0 (GE Healthcare, Amersham Biosciences, Piscataway, NJ, USA)

13.2.6 Protein Identification

1. Spot picker (Serva, Heidelberg, Germany)
2. Ultrasonic bath
3. 100 mM ammonium bicarbonate (ABC 100), pH = 8.5
4. Destaining solution: 50 % Potassium hexacyanoferrate (30 mM), 50 % Sodium thiosulfate (100 mM)
5. Dehydrating solution: Acetonitrile
6. Reducing solution: Dithiothreitol (10 mM in 25 mM ammonium bicarbonate)
7. Alkylating solution: Iodoacetamide (54 mM in 25 mM ammonium bicarbonate)

8. Trypsin solution: 1 µg/µl trypsin in 50 mM acetic acid (Trypsin Gold, Promega)
9. Peptide extraction: 50 % (v/v) acetonitrile, 45 % (v/v) water, 5 % (v/v) trifluoroacetic acid (TFA)
10. Derivatization: 4-sulfophenyl isothiocyanate (SPITC)
11. Matrix for MALDI ionization: α-cyano-4-hydroxycinnamic acid
12. Pre-spotted anchor chip MALDI target plate (PAC target, Bruker Daltonics)
13. Solvent evaporation: Vacuum centrifugation (SpeedVac, Eppendorf)
14. Desalting of peptides: ZipTips C18 (microbed, Millipore)
15. Derivatization of peptides: ZipTip C18 pipette tips (Millipore)
16. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Ultraflex II; Bruker Daltonics)
17. Spectra processing and peak annotation: FlexAnalysis and BioTools (Bruker Daltonics)
18. Mascot Server version 2.3 (Matrix Science, Boston, MA), ProteinScape 2.1 (Bruker Daltonics)

13.3 Methods

13.3.1 *Sample Preparation and Protein Extraction*

Plant tissues should be ground into a very fine powder using liquid nitrogen.

1. Weigh 100 mg plant tissue and cut it in several pieces; place the tissue pieces in a 2 ml Eppendorf tube.
2. Grind the selected tissues with a Retsch Mill into a fine powder using liquid nitrogen because they generally contain low levels of proteins, tightly linked to the cell structures.

13.3.1.1 TRIZOL Protein Isolation Protocol

A number of methods for protein extraction from *J. curcas* tissues for proteomics applications were compared in our lab (PBU), and the TRIZOL reagent was selected for protein isolation.

1. Add 1 ml of TRIZOL to 100 mg pulverized tissue; mix well and keep for 5 min at RT.
2. Add 0.2 ml of chloroform per 1 ml of TRIZOL reagent used, and shake samples by hand vigorously.
3. Centrifuge at $12,000 \times g$ for 5 min at RT. The mixture separates into the three phases; upper (aqueous) phase that contains RNA, interphase (DNA and proteins), and lower phenolic phase.
4. Remove the upper (aqueous) phase.

5. Add 0.3 ml of ethanol to the lower phases, mix by inversion, and keep for 2–3 min at RT (this precipitates the DNA).
6. Centrifuge at $2000 \times g$ for 15 min at 4 °C.
7. Collect the supernatant and add 1.5 ml isopropanol; mix and store the samples at RT for 10 min (this precipitates the proteins).
8. Centrifuge at $12,000 \times g$ for 10 min at 4 °C.
9. Remove the supernatant, and wash the protein pellet three times by adding 2 ml solution of 0.3 M guanidinium hydrochloride in 95 % ethanol for 20 min at RT (keep the samples in washing solution for 20 min at RT before centrifuging).
10. Centrifuge at $7500 \times g$ for 5 min at 4 °C; remove and discard the supernatant.
11. Vortex the protein pellet in 2 ml of 100 % ethanol, store the protein pellet in ethanol for 20 min at RT, and centrifuge at $7500 \times g$ for 5 min at 4 °C.
12. Remove ethanol and dry protein pellet briefly (do not prolong storage of the protein pellet, which can make subsequent solubilization difficult).
13. Dissolve pellet in 8 M urea.
14. Determine protein concentration (*see* Sect. 13.3.2).
15. Aliquot the samples and store at -80 °C until further use.

13.3.2 Determination of the Protein Concentration

1. Determine the protein concentration by the BCA method using a freshly prepared BSA standard curve. Typical protein concentrations range from 2 to 2000 µg/ml.
2. Prepare working reagent (WR) by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).
3. Add 25 µg of each standard or extracted sample into a microplate well, and add 200 µl of WR, mix by careful vortexing.
4. Cover the plate and incubate for 30 min at 37 °C.
5. Cool plate at RT and measure the absorbance at 595 nm on a plate reader.

13.3.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Prepare the Novex[®] 4–20 % Tris–Glycine Mini Gels by removing the comb and rinse the gel wells three times using running buffer (1×).
2. Remove the white tape near the bottom of the gel cassettes to give contact between buffer and gel.
3. Place the gels in the Mini SDS-PAGE electrophoresis.
4. Fill the gel wells with 1× running buffer.
5. Prepare 10 µl of each sample in 2× SDS-PAGE sample loading buffer.

6. Spin down samples and heat them for 10 min at 90 °C to monomerize possibly present complex proteins. Keep samples at 4 °C until ready to use in order to avoid polymerization.
7. Load the appropriate volume of samples and protein marker onto the gel.
8. Run for 2 h at 125 V.
9. At the end of the run, use the gel either immediately for western blotting or for staining with Coomassie brilliant blue (*see* Sect. 13.2.4.3.3).

13.3.4 Separation of Protein by 2-DE

13.3.4.1 First-Dimension Isoelectric Focusing (IEF)

1. Add rehydration solution (prepare it 1 day before isoelectric focusing) to the suitable volume of sample (μg of protein, *see* **Note 2**). Vortex the solution shortly and spin off the vial with a table centrifuge.
2. Open the 13 cm IPG strip (GE Healthcare Immobiline™ DryStrip pH 3–10 NL) package from the acidic end (*see* **Note 3**).
3. Add 250 rehydration solution-containing samples in the middle of the IPG strip in the ceramic strip holder (Ettan IPGphor 3 strip holders).
4. Put the IPG strip onto the strip holder with the gel side down to fit into the holder and to make its code number easily readable. Placing the strip starting from the pointed end helps to avoid bubbles. Move the strip forward and backward along the surface of the solution to ensure the complete and evenly wetting of the strip.
5. Cover the strip by adhesive tape or paraffin oil (to avoid evaporation of solution and crystallization of urea) and incubate at RT for 16 h.
6. Place the strip in the focusing tray (Ettan IPGphor 3 IEF System), with the gel side up. The “+ end” of the IPG strip has to be at “+” pole.
7. Moisturize two filter paper blocks (avoid dust and dirt) with 10 μl of Milli-Q water. Put each wet paper block on both the “+” and “-” ends of the gel on the strip (the pointed end of the IPG strip is at the anode (positive end)). Take care, not to damage the gel.
8. Place electrodes over the paper blocks and fix them in the focusing tray. Cover each strip with 2–3 ml of paraffin oil, close the lid, and switch on the instrument.
9. Start focusing the rehydrated strip with 500 W and higher than 50 μA and continue as described in Table 13.1.
10. Take out the IPG strips and discard the paraffin; transfer the IPG strips into the rehydration tray.
11. Wash the IPG strips very shortly with Milli-Q water to remove the residual paraffin.
12. After isoelectric focusing, the IPG Strip gels should either proceed immediately to the second-dimension separation or to store at -80 °C.

Table 13.1 Program outline for 13 cm IPG strip (3–10 NL) focussing

Step	Voltage	Time (h:min)	kVh
1st step and hold	200	2:30	0.5
2nd step gradient	1000	1:20	0.8
3rd step gradient	8000	2:30	11.25
4th step and hold	8000	0:40	5.2

13.3.4.2 2-DE

Equilibration

1. Incubate the IPG strip for 12 min at RT in 2 ml of equilibration solution A.
2. Discard the solution A (work quickly to avoid unwilling contamination of the samples).
3. Incubate the IPG strip for 12 min at RT in 2 ml of equilibration solution B.
4. The strips can be now transferred to the second-dimension gel.

Gel Preparation

1. Clean the glass plates with ethanol or isopropanol using a lint-free cloth to remove dust and fibers.
2. Prepare 12 % acrylamide solution (acrylamide is neurotoxin, suitable precautions should be taken) for each 2D gel of 14 × 16 cm (1 mm thick) before the strip equilibration, and prepare 1 % agarose during the strip equilibration.
3. Pour the acrylamide solution between the glass plates until the solution is about 5 mm below the top of glass plate, carefully avoiding air bubbles.
4. Add 200 µl saturated isobutanol on top of the acrylamide gel to create a flat gel end.
5. Leave the gel to polymerize for at least 1 h at RT.
6. Remove isobutanol by washing the gel with running buffer (Sect. 13.2.4.2, step 7).
7. If the gel is not required immediately, store the gel in the casting apparatus at +4 °C for 72 h covered with running buffer and sealed with Parafilm.
8. Transfer the re-equilibrated IPG strips with forceps to the “+” end, cut the other gel strip end, place the cut end on the large glass plate near the (–) pole, and push the strip down using the flat side of a spatula till the strip touches the surface of the acrylamide gel (without damaging the gel).
9. Fill the strip slot with hot agarose to the edge of the glass plate avoiding air bubbles. Let the agarose polymerize completely before transferring the gel cassette to the electrophoresis chamber device.
10. Fill the buffer chamber with 1 × running buffer. Run the gels for 5 h at 160 V, 35 mA, and 5 W. Cool the chamber at 15 °C during the run.

11. Finish the run, when the bromophenol blue front reaches nearly the end of the gel (about 0.5 cm to the bottom).
12. Stop the run, remove the running buffer, disassemble the electrophoresis chamber device, and open the glass plates.

13.3.4.3 Protein Staining

Silver Staining for Analytic Gels (*see Note 4*)

1. Fix the gels before staining in fixing solution (*see Sect. 13.2.4.3.1, step 1*) for 30 min at RT or up to 72 h at 4 °C.
2. Soak the gels in glutaraldehyde solution for 30 min.
3. Wash the gel (2×) in Milli-Q water for 10 min.
4. Soak the gel in naphthalene disulfonic acid solution for 15 min, in order to obtain homogeneous dark brown staining of proteins.
5. Wash the gel (4×) in Milli-Q water for 15 min.
6. Stain the gel with freshly prepared ammoniacal silver nitrate solution for 30 min.
7. Wash the gel (4×) in Milli-Q water for 4 min.
8. Develop the gel with formaldehyde solution from 5 to 10 min or even shorter, depending on the protein load and background levels until the desired spot intensity is achieved.
9. Stop the process with stop solution and wash the gels (2×) in water for 10 min.

Silver Staining Compatible with Mass Spectrometry

Silver staining compared to Coomassie staining increases the sensitivity from 10 to 100-fold; however, glutaraldehyde from normal silver staining is not compatible with mass spectrometry, which causes protein–protein cross-linkage.

1. Remove the fixing solution (*see Sect. 13.2.4.3.2, step 2*) and wash the gel in 30 % ethanol for 10 min.
2. Remove the 30 % ethanol solution and wash the gel in Milli-Q water for 10 min or for 72 h in Milli-Q water.
3. Remove the water and incubate the gel in the sensitizer solution for 10 min.
4. Remove the sensitizer solution and wash the gel (2×) in Milli-Q water for 10 min.
5. Remove the water and equilibrate the gel in silver stain solution for 10 min.
6. Remove the silver stain solution and wash the gel in Milli-Q water for 1 min (washing longer than 1.5 min decrease sensitivity).
7. Remove the water and develop the gel in developer solution (*see Sect. 13.2.4.3.2, step 6*) from 3 to 7 min (*see Note 5*).

8. Add 15 ml of the ProteoSilver™ Stop Solution (see Sect. 13.2.4.3.2, step 7) to stop the developing reaction and incubate for 5 min. Bubbles of CO₂ gas will form in the mixture.
9. Remove the developer/stop solution and wash the gel in Milli-Q water for 15 min.
10. Store gel in Milli-Q water at 4 °C.

Coomassie Staining

1. Directly after electrophoresis, rinse gel in Milli-Q water for 1 min.
2. Incubate the gel in Coomassie staining solution at RT for 3–4 h with gentle agitation on an orbital shaker.
3. Incubate gel in destaining solution until spots are visualized.
4. Incubate the gels in 5 % acetic acid with gentle agitation and change the solution until the background is transparent.
5. Store the gel in Milli-Q water at 4 °C.

Post-fluorescence Gel Staining (SYPRO® Ruby Protein Gel Stain) (see **Note 6**)

SYPRO® Ruby protein gel stain is an irritant; therefore, it should be handled with care and using powder-free gloves.

1. Perform all fixation, staining, and washing steps with continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm).
2. Place the gel into a clean polypropylene container (one gel per container, see **Note 7**) with 100 ml of SYPRO fixing solution for at least 15 min (2×) on orbital shaker.
3. Wash the gel in Milli-Q water for 10 min (3×). Incubate gels in SYPRO® Ruby gel stain for at least 3 h or overnight on an orbital shaker (use a sufficient staining volume so that staining solution flows over the surface of the gel).
4. Cover the gel container during staining and subsequent wash steps to exclude light, as with any other fluorescent stain.
5. Transfer the gel to a clean container with 100 ml of SYPRO wash solution and wash for 30 min.
6. Rinse the gel with Milli-Q water for 5 min (2×).
7. Visualize the gels stained with the SYPRO® Ruby protein gel stain are using imaging systems Typhoon at 450, 473, 488, or 532 nm and evaluate with 2-D gel evaluation software (e.g., ImageMaster 2D Platinum).
8. Store the gel wet, seal the stained gel in a small volume of fresh SYPRO® Ruby stain (approximately 5–10 ml, enough to keep the gel wet) containing a preservative, press out any air, and store at 4 °C.

13.3.5 Gel Scanning and Image Analyses

1. Transfer the gel to a suitable scanner, and set the scanner for generation of 16 Bit positive images with a resolution of 400 dpi in TIFF format.
2. Import the gel images into ImageMaster 2D Platinum software for spot matching and evaluation. Use viewing tools like contrast, overview, and profile, 3-D to assess image quality (poor quality images can cause insufficient spot detection and/or matching).
3. Place one or several landmarks to support automatic matching. Keep landmarks at a minimum. Place the landmark on well-defined smaller spots clearly identifiable on all gels in the dataset.
4. Use the ratio between means and gaps to find the most interesting potential marker proteins or use statistical analyses such as ANOVA, Wilcoxon, or Kolmogorov. They are automatically calculated within classes (experimental groups) and presented in the class analysis table (*see Note 8*).

13.3.6 Protein Identification and Data Interpretation

13.3.6.1 In-Gel Digestion of Proteins

1. Excise selected spots from the gel using a spot picker to minimize the amount of unstained gel area around the spot in order to minimize the amount of contaminants from the gel.
2. Transfer the excised gel plug into a clean, dust-free, correctly labeled 500 μ l Eppendorf tube, and add enough Milli-Q water to cover the gel piece. The spots can be stored at 4 °C at this point.
3. Remove the Milli-Q water, and wash each spot with 100 μ l ABC 100 using an ultrasonic bath (US) for 5 min. Remove the supernatant.
4. Destain the silver-stained spots by adding 30 μ l of destaining solution (Sect. 13.2.6 protein identification). After about 2 min, supernatant can be discarded (Gharahdaghi et al. 1999).
5. Wash the destained gel pieces with 100 μ l Milli-Q water followed by 100 μ l ABC 100 (US, 5 min each).
6. Prior to the reduction step, dehydrate the gel pieces using 100 μ l acetonitrile (US, 5 min), remove the supernatant, and evaporate the remaining acetonitrile in a SpeedVac (RT, 5 min)
7. Reduce disulphite bonds adding 50 μ l dithiothreitol (10 mM in 25 mM ammonium bicarbonate) to the sample and incubate at 56 °C for 60 min on a thermo mixer (*see Note 9*).
8. For the alkylation (*see Note 10*), add 50 μ l of 54 mM iodoacetamide solution after removing the dithiothreitol and incubate for 45 min at RT in the dark by covering the thermo mixer with a dark lid (Jiménez et al. 2001).
9. After alkylation, wash the spots with 100 μ l ABC 100 as before (US, 5 min).

10. Dehydrate the gel pieces again using 100 μl acetonitrile (US, 5 min) and evaporate the remaining drops in the SpeedVac.
11. Reconstitute the lyophilized trypsin (*see Note 11*) powder (Trypsin Gold, Promega, Germany) in 50 mM acetic acid to a concentration of 1 $\mu\text{g}/\mu\text{l}$ (trypsin stock). Before use, dilute 5 μl of trypsin stock solution with 120 μl water, 120 μl ABC 100, and 10 μl calcium chloride (120 mM) to a final concentration of 20 ng trypsin per μl . Perform the in-gel digest using 5 μl of this 20 ng/ μl solution (Schevchenko et al. 1999).
12. Allow the gel piece to swell in the trypsin solution at 4 $^{\circ}\text{C}$ for 20 min and remove the remaining solution from the gel spot.
13. Cover the rehydrated pieces with 30 μl of a 50 mM ABC for the digest and put them on the thermoMixer.
14. For the digestion procedure, program the thermoMixer at 37 $^{\circ}\text{C}$ for 8 h and then cool down to 4 $^{\circ}\text{C}$.
15. Extract the peptides from the gel three times using 30 μl of acetonitrile: water: TFA (50:45:5 v/v) supported by ultra-sonication for 15 min each time. Pool extracted peptides in a fresh 500 μl Eppendorf tube, and dry down the solution in a SpeedVac at 45 $^{\circ}\text{C}$ for 150 min.

13.3.6.2 ZipTip Purification

To eliminate salts from the sample and to concentrate the extracted peptides, carry out a ZipTip purification as follow (*see Note 12*):

1. Resuspend the dried samples in 10 μl 0.1 % TFA.
2. Freshly prepare a solution by mixing 350 μl ACN with 150 μl 5 % TFA for eluting peptides.
3. The pipette plunger is depressed to a dead stop. Using the maximum volume setting of 10 μl , elution solution is aspirated into the tip and dispensed to waste.
4. Equilibrate the ZipTip by aspirating and dispensing 3.3 μl of 0.1 % TFA and discard to waste container (*see Note 13*). Repeat this step three times (total 10 μl).
5. Bind the peptides to the ZipTip pipette tip by aspirate and dispense the sample ten times for maximum binding of peptides to the reversed phase material.
6. In a further step, wash the tip 3 \times with 3.3 μl 0.1 % TFA (total 10 μl).
7. Elute the peptides from the tip by carefully aspirating and dispensing 2 μl elution solution through the ZipTip pipette tip 2 \times without introducing air into the sample. Repeat once and add the second 2 μl eluent to the first vial (total 4 μl eluted peptides).
8. Spot desalted peptides (0.5 μl) onto a disposable PAC target (MALDI target plate pre-spotted with α -cyano-4-hydroxycinnamic acid) (*see Note 14*).

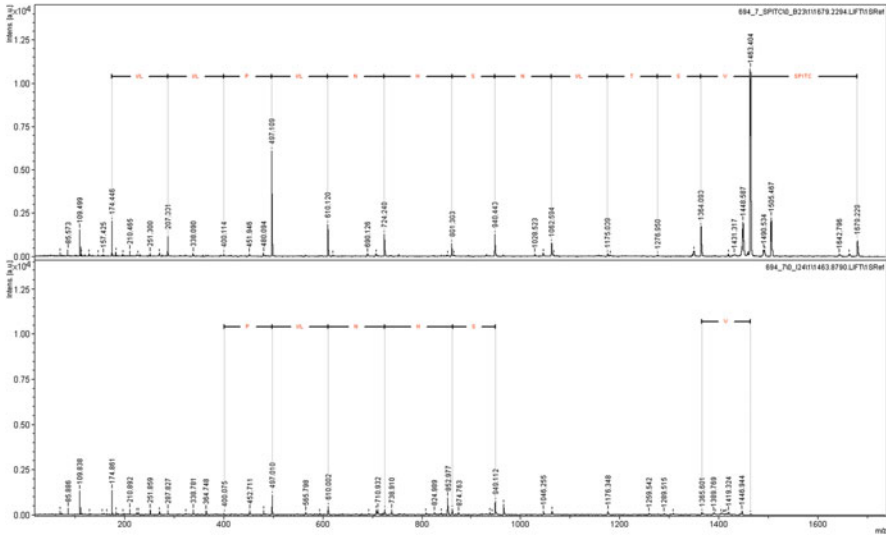


Fig. 13.2 Derivatization of internal peptides with SPITC

13.3.6.3 Derivatization of Peptides for De Novo Sequencing

In case of organisms or species with insufficient or poor database entries and spectral libraries, the only way to identify the proteins is to perform de novo sequencing. In order to improve the identification rate and obtain complete peptide sequences, the proteolytic peptides are derivatized by 4-sulphophenyl isothiocyanate (SPITC). This N-terminal chemical derivatization increases the structural information and the efficiency of post-source decay (PSD) fragmentation (Fig. 13.2). The reaction delivers a negative sulfonic acid group to the N-terminus of the proteolytic peptides leading to suppression of b-ions (*see Note 15*). Consequently, only positively charged ions (y-ions) are observed in MALDI-TOF/TOF spectra (Fig. 13.3) (Joss et al. 2006; Gevaert et al. 2001).

1. After the first spotting, dry the remaining 3.5 μl of the eluted peptides in the SpeedVac and dissolve in 10 μl 0.1 % TFA.
2. To avoid a too low pH for derivatization, repeat the ZipTip procedure without TFA in the elution solution.
3. Dry the eluted peptides again in the SpeedVac.
4. Prepare a 10 mg/ml solution of SPITC (*see Note 40*) in 20 mM NaHCO_3 buffer (pH 9.5) (Wang et al. 2004).
5. Add 4 μl the SPITC solution to the dried peptides and incubated for 30 min at 56 $^\circ\text{C}$.
6. Stop the reaction by acidification of the solution with 1 μl 5 % TFA.

7. Desalt the derivatized peptides using μ ZipTips C18 (see Sect. 13.3.6.2).
8. Spot the eluted, derivatized, and purified peptides on a PAC target as described above.

13.3.6.4 MALDI-TOF/TOF Analysis of Peptides

1. Acquire the MS and MS/MS spectra on an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, Germany) in positive reflector mode with a nitrogen laser operating at 337 nm.
2. Prior to each measurement, calibrate the mass spectrometer using the pre-spotted calibration spots on the PAC target with an accuracy of 10 ppm (see Note 16–17).
3. Measure MS spectra in a mass range from 500–4400 Da.
4. Acquire MS/MS spectra of the peaks with the highest signal-to-noise ratio using the LIFT mode using the instrument default calibration.
5. Analyze spectra processing and peak annotation using FlexAnalysis 3.0 (Bruker Daltonics, Bremen, Germany).
6. For standard database searches, processed spectra are analyzed using a Mascot Server version 2.3 (Matrix Science, Boston, MA) enabling the search in customized databases (in this case genome sequences of *J. curcas*) and the software ProteinScape 2.1 (Bruker Daltonics, Germany) in a generic database built on genomic data of the organism using the following search parameters: (a) taxonomy all entries, (b) global modifications carbamidomethylation on cysteine, (c) variable modifications oxidation on methionine, (d) deamidation of asparagine and glutamine, (e) N-terminal pyro-glutamic acid and a N-terminal SPITC derivatization, (f) enzyme specificity trypsin, (g) charge state $z = 1$, (h) MS tolerance 100 ppm; MS/MS tolerance 1 Da, and (i) one missed cleavage allowed; significance threshold $p < 0.05$.
7. Carry out manually peptide de novo sequencing using FlexAnalysis. Use these sequences for a homology search by MS-homology search (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology>) (Zakharova et al. 2014).

13.4 Notes

1. Add the ammonium/sodium hydroxide solution to the silver nitrate solution; a transient brown precipitate will form. After it cleared, bring to 500 ml with water. Drop the silver nitrate solution carefully into the solution.
2. Calculate the volume of sample solution that will contain the required amount of protein. Recommended sample loads for 13 cm Immobiline™ DryStrip

pH 3–10 NL for silver (analytic, 10–20 µg of protein) and Coomassie (preparative, 50–240 µg of protein) staining.

3. This end of the gel is usually more stable and less prone to damage. In all steps, move strip with the forceps. Strictly avoid touching the strip on the surface, not to damage the gel.
4. Silver nitrate is extremely sensitive and dirty. Be very careful in weighing and pouring solutions. After staining, wash both the inside and the outside of trays very carefully with deionized water to avoid tracking silver.
5. Three to seven min is sufficient to produce the desired staining intensity for most gels. Development times as long as 10–12 min may be required to detect band or spots with very low protein concentrations (0.1 ng/m²).
6. Post-fluorescence staining using SYPRO[®] Ruby or Orange stains can be used subsequent to staining with other fluorescence protein stains. However, SYPRO[®] Ruby stain should always be used the last, because its bright fluorescent signal tends to dominate the signal from other stains. SYPRO[®] Ruby stain does not work well for colorimetric stains such as Coomassie[®] and silver stains. It provides challenging characteristics like linear quantitation range from 0.25 to 1 ng and is capable of visualization of different protein types including glycoproteins, phosphoproteins, lipoproteins, calcium-binding proteins, fibrillary proteins, and proteins that are difficult to stain. SYPRO[®] Ruby stain can be multiplexed with other gel stains to obtain more information from one gel (Pro-Q[®] Diamond phosphoprotein gel stain, Pro-Q[®] Emerald 300 glycoprotein gel stain, Pro-Q[®] Sapphire and InVision[™] oligohistidine-tag gel stains, and Pro-Q[®] Amber transmembrane protein gel stain). Further, it is compatible with subsequent analysis of proteins by Edman-based sequencing or mass spectrometry.
7. Polypropylene or polycarbonate containers adsorb minimal amounts of the dye.
8. There are different tables providing the result of the analysis. To find the appropriate information, check the appropriate table that summarizes the data needed.

Spot table: Gives summarized information about enabled spots.

Gel analysis table: Gel analysis table and histograms provide valuable tools for checking spot detection, verifying matches, and analyzing expression changes within a class.

Class analysis table: Provides information about expression changes between classes (experimental groups).

9. Disulfide bonds stabilize the 3D structure of proteins and are very important for their biological functionality. An important step in peptide chemistry is the reduction of disulfide bonds by using reducing agents like dithiothreitol.
10. The reduced disulfide bonds can spontaneously be oxidized. Iodoacetamide is used in order to stabilize the thiol groups and prevent the building of disulfide bonds.
11. Trypsin as proteolytic enzyme hydrolyses peptide bonds selectively at the carboxyl side of lysine and arginine amino acids. Its optimum pH is between

7 and 8 (pH = 7.6). However, in order to prevent auto-proteolysis, 50 mM acetic acid should be used as storage solution. This prevents generating fragments, which would interfere with peptide analysis during ionization in mass spectrometry.

12. ZipTip is a sample preparation strategy based on reversed phased chromatography. The pipette tips contain C18-modified silica material. This step is used for desalting and concentrating the peptides (volume: 1–10 μ l).
13. The equilibration of reversed phase material helps to reach a better adsorption of peptides to the C18 side chains.
14. Matrix in MALDI ionization plays a key role and needs to be of highest purity.
15. When using soft ionization techniques like MALDI or ESI, structural information by fragmentation of molecular ions of peptides is necessary. The fragmentation of peptides according to (Nishimura and Tojo 2014) is a post-source decay delivering predominantly b- and y-ions.
16. Since the reaction is pH-dependent, it is important to maintain a pH value of 9.5.
17. Calibration of mass spectrometer is a crucial point determining the accuracy of mass measurement and therefore the confident protein identification. Therefore, always calibrate the instrument prior to analyses.

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Part IV
Genotypic Screening

Chapter 14

Low-Cost Methods for DNA Extraction and Quantification

Owen A. Huynh, Joanna Jankowicz-Cieslak, Banumaty Saraye, Bernhard Hofinger, and Bradley J. Till

Abstract Nucleotide variation, whether induced or natural, is responsible for a vast majority of heritable phenotypic variation. Evaluation of genomic DNA sequence is therefore fundamental for both functional genomics and marker-assisted breeding. The starting point for this is the extraction of high-quality DNA of a suitable quantity for planned downstream applications. A myriad of kits and techniques exists, but not all are suitable for laboratories with limiting infrastructure due to high costs and reliance on toxic chemicals. We describe here a protocol for extraction of high-quality genomic DNA from leaves of a variety of plant species that uses self-prepared buffers and reagents and obviates the need for toxic organic chemicals such as chloroform. We also provide a protocol for using free image analysis software to quantify isolated DNA.

Keywords Chaotropic salts • DNA purification • ImageJ • TILLING

14.1 Introduction

While deoxyribonucleic acid (DNA) has been known to be a component of cells since the late 1800s, it wasn't until the pioneering work of Oswald Avery that it was understood that the molecule is the genetic material (Avery et al. 1944; Dahm 2005). Today, DNA is at the heart of many biological analyses. Molecular markers allow facile and rapid introgression of alleles into elite crop varieties, high-density variant analysis enables genomic selection, whole genome sequencing is revealing evolutionary dynamics in populations and Targeting Induced Local Lesions IN Genomes (TILLING) approaches allow reverse-genetics using induced mutations in plant and animal species (Jankowicz-Cieslak et al. 2011; Varshney et al. 2014).

O.A. Huynh • J. Jankowicz-Cieslak • B. Hofinger • B.J. Till (✉)
Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria
e-mail: b.till@iaea.org

B. Saraye
Food Agricultural Research and Extension Institute, Reduit, Mauritius

Many applications and methods for the molecular investigation of plants require extraction of genomic DNA. The ideal method balances throughput, per sample cost, yield per milligram of tissue and the use of toxic chemicals according to the priorities of a specific laboratory.

Commercial kits have been developed for DNA isolation from numerous tissues of plants and animals and also microorganisms. These kits are suitable for rapid and reliable DNA extractions of high purity and high quality and produce high yields. They have become the standard for sensitive molecular assays, but the cost per sample remains high and therefore DNA extraction is still a limitation for large-scale projects in some laboratories. Many commercial methods are based on the binding of DNA to silica in the presence of chaotropic salts. The silica in such kits is usually provided packed in ready-to-use spin columns. Lower-cost alternatives utilising self-made buffers exist but often have disadvantages such as the use of toxic organic phase separation. We present a protocol that utilises the DNA binding capacity of silica in the presence of chaotropic salts. Costs are reduced through the use of self-made buffers and avoiding pre-made spin columns. The principle of the silica-based DNA isolation method is the same as in commercially available kits. In the presence of high concentrations of chaotropic salt, the interaction of water with the DNA backbone is disrupted, and charged phosphate on the DNA can form a cationic bridge with silica. DNA-bound silica is separated from the other soluble cellular components by centrifugation. Washing the DNA-bound silica in the presence of a high percentage of alcohol removes excess salt. The subsequent addition of an aqueous solvent (water or buffer) drives the hydration of the DNA and its release from silica. The now soluble DNA can be separated from silica through a quick centrifugation step. The methods provided here include improvements and further cost reductions from our previously published protocol utilising chaotropic salts and silica binding (Till et al. 2015). Following DNA extraction it is necessary to assess the quality and quantity of DNA. There are many methods that can be used such as spectroscopy, fluorimetry and gel-based methods. Gel-based methods are quite common and have the advantage that only standard electrophoresis equipment common to most molecular biology labs is required. Further, gel-based methods allow the direct assessment of DNA degradation which is not easily measured using other approaches. While quantification of DNA on gels can be done visually, the precision of measurements is improved through the use of image analysis software. We describe a method utilising quantitative markers and the free image analysis software ImageJ (Schneider et al. 2012). This procedure includes the use of spreadsheet software (e.g. Microsoft Excel, LibreOffice Calc or Apache OpenOffice Calc) for rapid estimations of DNA concentrations of many samples.

14.2 Materials

14.2.1 DNA Extraction

1. Vortex mixer (e.g. Vortex-Genie 2, Scientific Industries, Inc., Cat. No. SI-0236).
2. Benchtop scale (e.g. Mettler Toledo Cat. No. XS2002S).
3. Microcentrifuge.
4. Micropipettes (1000 μ l, 200 μ l, 20 μ l).
5. Microcentrifuge tubes (1.5 ml, 2.0 ml).
6. 50 ml conical tube with cap.
7. Metal beads (e.g. tungsten carbide beads, 3 mm, Qiagen 69997, *see Note 1*).
8. Distilled or deionised water (dH₂O).
9. Celite 545 silica powder (e.g. Celite 545 AW, Sigma-Aldrich, 20,199-U).
10. Sea sand (optional, *see Note 2*).
11. RNase A (10 μ g/ml).
12. 6 M KI (potassium iodide) or alternative buffers (*see Notes 3 and 4*).
13. SDS (sodium dodecyl sulphate).
14. Sodium acetate anhydrous (prepare 3 M sodium acetate (pH 5.2) solution).
15. NaCl (sodium chloride).
16. Absolute ethanol.
17. Wash buffer (0.05 M NaCl in 95 % EtOH) (*see Note 5*).
18. 10 \times TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.0).

14.2.2 DNA Quantification

1. Equipment for horizontal gel electrophoresis (combs, casting trays, gel tank, power supply)
2. Agarose (for gel electrophoresis)
3. 0.5 \times Tris/Borate/EDTA (TBE) buffer (for gel electrophoresis)
4. Ethidium bromide, SYBR or equivalent double-stranded DNA staining dye
5. Lambda DNA (e.g. Invitrogen 25250-010) for the preparation of concentration standards (*see Note 6*)
6. DNA gel loading dye (containing Orange G or bromophenol blue) (*see Note 7*)
7. Gel photography system (digital camera, light box)
8. Computer and monitor (*see Note 8*)
9. ImageJ software (<http://rsbweb.nih.gov/ij/>) (*see Note 9*)
10. Spreadsheet software (e.g. Microsoft Excel, LibreOffice Calc or Apache OpenOffice Calc)

14.3 Methods

14.3.1 DNA Extraction

14.3.1.1 Preparation of Silica Powder DNA Binding Solution

1. Transfer 800 mg Celite 545 silica into a 50 ml conical tube.
2. Add 30 ml dH₂O.
3. Vortex and invert 15 times or until a hydrated slurry forms.
4. Let stand for 15 min until all solid material has settled.
5. Remove (pipette off) the liquid. It is okay to leave a small amount of liquid in the tube.
6. Repeat steps 2–5 to a total of three washes.
7. Suspend the hydrated silica in a volume of dH₂O equal to the volume of silica. This is the liquid silica stock (LSS) and can be stored at room temperature (RT) for up to 1 month (*see Note 10*).
8. Suspend LSS by vortexing for 30 s or until a homogenised slurry is formed. For each tissue sample, transfer 50 µl into a fresh 2 ml tube.
9. Add 1 ml dH₂O per tube to perform a final wash step.
10. Mix by vortexing for 15 s or until silica is fully suspended.
11. Centrifuge at the full speed (16,000 × *g*) for 20 s.
12. Pipette off liquid.
13. Add 700 µl 6 M KI (*see Note 3*).
14. Suspend the silica in 6 M KI by vortexing 15 s. This is the silica binding solution (SBS, *see Note 11*).

14.3.1.2 Low-Cost Extraction of Genomic DNA from Plant Tissues

1. Prepare an ice bath.
2. Label 2 ml tubes containing three metal beads (*see Note 1*) and optional sea sand with sample names. Grinding is facilitated by addition of purified sea sand (*see Note 2*).
3. Add tissue to the appropriate tube (*see Note 12*).
4. Tape tubes onto a vortex mixer (Fig. 14.1) and vortex on high setting for 30 s or until material is ground to a fine powder.
5. Add 800 µl of lysis buffer (0.5 % SDS (w/v) in 10× TE) and 4 µl RNase A (10 µg/ml) to each tube (*see Note 13*).
6. Vortex 2 min until ground tissue is fully hydrated and mixed with buffer.
7. Incubate for 10 min at RT.
8. Add 200 µl 3 M sodium acetate (pH 5.2). Mix by inversion of tubes and incubate on ice for 5 min.
9. Centrifuge at 16,000 × *g* for 5 min at RT to pellet the leaf material.
10. Label the tubes with aliquots of silica binding solution (SBS, 700 µl) with the sample name.



Fig. 14.1 Grinding of plant tissues. Leaf tissue is placed in a 2 ml tube in the presence of metal balls and optional sea sand (*left panel*). Use of desiccated tissue often results in higher-quality DNA compared to fresh tissue. Multiple tubes are then attached to a vortex mixer using tape (*middle panel*). Grinding is complete when a fine powder is produced (*right panel*). The presence of unground tissue with the powder does not affect the quality of extracted DNA

11. Transfer liquid into appropriately labelled SBS-containing tubes (*see Note 14*).
12. Completely suspend the silica powder by vortexing and inversion of tubes (approximately 20 s).
13. Incubate 15 min at RT (on a shaker at 400 rpm or invert tubes every 3 min by hand).
14. Centrifuge at $16,000 \times g$ for 3 min at RT to pellet the silica.
15. Remove the supernatant with a pipette and discard (the DNA is bound to the silica at this stage).
16. Add 500 μl of freshly prepared wash buffer to each tube (*see Note 5*).
17. Completely suspend the silica powder by vortexing or inverting tubes (approximately 20 s).
18. Centrifuge at $16,000 \times g$ for 3 min at RT to pellet the silica. Remove supernatant and keep the pellet.
19. Repeat steps 16–18.
20. Centrifuge pellet for 30 s and remove any residual wash buffer with a pipette.
21. Open the lid on tubes containing silica pellet and place in fume hood to fully dry pellets for 30 min (*see Note 15*).
22. Add 200 μl TE buffer to each tube to elute the DNA. The DNA is now in the liquid buffer (*see Notes 16 and 17*).
23. Completely suspend the silica powder by vortexing and inversion of tubes (approximately 20 s).
24. Incubate at RT for 5 min.
25. Centrifuge at $16,000 \times g$ for 5 min at RT to pellet the silica.
26. Label new 1.5 ml tubes with sample information.
27. Collect liquid containing genomic DNA and place into new tubes.
28. Store DNA temporarily at 4 °C before checking quality and quantity. The approximate cost per sample is between 42 and 64 cents depending on the chaotropic buffer used (*see Table 14.1*). Modifications can be made to the protocol to increase throughput (*see Note 18*).

14.3.2 DNA Quantification

14.3.2.1 Preparation of DNA Concentration Standards and Running the Gel

1. Prepare DNA concentration standards. Dilute lambda DNA to a set of DNA concentrations covering the expected range of the genomic DNA samples being assayed (*see Note 6*).
2. Prepare a 1.5 % agarose gel in 0.5× TBE buffer with 0.2 µg/ml ethidium bromide or alternative staining dye (*see Note 19*).
3. Add 3 µl of DNA sample plus 2 µl DNA loading dye. Use the same volumes for the DNA concentrations standards.
4. Load samples and concentration standards on the gel.
5. Run gel at 5–6 V/cm for 30–60 min. The DNA sample (genomic DNA band) should be completely out of the well and into the gel at least 0.2 cm. Do not run the gel too long as the genomic DNA band will become diffuse and hard to quantify. You may be able to accurately quantify degraded samples by altering electrophoresis conditions. Modifications can be made to increase throughput (*see Notes 18 and 20*).

Table 14.1 Estimated costs for DNA extractions using different chaotropic salts

Material	Use per sample	Cost per sample using KI (USD) ^a	Cost per sample using NaI (USD)	Cost per sample using guanidine thiocyanate (USD)
Celite 545	0.016 g	<0.01	<0.01	<0.01
SDS	0.004 g	<0.01	<0.01	<0.01
Sodium acetate	0.05 g	<0.01	<0.01	<0.01
NaCl	0.003 g	<0.01	<0.01	<0.01
RNase A (mg)	1×10^{-7} mg	<0.01	<0.01	<0.01
Ethanol (ml) ^b	1 ml	0.13	0.13	0.13
Potassium iodide	0.7 g	0.51		
Sodium iodide	0.6 g		0.31	
Guanidine thiocyanate	0.5 g			0.29
10× TE buffer (ml)	0.82 ml	<0.01	<0.01	<0.01
Total (USD)		0.64	0.44	0.42

^aCosts based on 2015 US catalogue prices

^bEthanol is typically not supplied in kits but supplied by the user. This should be considered when comparing costs with commercial kits

14.3.2.2 Photographing the Gel and Quantification

1. Adjust the image and take the longest possible exposure that does not saturate the image of any of the samples being assayed (*see Note 21*).
2. Save this image in TIFF format (*see Note 22* and Fig. 14.2).
3. Start the ImageJ software.
4. Open the TIFF image to be analysed (*File > Open*) (*see Note 23* for an example image).
5. Open the rotate dialogue box to straighten the image (*Image > Transform > Rotate*).
6. In the rotate dialogue box, select 'preview', set the grid lines number to '30', select one of the interpolation features (either 'bilinear' or 'bicubic') and adjust the angle in degrees until the bands are in line with the grid lines (*see Note 24*).
7. When finished, click OK.
8. Subtract background noise (*Process > Subtract Background*). Deselect 'light background'. Ensure that rolling ball radius is set properly. A default of five pixels works for most mini gels (*see Note 25*).
9. Select the magnifying glass tool in the ImageJ toolkit dialogue box and make the gel image as large as reasonable.
10. Select the rectangle selection tool in the ImageJ toolkit dialogue box.
11. Find the highest intensity or largest band on the gel to be analysed and draw a box around it. Partially degraded samples can still be quantified (*see Fig. 14.3* and *Notes 20, 26 and 27*).
12. Left click and hold the mouse over the box and move it so that it is positioned around lane 1.
13. Measure the box by hitting the 'm' key. A results table should appear with columns for sample number, area, mean, min and max values (*see Note 28*).
14. Minimise the table so that you can again view the gel image.
15. Move the box to lane 2 and hit the 'm' key (*see Note 29*).
16. Continue to move the box to the next lane and hit the 'm' key until all the lanes in a gel tier are measured, including the standards.
17. Evaluate the table to ensure every sample has the same area value. If not, redo the analysis.

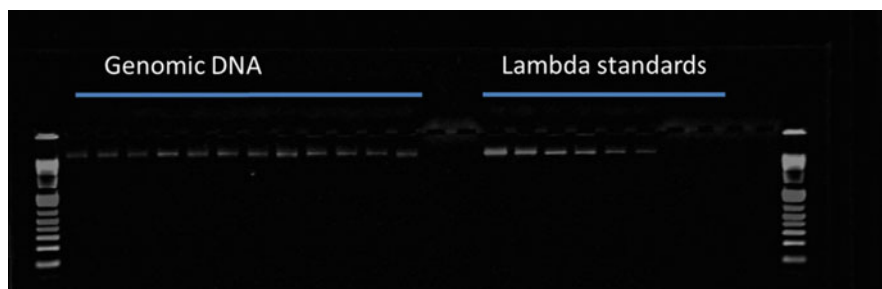


Fig. 14.2 Example of genomic DNA with lambda DNA standards

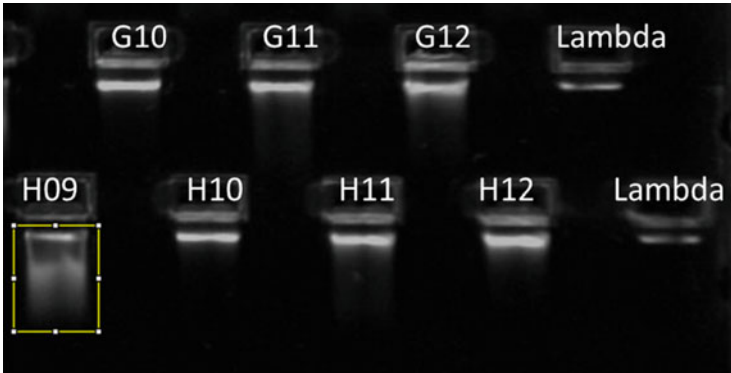


Fig. 14.3 ImageJ analysis of cassava genomic DNA samples assayed on a high-throughput E-Gel 96 2% agarose gel. Seven of 96 samples assayed are shown. Well positions on the 96-well sample plate are indicated for each sample along with the location of lambda DNA standards. Sample H09 shows extensive smearing indicating DNA degradation. This sample can be quantified by enlarging the size of the analysis box. Care should be taken when quantifying degraded DNA samples (see **Notes 18** and **20**). This gel image was produced in the Plant Breeding and Genetics Laboratory by Daniel Tello and Lina Kafuri

18. Check that the number of samples equals the number of lanes on the gel. If not, you either missed a lane or counted a lane more than once. If so, you need to start over.
19. When you are satisfied that the table is correct, select the entire contents of the table (Ctrl + A), copy and paste into a spreadsheet program (e.g. Microsoft Excel, OpenOffice or LibreOffice Calc) (see **Note 23**).
20. Prepare a standard curve (*Insert > Charts > Scatter*) using the ‘Mean’ values of the lambda standards from the table.
21. Add a trend line (*Chart > Add Trendline > Polynomial*). Under type, click polynomial and select second order. Typically, the second-order polynomial is suitable (Fig. 14.4) (see **Note 30** for the use of higher-order polynomial).
22. Click Options and select *Display equation on chart* and *Display r-squared value on chart*.
23. Click OK.
24. Inspect the graph: are there any points that are clearly off of the trend? If so, consider removing this data point and redrawing the graph (Fig. 14.5).
25. Use the equation in the graph to solve for the y value (DNA concentration). The X-axis is your density data collected from ImageJ.
26. Use the spreadsheet formula tools to calculate the DNA concentrations in the samples (see **Note 31**). The results will be the DNA concentrations in ng/ μ l. Use the autofill function for the other samples.
27. Visually compare the band intensities on the image with the concentrations estimated from the standard curve. Bands with lower intensity by visual inspection should have lower concentrations listed on the worksheet. If not, consider repeating the measurement.

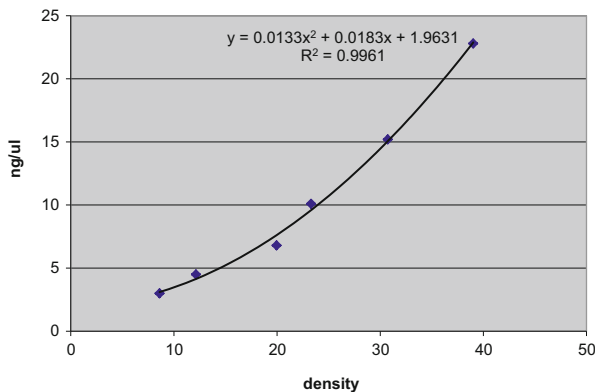


Fig. 14.4 Example of standard curve with second-order polynomial trend line and equation

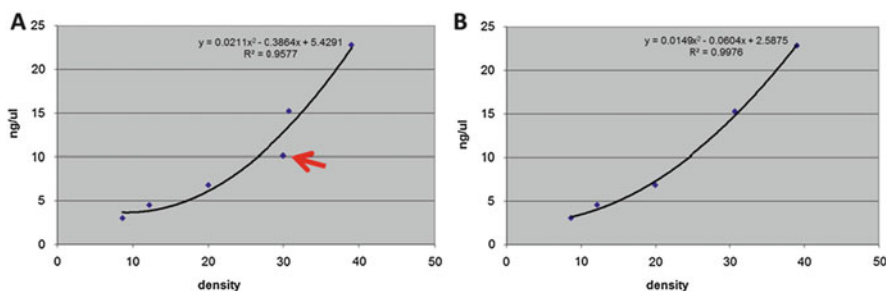


Fig. 14.5 Example of correcting a standard curve when there is one erroneous data point. (a) The intensity value of the 10 ng/μl data point (*red arrow*) is considered to be wrong because the density value is much higher than expected (based on the trend line in Fig. 14.4, the 10 ng/μl should have a value closer to 23). (b) A correction is made by removing this data point and redrawing the standard curve. A curve more similar to that shown in Fig. 14.4 is achieved and concentration accuracy is improved

14.4 Notes

1. While costly, tungsten carbide beads are durable and can be washed and reused many times, making the actual cost per extraction extremely low. An alternative is to use 3-mm steel ball bearings. Ball bearings can be purchased in bulk at a low cost allowing for disposal of bearings after a single use (cost per sample is less than 1 cent).

2. Adding approximately 0.1 g (a pinch) of purified sea sand (e.g. Sigma-Aldrich Cat. No. 274,739) per sample can greatly improve tissue grinding.
3. It takes several hours to prepare 6 M KI. Prepare in advance and avoid exposure to light by storing in brown bottle or foil-covered bottle. The buffer will eventually turn yellow but is still suitable for use.
4. Alternative chaotropic buffers can be used at the same molarity. Examples include guanidine thiocyanate and sodium iodide. We have not observed any difference in DNA quantity or quality recovered using different buffers. The buffer represents the most expensive part of the assay (Table 14.1). We therefore recommend testing each buffer and choosing the one that can be purchased at lowest cost.
5. Prepare fresh because salt precipitates during storage.
6. Lambda DNA is a high molecular weight DNA at a precise concentration. Dilute this lambda stock in $1 \times$ TE to about ten different concentrations in the range between 2.5 and 150 ng/ μ l to serve as DNA concentration standards. The choice of the optimal DNA concentration standards depends on the concentrations of the sample DNAs. Calculate dilutions based on the information printed on the stock tube of the lambda DNA (note that the concentration of lambda DNA may vary from batch to batch). Store stocks at 4 °C.
7. Prepare gel loading dye containing 30 % glycerol and colour dye. Do not use xylene cyanol as it migrates near the genomic DNA fragment and can interfere with quantification.
8. Most computer operating systems are suitable (Windows, Macintosh, Linux).
9. Download and install ImageJ software on your computer. Follow provided instructions for your operating system. The version used for this chapter was 1.49 v (Java version). The software is a free public domain program developed by Wayne Rasband of the National Institutes of Health, USA. Full documentation can be obtained from the website.
10. Long-term storage of LSS is to be avoided to reduce proliferation of microorganisms that may lead to degradation of genomic DNA. Longevity may be prolonged by addition of an antimicrobial agent such as sodium azide. However, due to the low cost of silica and the ease of preparation, we suggest making fresh preparations monthly and avoiding the addition of toxic chemicals.
11. Tubes containing SBS are typically prepared within 24 h of use. The effect of long-term storage has not been tested.
12. The type and condition of starting tissue is a key component of the quality and quantity of extracted genomic DNA. We suggest tissue desiccation of leaf material using silica gel as outlined in Till et al. (2015).
13. Alternative lysis buffers can be used if yields are low or sample degradation is observed. A description of alternative lysis buffers is provided in Till et al. (2015).
14. Transfer liquid only. Avoid transferring solid material. We have observed that transfer of a minor amount of particulate from leaf preparations does not negatively affect quality or quantity of resulting genomic DNA.

15. The colour of pellets turns from grey to white when dried. If a fume hood is not available, pellets can be dried for a longer period of time on the benchtop.
16. A buffered solution is preferred over water to prevent sample degradation, e.g. TE buffer (Tris buffer maintains pH, while presence of EDTA chelates divalent cations and inactivates nucleases that could damage DNA).
17. The amount of TE buffer used in elution can be adjusted if a higher sample concentration is desired. This should be determined empirically.
18. Various modifications can be made to increase the throughput of sample processing. Reactions can be scaled to a 96-well format. Specialised equipment exists for grinding tissue in 96-well plates (e.g. the TissueLyser from Qiagen). In addition, modular 96-well rapid agarose gel electrophoresis such as the E-Gel 96 system (Thermo Fisher Scientific) can be loaded with multichannel pipettors and be used to electrophorese hundreds of samples in less than 10 min (see Fig. 14.3). Images from these gels are suitable for the quantification method described here. It is important to note that scaling reactions to a 96-well plate format will likely result in lower yields of DNA due to physical limitations on the weight of tissue that will fit into a well of a plate.
19. **Caution:** Ethidium bromide is mutagenic. Wear gloves, lab coat and goggles. Dispose of gloves in toxic trash when through. Avoid contaminating other lab items (equipment, phones, door handles, light switches) with ethidium bromide. SYBR Safe DNA gel stain can be used instead of ethidium bromide.
20. The genomic DNA band should be clear of the gel well for accurate quantification. If a sample is still partially in the gel well, quantification will be less accurate due to fluorescence that can occur at the well interface. Partially degraded samples can be quantified by extending the size of the analysis box (Fig. 14.3). However, it is often best to improve gel images by running samples again on a 3 % MetaPhor agarose gel (~10.5 g MetaPhor (Cambrex) in 350 ml 0.5× TBE) following manufacturer's instructions. This allows for a sharper band to be produced for more accurate quantification. The aim is to quantify the entire DNA that can be used productively in PCR reactions. It is advisable to test PCR performance of degraded DNA prior to use as excessively nicked DNA may inhibit PCR amplification. If a high percentage of samples are degraded, consider changing lysis buffer (see Note 13) and make sure tissue is properly handled prior to extraction (Till et al. 2015).
21. It is important to get a proper exposure of the gel that shows a difference in ethidium bromide staining in the concentration ranges you are assaying. The image of a reference sample of a higher concentration than any of the samples being assayed can be saturated. For example, if all of your samples are at 20 ng/μl, you should be able to observe a noticeable difference in the concentration standards around 20 ng/μl.
22. **Caution:** Do not use compressed file formats such as JPEG.
23. Go to <http://www-naweb.iaea.org/nafa/pbg/public/manuals-pbg.html> to download an example of DNA gel image and an example of DNA concentration workbook with data added. The example of DNA concentration workbook shows how a spreadsheet can be used to semiautomate the task of concentration

determination. The second worksheet in this workbook shows how formula can be applied to group ('bin') samples of similar concentration together. This is especially useful when normalising DNA samples to a common concentration prior to sample pooling (such as in TILLING assays). Binning samples can dramatically reduce the number of pipetting volumes to adjust samples to a common concentration.

24. You can use positive values for clockwise rotation and negative values (by placing a minus '-' sign) for counterclockwise rotation. You may have to use a decimal setting to get the lanes to line up.
25. Information on rolling ball radius settings can be found at <http://imagej.nih.gov/ij/docs/menus/process.html#background>. In general, your image should be completely black with the exception of DNA-dye-stained bands. Try several different pixel radii to see how your gel looks. Notice that when the number is very high, you can see the outline of your gel. If you do not fully subtract these pixels, they will be counted and your DNA concentration calculations will be less accurate.
26. ImageJ reports the pixels in the box. Variation from counting background pixels is controlled for by keeping the box size constant.
27. **Caution:** Make sure that the box surrounds the entire signal but does not overlap with the signal from another band. Failure to do so will lead to an inaccurate reading.
28. 'Area' is area of selection in square pixels or in calibrated square units. 'Mean' is the average grey value within the selection (sum of the grey values of all the pixels in the selection divided by the number of pixels). 'Min' and 'max' values are minimum and maximum grey values within the selection.
29. **Caution:** Do not change the size of the box. You must measure the same volume of the box for each lane. If you accidentally change the size of the box while measuring lanes, start over.
30. **Optional:** You may try a higher-order polynomial to evaluate how differences in curve fitting can affect your concentration estimation. For example, the third-order polynomial for the graph in Fig. 14.4 is the formula:

$$y = -0.0002 \times x^3 + 0.0244 \times x^2 - 0.2174 \times x + 3.3653.$$
31. For example, using the graph in Fig. 14.4, if the density data for your sample is in cell D5, select an empty cell in row 5 and enter the following formula:

$$y = 0.0133 \times D5 \times D5 + 0.0183 \times D5 + 1.9631.$$

The result will be the concentration in ng/ μ l. Select the cell and drag the right corner down to autofill for other samples. See **Note 23** for examples.

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Chapter 15

A Protocol for Benchtop Extraction of Single-Strand-Specific Nucleases for Mutation Discovery

Bernhard J. Hofinger, Owen A. Huynh, Joanna Jankowicz-Cieslak, and Bradley J. Till

Abstract Single-strand-specific nucleases are used to cleave mismatches in otherwise double-stranded DNA. Assays typically involve PCR amplification followed by a denaturing and annealing step to generate heteroduplexed DNA molecules from PCR products containing nucleotide polymorphisms. This is followed by digestion with nucleases that cleave at the site of the mismatch. The molecular weights of the cleavage products indicate the approximate position of nucleotide polymorphisms in PCR amplicons. Cleaved DNA products are observed by one of several readout platforms such as native gel electrophoresis, denaturing gel electrophoresis, capillary electrophoresis, or denaturing high-performance liquid chromatography (DHPLC). This approach is highly suitable for accurate discovery of natural and induced single-nucleotide polymorphisms (SNPs) and also small insertions and deletions (indels). The use of self-extracted single-strand-specific nucleases, typically prepared from crude extracts of celery (*Apium graveolens*), is common for reverse-genetics (e.g. Targeting Induced Local Lesions IN Genomes (TILLING)) and studies of natural nucleotide polymorphisms (e.g. Ecotilling). While protocols have been published describing the preparation of single-strand-specific nuclease from plant tissues, many rely on toxic chemicals, dialysis and large-volume preparatory centrifuges and also require steps to be performed at 4 °C. We provide here a streamlined room temperature extraction protocol that uses standard microcentrifuges and eliminates toxic chemicals and traditional dialysis.

Keywords Enzymatic mismatch cleavage • Polymorphism discovery • TILLING • Ecotilling • Enzyme purification

B.J. Hofinger • O.A. Huynh • J. Jankowicz-Cieslak • B.J. Till (✉)
Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria
e-mail: b.till@iaea.org

15.1 Introduction

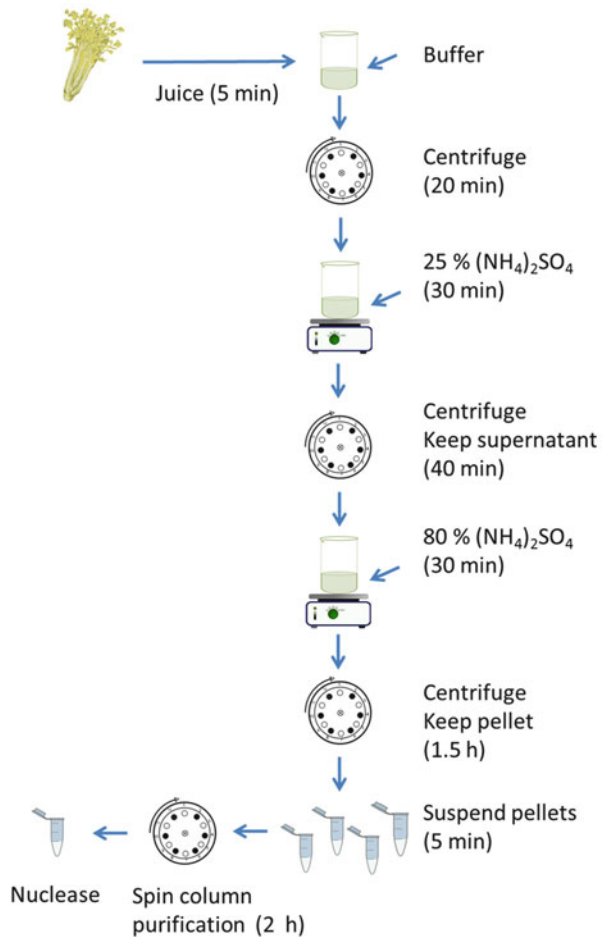
Natural and induced mutations are powerful tools for functional genomics and plant breeding. Methods for rapid discovery and genotyping of mutations are important for a variety of applications including marker development, marker-assisted selection, population genetics, and reverse-genetics. Single-nucleotide polymorphisms (SNPs) represent the most common type of natural nucleotide variation in plants. While the spectra of mutations induced by treatment with either chemical or physical mutagens vary depending on the mutagen and dosage of mutagen used, many treatments with chemical mutagens produce predominantly SNP or small indel mutations. For example, data collected from Targeting Induced Local Lesions IN Genomes (TILLING) projects where species were treated with either ethyl methanesulfonate (EMS), sodium azide or a combination of sodium azide, and N-methyl-N-nitrosourea (MNU) show that these mutagens create predominantly single-base-point mutations (Jankowicz-Cieslak et al. 2011). Furthermore, treatment with EMS produces greater than 90 % GC to AT transition mutations for many crop species (Greene et al. 2003; Slade and Knauf 2005; Jankowicz-Cieslak et al. 2011; Jankowicz-Cieslak et al. 2012). There is a broader diversity of effects reported of plants treated with physical mutagens. For example, fast neutron irradiation of plants has been reported to generate genomic deletions ranging in size from less than 56 base pairs to nearly three million base pairs (Li et al. 2001; Rogers et al. 2009; Bolon et al. 2011; Belfield et al. 2012). The most common mutagen used in the development of the ~3200 officially released mutant crop varieties curated in the IAEA's Mutant Variety Database (<http://mvd.iaea.org/>) is gamma irradiation. Reports on the type of DNA lesions induced by gamma irradiation are quite variable. In rice, data suggest that >80 % of mutations induced via gamma irradiation are small deletions up to 16 base pairs, with the remainder being large deletions (Sato et al. 2006; Bruce et al. 2009). Yet in maize only deletions greater than 200 kilobases were reported (Yuan et al. 2014). Some caution is needed when trying to draw broad conclusions from these datasets as many technologies employed to discover mutations have strong ascertainment biases. For example, enzymatic mismatch cleavage of PCR products is highly efficient for discovery of SNPs and deletions of up to approximately 50 base pairs, but variations involving a larger number of nucleotides go undetected (Till et al. 2003; Comai et al. 2004). Discovery of large insertions and deletions requires more technically complex and expensive approaches (Bolon et al. 2011; Henry et al. 2014).

Enzymatic-based approaches for the discovery and characterization of smaller nucleotide variation date back to the 1970s. A class of enzymes known as single-strand-specific nucleases was first shown to preferentially cleave single-stranded regions of otherwise double-stranded DNA (Shenk et al. 1975; Kroeker and Kowalski 1978; Desai and Shankar 2003). By the late 1990s, PCR and capillary Sanger sequencing allowed direct identification of single-nucleotide variation. This remains cost prohibitive for large-scale screening, and assignment of heterozygous indels using this technique is challenging. In 1998, a single-strand-specific nuclease

related to S1, named CEL I, was described that could cleave all types of single-base mismatches (Oleykowski et al. 1998). Using this enzyme purified from celery, efficient PCR-based screening approaches were developed for discovery of EMS-induced mutations in TILLING reverse-genetic screens (Colbert et al. 2001). Further studies and optimizations showed that crude enzyme extracts from celery or mung bean sprouts could be prepared that contain a high single-strand-specific nuclease activity and could be used for accurate discovery of both SNP and small indel nucleotide variation (Till et al. 2004). Crude extracts from celery have been commonly used in TILLING assays and also for the detection of natural nucleotide variation (known as Ecotilling), and thousands of nucleotide variants have been discovered (Jankowicz-Cieslak et al. 2011; Till 2014). The use of crude enzyme extracts was shown to result in accurate recovery of DNA variation. In studies of both human samples and highly heterozygous polyploid bananas, false-negative and false-positive discovery errors were reported below 5 % (Till et al. 2006a, b; Till et al. 2010). While many TILLING and Ecotilling projects have utilized fluorescence-based detection of DNA to increase sensitivity and throughput, the development of a more simple and low-cost method employing PCR and standard gel electrophoresis has made mutation discovery suitable for any laboratory equipped for basic molecular biology (Dong et al. 2009; Uauy et al. 2009; Lee et al. 2014).

Assay costs are further reduced by using single-strand-specific nucleases that are self-prepared from plant materials, typically celery. The standard procedure involves two ammonium sulfate cuts carried out at 4 °C in the presence of the toxic protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Till et al. 2006a, b). Following precipitation, the sample is desalted using 10,000 molecular weight cutoff dialysis membrane. While efficient and low cost, this standard protocol requires all steps to be performed at 4 °C, including large-volume centrifugation. We have simplified this procedure so that nuclease can be prepared at room temperature using a benchtop microcentrifuge common to most molecular biology laboratories. We have also eliminated the use of toxic chemicals. This streamlined approach is diagrammed in Fig. 15.1. Celery is first juiced and buffer added to maintain pH. Particulate matter in the juice is removed by centrifugation. Ammonium sulfate is then added to the clarified juice to a saturation of 25 %. Single-strand-specific nucleases remain in solution at this saturation, but other proteins are precipitated and removed by centrifugation. The supernatant is collected, and ammonium sulfate is added to 85 % saturation, where the desired single-strand-specific nucleases precipitate and are collected by centrifugation. Following removal of the supernatant and suspension of the pellet in buffer, residual salt from the ammonium sulfate precipitation, which is inhibitory to mutation discovery, is removed. This is through the use of specialized filter columns that retain the nuclease. A total of four buffer washes are performed to remove salt. This process also concentrates proteins. The purified enzyme is then collected and activity is tested. The whole procedure can be completed in 1 day. Enzyme for approximately 3000 reactions can be produced from 18 ml of celery juice. The cost of enzyme for one reaction is less than 0.06 euro cents.

Fig. 15.1 Overview of the steps involved in preparation of crude enzyme extracts from celery containing single-strand-specific nuclease activity. Approximate time for each step is indicated



15.2 Materials

1. Vegetable juicer (e.g. L'Equip) (*see Note 1*).
2. Celery (*see Note 2*).
3. Buffer A: 1 M Tris-HCl, pH 7.7.
4. Buffer B: 0.1 M Tris-HCl, pH 7.7, 0.5 M KCl.
5. Ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$.
6. Amicon Ultra 0.5 ml 10 K centrifugal filters and collection tubes (EMD Millipore Cat. No. UFC501024).
7. Micropipettes (1000 μl , 200 μl , 20 μl).
8. 2.0 ml microcentrifuge tubes.
9. Standard bench microcentrifuge.
10. Magnetic stirrer.

15.3 Methods

15.3.1 Enzyme Preparation

1. Rinse celery with water. Remove any leaves and cut off tough tissue at base of stalk. The amount of juice produced depends on the type of juicer or blender used. An efficient juicer will produce ~250 ml from 0.5 kg of celery (*see Notes 2 and 3*).
2. Juice the celery. Place 18 ml into a clean beaker or tube. Excess juice can be stored at -80°C for years (*see Note 4*).
3. Add 2 ml of buffer A to 18 ml of celery juice.
4. Distribute liquid equally into twelve 2.0 ml microcentrifuge tubes.
5. Spin the juice for 20 min at $2,600 \times g$ to pellet debris at room temperature (RT).
6. Pour supernatant into a 25 ml graduated cylinder and record volume.
7. Bring the supernatant to 25 % $(\text{NH}_4)_2\text{SO}_4$ by adding 144 g per liter of solution. Mix gently for 30 min using stir plate and magnetic stir bar (Fig. 15.2). (*e.g. if total volume is 18.5 ml, add 2.66 g $(\text{NH}_4)_2\text{SO}_4$*).
8. Distribute liquid into 2.0 ml microcentrifuge tubes. Spin at $15,000 \times g$ at room temperature for 40 min.
9. Pour supernatant into clean graduated cylinder. Record volume. Discard pellet (Fig. 15.3a).
10. Bring the supernatant from 25 to 80 % $(\text{NH}_4)_2\text{SO}_4$ by adding 390 g per liter of solution. Mix gently for 30 min in a beaker with magnetic stir bar. (*e.g., if total volume is 18 ml, add 7.02 g $(\text{NH}_4)_2\text{SO}_4$*).
11. Distribute liquid into 2.0 ml microcentrifuge tubes. Spin at $15,000 \times g$ for 1.5 h. Save the pellet and discard the supernatant, being careful when decanting the supernatant. The pellet can be stored at -80°C for months (Fig. 15.3b).
12. Suspend the pellets with buffer B. Add 0.5 ml of buffer to the first tube and pipette until pellet is in solution. Transfer this liquid to the next tube and suspend the pellet. If a large amount of foam is produced, add 0.5 ml of buffer to a new tube. Do not exceed a total of 1.5 ml of buffer. Combine liquid into one tube when all pellets are in the solution (Fig. 15.4a).
13. Desalting. Distribute liquid into four Amicon filter devices, making sure not to exceed 500 μl in any filter (Fig. 15.4b). Attach collection tube and spin at $14,000 \times g$ for 30 min.
14. When complete, remove liquid from collection tube, replace filter onto the same tube, adjust volume to 500 μl with fresh buffer B, pipette up and down $5\times$, and spin at $14,000 \times g$ for 30 min.
15. Repeat the procedure in step 14 for a total of four times (Fig. 15.5a and b). The removal of salts can be calculated (Table 15.1, *see Note 5*).
16. To elute sample, invert the filter and place into a new collection tube. Centrifuge at $1000 \times g$ for 2 min.



Fig. 15.2 Protein precipitation with $(\text{NH}_4)_2\text{SO}_4$

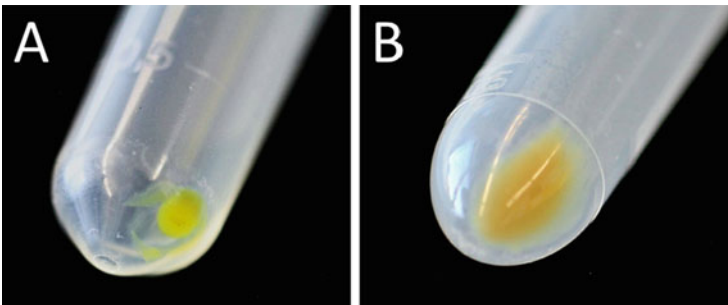


Fig. 15.3 Protein pellets after precipitation with ammonium sulfate. (a) Protein pellet after 25 % $(\text{NH}_4)_2\text{SO}_4$ precipitation. At this stage the enzyme remains in the aqueous phase. (b) Protein pellet after 80 % $(\text{NH}_4)_2\text{SO}_4$ precipitation. This pellet contains nucleases for mutation discovery

17. Combine all eluates into a single tube. Approximately 85 μl should be recovered (Fig. 15.5c). This tube contains crude celery juice extract (CJE) consisting of CEL I and other enzymes.
18. Centrifuge for 30 min at $10,000 \times g$ to remove any residual solid material. Transfer to a new tube and perform activity test.

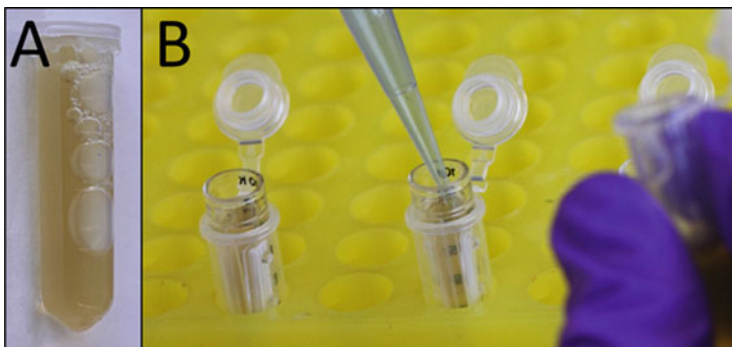


Fig. 15.4 Suspension and desalting of protein precipitate. (a) Approximately 1.5 ml liquid is recovered after resuspension and combination of all pellets (derived from 80 % $(\text{NH}_4)_2\text{SO}_4$ precipitation). (b) Liquid is transferred to filter devices for desalting

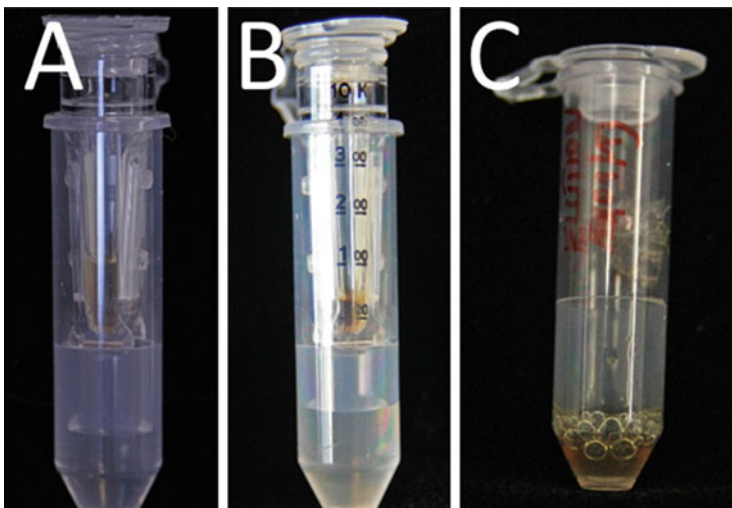


Fig. 15.5 Stages of enzyme purification and desalting. (a) After first centrifugation ($\sim 100 \mu\text{l}$ liquid is retained in the filter device and $\sim 400 \mu\text{l}$ flow through). (b) After fourth centrifugation ($\sim 35 \mu\text{l}$ liquid retained in the filter and $\sim 465 \mu\text{l}$ flow through). (c) Recovered eluates after centrifugation of the inverted Amicon filter devices

Table 15.1 Volumes of retained liquid in filter devices after centrifugation steps and calculated desalting factors

Centrifugation step (30 min at 15,000 × g)	Starting volume (μl)	End volume (after centrifugation)	Buffer added (μl)	Desalting factor for each step	Combined desalting factor (calculated)
1	500	100	400	5×	5×
2	500	~50	450	10×	50×
3	500	~35	470	14×	700×
4	500	~30	475	17×	11,900×

Table 15.2 Digestion mixes of CJE dilutions for testing enzyme activity

Relative enzyme concentration	CJE (μl)	CJE buffer (μl)	Water (μl)
0×	0	1.5	8.5
0.1×	0.25 (1:100)	1.5	8.25
1×	0.25 (1:10)	1.5	8.25
5×	1.25 (1:10)	1.5	7.25
10×	2.5 (1:10)	1.5	6.0
20×	0.5	1.5	8.0

15.3.2 Activity Test

1. Test a range of amounts of extracted enzyme using samples with known mutations. Follow the protocol for enzymatic mismatch cleavage used in Chap. 16 of this book. If you do not have positive control material containing known nucleotide polymorphisms, you can request a kit from the Plant Breeding and Genetics Laboratory that contains genomic DNA, PCR primers, buffers, and nuclease with validated activity (*see Note 6*).
2. The typical activity of enzyme produced with this protocol is 0.025 μl per reaction (*see Note 7*). To assay activity, perform a standard titration curve with the outermost points flanking the target range on either side following Table 15.2. Enzyme is diluted with buffer B where indicated. For 1:10 combine 1 μl of enzyme with 9 μl buffer. For 1:100 combine 1 μl enzyme with 99 μl buffer. Mix before use. Perform reactions as in the protocol referenced above by adding 10 μl PCR product with 10 μl of enzyme mix from Table 15.2. Example data are shown in Fig. 15.6.

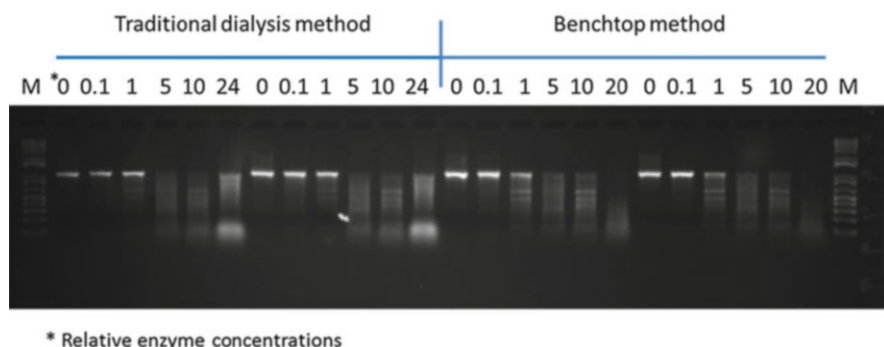


Fig. 15.6 Enzyme activity tests comparing traditional dialysis-based purification (*left*) and the benchtop protocol described in this chapter (*right*). *M* indicates lanes containing 1 Kb Plus DNA Ladder (Invitrogen). Mutations are easily identified at the 1× concentration

15.4 Notes

1. Any instrument capable of extracting juice from plant tissues may be used. If using a blender, consider pre-chopping the celery to avoid long fibrous strands that entangle the blender blades.
2. A variety of different plant species and tissues can be used including spinach, mung bean sprouts, and weedy plants growing wild (Oleykowski et al. 1998; Till et al. 2004; Till et al. 2015).
3. No difference in enzyme activity was observed between extraction performed at 4 °C and room temperature.
4. Juice can be stored at −80 °C for at least 2 years. Store juice in aliquots. Activity will reduce due to repeated freeze-thaw cycles.
5. It is important to remove excess salt from the nuclease preparation. Excess salt can cause smearing of gel lanes resulting in higher assay noise and inflated false-negative error rate.
6. A description of the positive controls can be found at <http://www-naweb.iaea.org/nafa/pbg/public/manuals-pbg.html>. Email Official.Mail@iaea.org with “plant breeding positive controls” in the subject line to request a kit.
7. The optimal amount of nuclease varies depending on the activity of the batch being used. If degradation of the PCR product is not observed at 20×, try 50× and 100× concentrations of enzyme.

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Chapter 16

A Protocol for Validation of Doubled Haploid Plants by Enzymatic Mismatch Cleavage

Bradley J. Till, Bernhard J. Hofinger, Ayşe Şen, Owen A. Huynh, Joanna Jankowicz-Cieslak, Likyelesh Gugsu, and Jochen Kumlehn

Abstract Doubled haploidy is an important tool for plant breeders. It provides a rapid means of developing recombinant populations consisting of individuals that are homozygous and therefore genetically fixed. Homozygosity is also important in plant mutation breeding where many induced mutations are predicted to be recessive and mutant alleles need to be in a homozygous state before new traits are expressed. While production of doubled haploids has been described for many plant species, efficient means to validate that produced materials are indeed homozygous are needed. Polymorphism discovery methods utilizing enzymatic mismatch cleavage are ideally suited for validation of doubled haploid plants. We describe here a low-cost protocol that utilizes self-extracted single-strand-specific nucleases, standard PCR reactions and agarose gel electrophoresis that can be applied to most plant species.

Keywords Homozygosity • Genetic fixation • Single-strand-specific nuclease • Polymorphism discovery • TILLING • Ecotilling • *Hordeum vulgare* • *Eragrostis tef*

B.J. Till (✉) • B.J. Hofinger • O.A. Huynh • J. Jankowicz-Cieslak
Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria
e-mail: b.till@iaea.org

A. Şen
Plant Breeding and Genetics Laboratory, Joint FAO/IAEA Division, International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria
Faculty of Science, Department of Biology, Istanbul University, 34134, Vezneciler, Istanbul, Turkey

L. Gugsu
Ethiopian Institute of Agricultural Research, HARC, PO Box 2003, Holetta, Ethiopia
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Plant Reproductive Biology, Corrensstrasse 3, 06466 Seeland, OT Gatersleben, Germany

J. Kumlehn
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Plant Reproductive Biology, Corrensstrasse 3, 06466 Seeland, OT Gatersleben, Germany

16.1 Introduction

First reported in the early 1920s, methods for the production of haploid plants have now been described for more than 250 species (Blakeslee et al. 1922; Maluszynski et al. 2003). In many cases, haploid plants either spontaneously become diploid or this state can be induced by treatment with chemicals such as colchicine. Doubled haploidy remains a popular and powerful tool in plant sciences and breeding because once plants are doubled haploid (DH), they are homozygous, genetic variants are fixed and inbreeding plants are true breeding. A wide range of methods have been described for producing DH plants, and efficiencies can vary dramatically (Maluszynski et al. 2003; Forster et al. 2007; Geiger and Gordillo 2009; Ravi and Chan 2010). Doubled haploids are extremely valuable commercially as they can accelerate the breeding process; they can be developed directly into uniform and stable cultivars of inbreeding species and provide parental lines to be used in hybrid breeding. For successful and efficient research and breeding, it is therefore necessary to validate that materials are truly DH and homozygous before plants are selected for further evaluation and use. This holds particularly true in the context of the development of new methods of haploid formation, be it the implementation of a novel principle or just the adoption of a known method in another species.

Enzymatic mismatch cleavage for discovery and genotyping of single-nucleotide polymorphisms (SNPs) and small insertion/deletions (indels) is ideally suited to detect loss of heterozygosity in putative doubled haploid plants in comparison to the progenitor lines. Widely used in Targeting Induced Local Lesions IN Genomes (TILLING) reverse-genetic studies, the procedure begins with the selection of gene-specific primers for PCR amplification of ~1–1.5 kb regions. This is followed by denaturation and annealing that creates heteroduplexed DNA in samples where heterozygous variation exists. Samples are then treated with a self-prepared mixture containing single-strand-specific nucleases that cleave DNA at mismatched regions. The resulting products are electrophoresed, and the presence of cleaved DNA fragments of lower molecular weight than the original PCR product indicates the presence and approximate location of heterozygous polymorphisms (Till et al. 2006a, b). The process is made low cost by self-extraction of nuclease and the use of standard agarose gel electrophoresis as a gel readout platform (Till et al. 2015).

Enzymatic mismatch cleavage has also been widely used in Ecotilling studies for discovery of natural nucleotide variation in populations (Till 2014). This approach has been shown to be highly accurate with less than 5% false discovery and false-negative error rates, even in highly heterozygous polyploids (Till et al. 2006a, 2010).

The method can be easily adapted to evaluate homozygosity in putatively haploid or DH plants. As a proof of principle, Hofinger et al. (2013) developed this method for barley and compared it to another approach for validation of DH plants: simple sequence repeat (SSR) markers (Hofinger et al. 2013). In this work, the authors showed that 11/26 primer pairs were suitable for DH validation by

enzymatic mismatch cleavage, while only 3/32 previously characterized SSR primer sets were suitable. Thus the enzymatic mismatch cleavage approach had an $\sim 4.5\times$ higher success rate. Furthermore, because enzymatic mismatch cleavage utilizes gene- or target-specific primers, the approach allows for accurate DH production error estimations per plant and per experiment taking into account genetic linkage. In addition, using gene-specific primers allows selection of specific alleles and for diversification of allele types in applications such as those involving F_1 hybrids. The entire process can be completed in one day (Fig. 16.1). Enzymatic mismatch cleavage has been used in over 20 plant and animal species for TILLING and Ecotilling, and thus this adaptation for haploid/DH validation is expected to be broadly applicable (Kurowska et al. 2011; Till 2014). Indeed, in addition to barley, we show data for screening putative DH *Eragrostis tef* plants (Fig. 16.3 and Hofinger et al. 2013). While the methods described in this protocol are straightforward and low cost, a key component of successful application is proper experimental design. Example data and a discussion of experimental design are provided along with a detailed protocol suitable for many crop species.

16.2 Materials

16.2.1 PCR Amplification

1. Taq DNA polymerase and Taq polymerase buffer (*see Note 1*)
2. dNTP mix
3. $MgCl_2$ (25 mM stock), if not supplied in Taq polymerase buffer
4. Forward and reverse primers (*see Note 2*)
5. Laboratory grade water (distilled or deionized and autoclaved)
6. Thermal cycler with heated lid and adjustable ramping

16.2.2 Enzymatic Mismatch Cleavage

1. Magnesium sulphate ($MgSO_4$)
2. Triton X-100
3. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).
4. Bovine serum albumin (BSA)
5. Potassium chloride (KCl)
6. $10\times$ Cleavage buffer (prepare a stock solution of 5 ml 1 M $MgSO_4$, 100 μ l 10 % Triton X-100, 5 ml 1 M HEPES (pH 7.4), 5 μ l 20 mg/mL BSA, 2.5 ml 2 M KCl, 37.5 ml water)
7. Single-strand-specific nuclease (*see Note 3*)
8. EDTA (ethylenediaminetetraacetic acid), 0.25 M

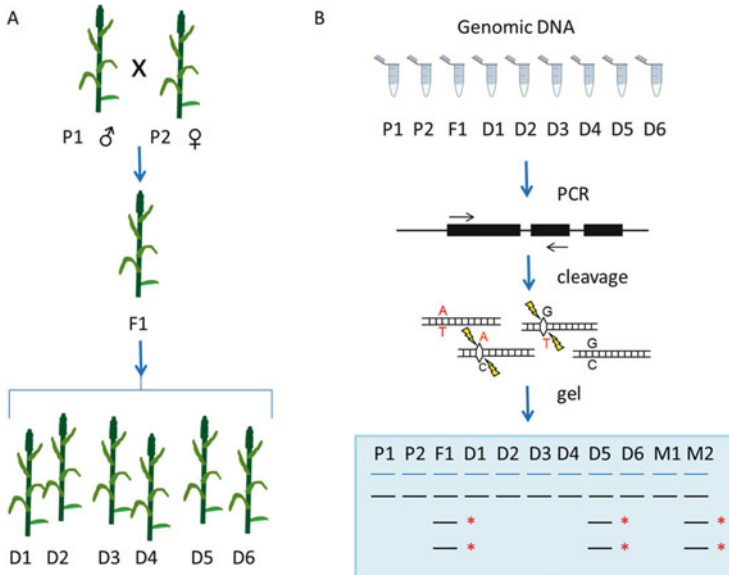


Fig. 16.1 The use of enzymatic mismatch cleavage for validation of doubled haploid plants in F_1 hybrid studies. (a) In a typical F_1 hybrid approach, two genetically diverse parental plants (P1 and P2) are crossed to create an F_1 hybrid (F_1). Putatively doubled haploids are produced from the F_1 plant (D1 through D6). (b) To validate the production of DH plants, genomic DNA is prepared from all materials. PCR is performed to amplify a specific target. PCR products are denatured and annealed to form mismatches where polymorphisms exist and samples are incubated with a nuclease that cleaves DNA that is not base-paired. DNA fragments are evaluated by agarose gel electrophoresis. Suitable primer pairs show cleavage products indicating heterozygosity in the F_1 sample (marked by *asterisks*). In the example drawn, all putatively DH plants are homozygous except sample D5. Lanes marked M1 and M2 represent mixtures of samples D1 and P1 and D1 and P2 (respectively). This allows assignment of the parental allele in the DH line. In this example D1 contains the allele from P1 because there was no heterozygosity observed in the mixture of the two samples. More primer pairs should be screened to increase confidence that the remaining plants are truly DH

16.2.3 Agarose Gel Electrophoresis

1. Standard agarose
2. TBE (tris/borate/EDTA) buffer
3. Ethidium bromide (10 mg/ml) (*see Note 4*)
4. Horizontal gel electrophoresis system with power supply
5. Gel imaging system
6. Gel loading dye (30 % glycerol plus Orange G) (*see Note 5*)
7. DNA molecular weight ladder (e.g. 1 kb ladder, Invitrogen 10787-018)

16.3 Methods

16.3.1 PCR Amplification

1. Select samples for analysis based on guidelines for experimental design (*see Note 6*).
2. Prepare a master mix according to manufacturer's guidelines for the Taq polymerase used. For example, for 10 samples with TaKaRa Ex Taq: 109.5 μl water, 20 μl 10 \times Ex Taq buffer, 16 μl 2.5 mM dNTP mix, 2 μl forward primer (10 μM), 2 μl reverse primer (10 μM), 0.5 μl TaKaRa Ex Taq (5 U/ μl).
3. Add 5 μL of DNA to each PCR tube (*see Note 7*).
4. Add 15 μl of PCR Master Mix to each PCR tube and mix by pipetting.
5. Centrifuge tubes for 1 min at 5000 $\times g$.
6. Incubate samples in thermal cycler with following conditions (*see Note 8*):

Step 1: Initial denaturation, 95 $^{\circ}\text{C}$, 2 min

Step 2: Denaturation, 94 $^{\circ}\text{C}$, 20 s

Step 3: Primer annealing, 73 $^{\circ}\text{C}$ ($-1^{\circ}\text{C}/\text{cycle}$), 30 s

Step 4: Ramp, 0.5 $^{\circ}\text{C}$ per second to 72 $^{\circ}\text{C}$

Step 5: Primer extension, 72 $^{\circ}\text{C}$, 1 min

Step 6: Cycling, repeat steps 2–5 for 7 cycles (8 cycles in total)

Step 7: Denaturation, 94 $^{\circ}\text{C}$, 20 s

Step 8: Primer annealing, 65 $^{\circ}\text{C}$, 30 s

Step 9: Ramp, 0.5 $^{\circ}\text{C}$ per second to 72 $^{\circ}\text{C}$

Step 10: Primer extension, 72 $^{\circ}\text{C}$, 1 min

Step 11: Cycling, repeat steps 7–10 for 44 cycles (45 cycles in total)

Step 12: Final extension, 72 $^{\circ}\text{C}$, 5 min

Step 13: Denaturation, 99 $^{\circ}\text{C}$, 10 min

Step 14: Cooling, 72 $^{\circ}\text{C}$, 20 s

Step 15: Cycling and touchdown, repeat step 14 for 69 cycles ($-0.3^{\circ}\text{C}/\text{cycle}$)

Step 16: Hold, 8 $^{\circ}\text{C}$, forever

7. Samples can be stored at -20°C for months.

16.3.2 Enzymatic Mismatch

1. Prepare an enzyme digestion master mix. For each sample add 4 μl 10 \times cleavage buffer, 7 μl single-strand-specific nuclease and 9 μl water (*see Note 3*).
2. Centrifuge PCR products for 1 min at 5000 $\times g$.
3. Add 20 μl of enzyme digestion master mix to PCR reactions (*see Note 3*).
4. Incubate reactions in a thermal cycler at 45 $^{\circ}\text{C}$ for 15 min (no heated lid) and then hold at 8 $^{\circ}\text{C}$.
5. Add 5 μl of 0.25 M EDTA to each tube to stop the reaction.

16.3.3 Agarose Gel Electrophoresis and Data Analysis

1. Prepare a 1.5 % agarose gel in 0.5x TBE buffer. Heat mixture in microwave until all agarose is dissolved. Take care to avoid boiling agarose.
2. Cool agarose to approximately 55 °C (at this temperature you can hold the flask in your hand comfortably for 5 s).
3. Add ethidium bromide to the warm gel solution to obtain a final concentration of 0.5 µg/mL in the gel. Mix by swirling.
4. Pour gel solution into gel apparatus and insert comb according to the instructions of the manufacturer.
5. Add 2 µl 6× loading dye to 10 µl PCR product.
6. Run gel at 100 V for 1 h or until suitable separation of bands is achieved (*see Note 9*).
7. Photograph the gel.
8. Evaluate data (*see Figs. 16.2 and 16.3 and Note 10*).

16.4 Notes

1. Most Taq polymerases should be suitable for this method. Compare your favourite Taq with the least expensive you can purchase. If the two produce

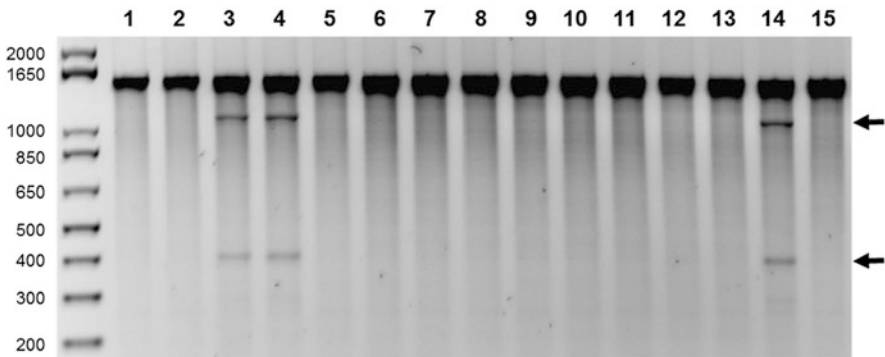


Fig. 16.2 Example data of validation of doubled haploid plant production in barley. A 1476-bp fragment of the barley *Mlo9* gene was PCR amplified and digested with a crude celery juice extract (CJE) containing single-strand-specific nuclease activity followed by agarose gel analysis. The top band in lanes 1–15 represents undigested PCR product. The cleavage products present in heterozygous samples are marked with *arrows*. Parental lines cv. Golden Promise (GP) and HOR1606 are homozygous for this gene region (*lanes 1 and 2*, respectively). A synthetic mixture of parental DNA and also the F₁ sample from crossing of the two parents show cleavage fragments resulting from a heterozygous SNP (*lanes 3 and 4*). Doubled haploid plants (*lanes 5–13*) are homozygous. Mixtures of genomic DNA from a DH plant and GP show cleavage products, while mixture of the same material with HOR1606 does not, indicating the DH harbours the HOR1606 allele (*lanes 14 and 15*). This figure and legend are reproduced from (Hofinger et al. 2013)

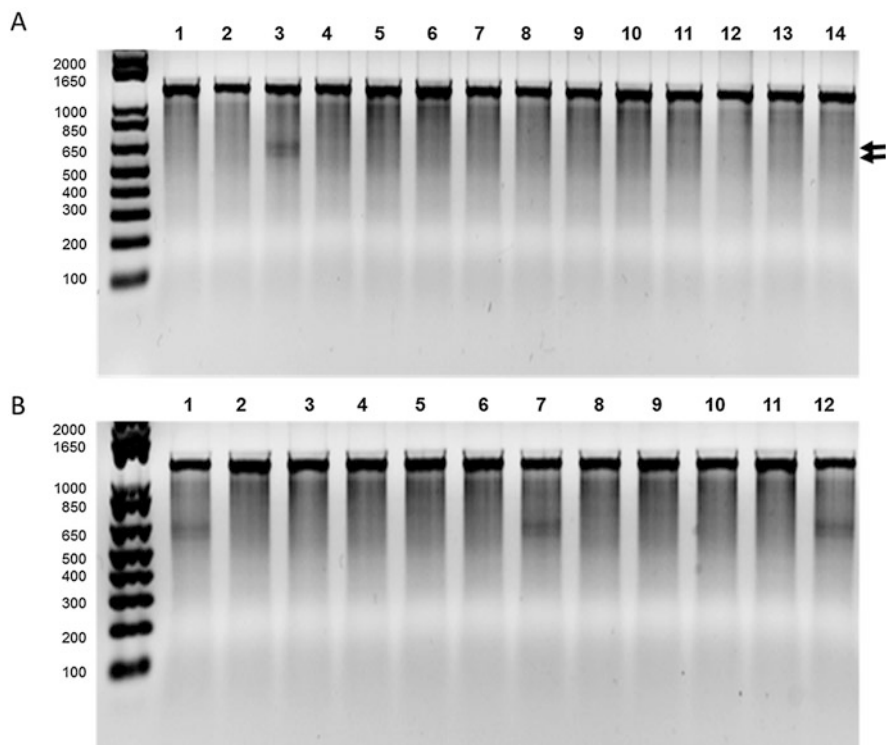


Fig. 16.3 Example data of validation of DH production in Tef primers were designed to amplify a 1400-bp region of the Tef genome. Screening for loss of heterozygosity was first performed with putative doubled haploid plants (panel **a**). Lanes 1 and 2 are parents. Lane 3 represents a synthetic F₁ hybrid created by mixing equal amounts of genomic DNA from each parent. Two cleavage products are produced indicating heterozygosity in the F₁ (marked by *arrows*). Lanes 4–14 represent putative DH plants. Experiments were then performed to determine the parental origin of the allele in putative DH plants. Panel **b** shows mixtures of samples with parent 1 (lane 1 of panel **a**). The first lane of panel **b** is the positive control. Lanes 2–12 are putative DH plants mixed with parent 1. This shows that two plants (lanes 7 and 12) inherited the allele from parent 2, while all others inherited the allele from parent 1. The reciprocal experiment was done by mixing samples with parent 2 (not shown)

similar results, use the cheaper version. Some Taq polymerases, like TaKaRa Ex Taq, come supplied with dNTPs and buffer containing MgCl₂.

2. Primer pairs should be selected to different genomic regions that are not genetically linked. The total number of primer pairs needed depends on the percentage of primer pairs where heterozygosity is observed. Primers are typically designed with the program Primer3 (Rozen and Skaletsky 2000) with a melting temperature of 70 °C (see **Note 8**).
3. The optimal amount of nuclease varies depending on the activity of the batch being used. Analysis of activity can be performed by screening PCR amplicons with heterozygous SNPs and titrating the amount of enzyme to produce clear

bands on the gel. See Chap. 15 for a protocol on enzyme preparation and activity optimization. When making master mixes, it is best to prepare more volume than is needed (e.g. for one additional sample) to compensate for minor pipetting errors. Volumes of reactions can be reduced from 40 to 20 μl in some cases.

4. *Caution:* Ethidium bromide is hazardous. Wear gloves and avoid contamination. Consult material safety data sheet (MSDS) for proper handling and disposal procedures. Alternative DNA stains can be used, e.g. SYBR Safe DNA gel stain, but detection may vary with different dyes.
5. Avoid loading dyes containing bromophenol blue or other dyes that migrate in a molecular weight range where you expect to observe DNA fragments. The presence of loading dyes can reduce the intensity of bands.
6. It is important to note that methods described here test only for homozygosity versus heterozygosity. Enzymatic mismatch cleavage does not distinguish between haploid and DH plants. An alternative method such as flow cytometry is required for this. The optimum experimental design includes DNA from the progenitor plant along with DNA from each putative DH plant produced from the progenitor. The only useful primer pairs will be those where heterozygosity is discovered in the progenitor plant. For example, if making doubled haploids from F_1 hybrid material, DNA from the F_1 hybrid is ideally evaluated along with DNA from the parents that were used to make the F_1 . If the F_1 harbours heterozygous SNPs at a particular locus, and the putative DH plants do not, this is evidence that the plant is DH (*see Note 10* for more on data analysis and interpretation). In this example, if F_1 material is not available, a synthetic F_1 sample can be prepared by mixing an equal concentration of DNA from each parent in a 1:1 ratio prior to PCR amplification. Screening the parental material alone is informative to learn if parents are heterozygous in any interrogated regions. It is not ideal to screen only putative DH material as it is difficult to estimate the probability that plants are truly DH rather than being homozygous because progenitor material was homozygous at that locus. In some cases, however, only putative DH material is available. In this circumstance it is advised to mix two to four putative DH samples together prior to PCR. If heterozygosity is observed in the mixture but not in individual samples, this suggests that the progenitor material was heterozygous. The set of primers used in this experiment would then be considered suitable (*see Note 10*).
7. The optimal amount of genomic DNA to be used should be determined empirically. Typically, a PCR amplicon yield of 10 ng/ μl is sufficient. The amount of genomic DNA needed to produce this amount can be roughly estimated by size of the genome (Till et al. 2006b). The yield of PCR product should be sufficiently high to produce cleavage fragments visible by agarose gel electrophoresis (see Figs. 16.2 and 16.3).
8. PCR conditions may need to be optimized. For example, primers with a melting temperature (T_m) of 70 $^\circ\text{C}$ were used to develop this protocol. Higher T_m primers increase specificity of amplification, but may not be necessary for all species. If lower T_m primers are used, the annealing temperature must be adjusted accordingly.

9. Optimal run time may vary. The Orange G DNA dye migrates at approximately 50 base pairs. A migration distance of 6–8 cm of Orange G from the comb typically provides suitable separation of fragments.
10. When analysing data, fragments observed of lower molecular weight than the full-length PCR product are typically the result of cleavage of heteroduplexed DNA at the site of a mismatch due to a nucleotide polymorphism. Truly doubled haploid plants are homozygous and therefore should not show cleavage products. However, cleavage fragments can also be observed due to a homopolymeric stretch of adenosine residues (Till et al. 2004). Evaluation of the parental material is therefore advised. Mixtures of parental material with putative DH plants allow assignment of alleles to a specific parent (see Figs. 16.2 and 16.3b). An estimation of the number of suitable primers can be made by calculating the probability that the data results by chance from self-fertilization. For example, the probability that the offspring of a self-fertilized heterozygous F_1 is homozygous for a specific locus is 0.5 (assume a Mendelian 1:2:1 ratio). The probability that two genetically unlinked loci are homozygous is $0.5^2 = 0.25$. By screening seven primer pairs from unlinked loci that show heterozygosity in the F_1 , one can achieve 99 % confidence.

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Chapter 17

Bioinformatics-Based Assessment of the Relevance of Candidate Genes for Mutation Discovery

Michał Słota, Mirosław Maluszynski, and Iwona Szarejko

Abstract The bioinformatics resources provide a wide range of tools that can be applied in different areas of mutation screening. The enormous and constantly increasing amount of genomic data obtained in plant-oriented molecular studies requires the development of efficient techniques for its processing. There is a wide range of bioinformatics tools which can aid in the course of mutation discovery. The following chapter focuses mainly on the application of different tools and resources to facilitate a Targeting-Induced Local Lesions in Genomes (TILLING) analysis. TILLING is a technique of reverse genetics that applies a traditional mutagenesis to create DNA libraries of mutagenised individuals that are then subjected to high-throughput screening for the identification of mutations. The bioinformatics tools have shown to be useful in supporting the process of candidate gene selection for mutation screening. The availability of bioinformatics software and experimental data repositories provides a powerful tool which enables a process of multi-database mining. The existing raw experimental data (genomics-related information, expression data, annotated ontologies) can be interpreted in terms of a new biological context. This may help in selecting the proper candidate gene for mutation discovery that is controlling the target phenotype. The mutation screening using a TILLING strategy requires a former knowledge of the full genomic sequence of the gene which is of interest. Depending on whether a fully sequenced genome of a particular species is available, different bioinformatics tools can facilitate this process. Specific tools can be also useful for the identification of possible gene paralogs which may mask the effect of mutated gene. Bioinformatics resources can also support the selection of gene fragments most prone to acquire a deleterious nucleotide change. Finally, there are available tools enabling a proper design of oligonucleotide primers for the amplification of a gene fragment for the purpose of mutation screening.

M. Słota • M. Maluszynski • I. Szarejko (✉)

Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

e-mail: iwona.szarejko@us.edu.pl

Keywords Candidate genes • Identification of gene paralogs • Gene expression repositories • TILLING

17.1 Introduction

17.1.1 *The Selection of Candidate Gene*

The proper selection of a suitable candidate gene for mutational analysis is a fundamental step which determines the chances of success. The identification and prioritisation of candidate genes for TILLING analysis employs different types of accessible in silico resources:

1. *Web-based tools for searching literature-derived data* for the general annotation and characterisation of the biological and molecular function of preliminary candidates
2. *Relevant repositories of genomics-related information* which can be used for retrieving of specific homologous genes from many species
3. *Gene expression repositories* which gather gene expression data: hybridisation arrays, chips, microarrays and RNA-seq data
4. *Gene ontology (GO) databases* which allow searching for genes according to their functional annotation, molecular characteristics or protein localisation

The selection strategy of suitable candidate genes involved in a course of specific developmental process or environmental stress reaction can be carried in different manners. The conventional approach is based on a presumptive knowledge or assumption about associated biological processes. This consists of a literature mining process that employs biological-oriented databases such as PubMed [www.ncbi.nlm.nih.gov/pubmed/], BioText [<http://biosearch.berkeley.edu/>] or other available services for general text mining as Google Scholar [<http://scholar.google.pl/>]. This attempt may be effective for such purpose if there is a known biological context associated with well-studied biological processes that were carried out on one of the model species. In the studies of more complex or under-examined traits for non-model species, there may be a lack of such presumptive knowledge. Limited knowledge about the genetic background of a complex trait can be overcome by collecting additional experimental data, as well as performing a wide range of in silico analyses. Gene expression databases such as Gene Expression Omnibus (GEO) [<http://ncbi.nlm.nih.gov/geo/>], ArrayExpress [<http://www.ebi.ac.uk/arrayexpress/>] or Genevestigator [<https://www.genevestigator.com/>] offer a publicly available repository of expression data for multiple gene sets. Browsing of gene expression database can lead to the identification of gene subsets that are differentially expressed across different experimental conditions. The identification of gene expression profiles can significantly reinforce the process of the selection of candidate genes associated with a distinct biological process or related to the reaction to environmental stimuli. Meta-analysis carried on database-derived data

can lead to the assortment of gene accessions displaying elevated or lowered expression levels in defined experimental conditions. Such genes may appear to be suitable candidates for the functional analysis using TILLING strategy. The proper description and classification of the gene expression and phenotype data is also crucial to enable meaningful comparisons. Gene ontology (GO) is a bioinformatics initiative aiming to introduce a unified vocabulary system for the biological terms. These terms are applied for the characterisation of the biological properties. GO can be applied for the hierarchical classification of gene products that can be divided into three main categories: cellular component, molecular function and biological process involvement (Carbon et al. 2009). Each annotation of GO term in database has a reference to associated information on related gene involvement. There are several different types of accessible web-based repositories that can be used for plant GO browsing, e.g. agriGO [<http://bioinfo.cau.edu.cn/agriGO/>], AmiGO Gene Ontology [<http://amigo.geneontology.org/>], Plant Ontology browser [<http://www.plantontology.org/amigo/go.cgi/>] and QuickGO browser [<http://www.ebi.ac.uk/QuickGO/>].

17.1.2 The Identification of the Genomic Sequences for Mutation Screening

Depending on the extent of available genomic sequence resources for a species, different approaches can be applied for sequence recovery. If the genome has already been sequenced, the genomic sequence of the gene of interest can be retrieved directly from a database. On the contrary, for a species for which the genome sequence is unknown or the genomic sequence is not available in accessible databases, the orthologous sequence can be identified using a closest relative as a query (e.g. *Arabidopsis* or rice). Basic Local Alignment Search Tool (BLAST) search may be carried, for example, against the rice genome repository to identify monocot orthologs of *Arabidopsis* genes [<http://blast.ncbi.nlm.nih.gov/>]. The identification of a complete coding sequence of a selected homologue requires the application of mRNA and/or amino acid sequences as a query in the search of expressed sequence tag (EST) databases. The assembling of identified and amplified sequences allows obtaining a coding sequence of the particular species. In the following step, the genomic sequence of an analysed homologue can be amplified with a use of designed exonic primers. The occurrence of paralogs (homologous genes created through a duplication event) in genomes should be taken into consideration during the selection of genes for TILLING analysis. Paralogous copies of a specific gene with redundant function can mask the effect of the mutation, thus complicating any gene knock-down strategies (Kurowska et al. 2011). In plants, paralogs are widespread among almost all plant lineages (Van Bel et al. 2011). There are accessible plant comparative genomics databases, such as GreenPhylDB [<http://www.greenphyl.org/>], Ensembl Plants [<http://plants>].

ensembl.org/] and Phytozome [<https://phytozome.jgi.doe.gov/>]. These resources group homologous genes into families using clustering useful for identifying possible orthologs. Gene Family Finder tool implemented within PLAZA 2.5 database [<http://bioinformatics.psb.ugent.be/plaza/>] enables the identification of gene families specific to one or more species and explores genomic information from search of different plants for structural and functional gene annotations (Van Bel et al. 2011). PLAZA package is an effective tool for the verification of the occurrence of possible gene paralogs within genome.

17.1.3 Selection of Gene Fragments for a Mutation Screening

The selection of a suitable gene region provides a higher probability to identify nonsense or useful missense mutations by TILLING screening (Chen et al. 2014). Depending on the applied detection method for mutation screening, a gene fragment length for TILLING should be limited to 1500 bp. The free web-based software Codons Optimised to Detect Deleterious Lesion (CODDLE) [<http://blocks.fhcrc.org/proweb/coddle/>] is a tool facilitating the choice of gene regions most suitable for TILLING analysis. CODDLE identifies the protein regions that are most likely to contain the largest percentage of deleterious lesions generated by G/C to A/T transitions. Also, the conservation-based sorting intolerant from tolerant (SIFT) software predicts whether an amino acid change is damaging for a protein [<http://sift.jcvi.org/>]. SIFT performs the multiple alignment for homologous sequences to predict whether the amino acid change is expected to have deleterious effects on the protein stability (Ng and Henikoff 2003). The determination of the region of the gene where the occurrence of point mutation has the highest probability to affect the gene function can be also carried out using a conserved domain prediction method (Till et al. 2007). On the basis of protein sequence obtained by in silico translation of a gene query, the highly conserved domains responsible for the reported catalytic properties can be identified using accessible repositories, such as conserved domain database (CDD) [<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/>].

17.1.4 Designing of Primers for the Amplification of a Gene Fragment

The classical TILLING protocol employs a mismatch-specific and sensitive endonuclease treatment followed by polyacrylamide electrophoresis and visualisation with a use of highly sensitive LI-COR gel analyser system (LI-COR Biosciences). This attempt requires an efficient amplification of a gene fragment selected for

mutational analysis (Till et al. 2006). The oligonucleotide primers flank a fragment of a length of optimally 1–2 kb or less. IR-labeled (labeled with infrared dye) primers which should have high (up to 70 °C) thermal stability can be designed using web-based primer design tools such as Primer3 [<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>] or Primer-BLAST [www.ncbi.nlm.nih.gov/tools/primer-blast/]. Designed primers should be analysed using BLAST tool against the full genomic DNA sequence to ensure that they target a single sequence. The same primer design and PCR parameters have been used for TILLING by sequencing (*see* Chap. 20).

17.2 Materials

17.2.1 Literature Mining

There are a variety of accessible web-based resources that can be applied for a scientific literature search. Depending on their repository content and applied indexing methods, these resources can be divided into specific categories (Table 17.1):

- (a) Search tools for scientific abstracts
- (b) Search tools designed to search beyond abstract, allowing access to the full-text articles
- (c) Image-oriented browsers which allow searching for figure/table content of specific articles
- (d) Databases which aim to enrich a literature search in terms of prioritising the relevance of records, gene ontology annotations and assignment of biological context

Table 17.1 Representative databases which can be applied for scientific literature mining

Category	Database	Website
Literature mining	PubMed	[http://www.ncbi.nlm.nih.gov/pubmed/]
	PubMed Central	[http://www.ncbi.nlm.nih.gov/pmc/]
	Google Scholar	[http://scholar.google.com/]
Figure/table browsers	BioText	[http://biosearch.berkeley.edu/]
	Yale Image Finder	[http://krauthammerlab.med.yale.edu/imagefinder/]
Text records prioritising	RefMed	[http://dm.postech.ac.kr/refmed/]
Ontology annotation	GOPubMed	[http://www.gopubmed.com/]
Context clusterisation	CiteXplore	[http://www.ebi.ac.uk/citexplore/]

17.2.2 Databases of DNA and Protein Sequences

The sequence of a specific gene can be retrieved for many species from publically accessible databases which gather information of nucleotide and protein sequences. As the species with the sequenced genomes are taken into consideration, there is a considerable support resulting from accessible genetic data browsers, such as NCBI GenBank. A repository search can be performed based on sequence query using a Basic Local Alignment Search Tool (BLAST) tool. BLAST searches for CoreNucleotide (main sequence collection), dbEST (expressed sequence tag collection) and dbGSS (genome survey sequences collection of unannotated short single read) can be carried independently. In the case of species for which the amount of genomic data is more limited, there are numerous locally developed search tools available which can be applied. ViroBLAST [<http://indra.mullins.microbiol.washington.edu/viroblast/viroblast.php/>] is a stand-alone BLAST web server for nucleotide and protein queries of multiple databases and user's defined datasets. ViroBLAST was implemented in various areas of plant research for browsing a raw data of sequenced plant genomes. If the genome of a particular species has not already been sequenced, there are alternative strategies for the identification of the sequence of a gene that is of interest, employing expressed sequence tag (EST) repositories. EST databases are widely used for the identification of a complete coding sequence of a new gene homologue using a related species query. Among different databases of DNA and protein sequences, the ones which are most commonly used for a gene sequence recovery are presented (Table 17.2).

17.2.3 Meta-analysis of Gene Expression Data

The use of gene expression databases can facilitate the process of searching for genes that are differentially expressed in different experimental conditions or tissues. This approach can contribute to the identification of up- or downregulated genes which can be suitable candidates for the functional analysis of their involvement in a particular process. A meta-analysis of gene expression profiles can be conducted with the application of gene expression repositories. Microarray databases aim to store the measurement of expression raw data, to process the content into a searchable index and to provide access to data in other applications for further analyses and interpretation. There are accessible databases which allow analysis of the primary data in order to develop a new data resource (Table 17.3).

Table 17.2 Representative databases which can be applied for a gene sequence mining

Category	Database	Website
Nucleotide databases	NCBI GenBank	[www.ncbi.nlm.nih.gov/genbank/]
	TAIR	[www.arabidopsis.org/]
Search tool	BLAST	[http://blast.ncbi.nlm.nih.gov/blast/]
ViroBLAST search tools	<i>Hordeum vulgare</i> ViroBLAST	[http://webblast.ipk-gatersleben.de/barley/viroblast.php/]
	<i>Populus trichocarpa</i> ViroBLAST	[http://popgenie.org/blast/]
	<i>Triticum turgidum</i> ViroBLAST	[http://wheat.pw.usda.gov/GG2/WheatTranscriptome/viroblast/]
	EST databases	NCBI dbEST
	PlantGDB	[http://www.plantgdb.org/cgi-bin/blast/PlantGDBblast/]
	CR-EST	[http://pgrc.ipk-gatersleben.de/cr-est/]

Table 17.3 Representative databases which can be applied for the meta-analysis of gene expression data

Database	Website	No. of experiments
ArrayExpress	[http://www.ebi.ac.uk/arrayexpress/]	63,450
ArrayTrack	[http://www.fda.gov/ScienceResearch/BioinformaticsTools/Arraytrack/]	1622
Gene Expression Omnibus (GEO)	[http://www.ncbi.nlm.nih.gov/geo/]	65,361
Genevestigator	[https://www.genevestigator.com/]	2621

17.2.4 Gene Ontology (GO) Analysis

The identification of candidate genes can be aided by using gene ontology-based criteria of semantic similarity. The functional interpretation of a raw experimental data can be accomplished by using the GO, for example, via an enrichment analysis. The annotation of GO terms for target genes can also contribute to achieve a better understanding of the biological context among three domains which are attributed: cellular component, molecular function and biological process. Gene ontology (GO) and plant ontology (PO) data can be browsed using various textual and graphical views and filtered for species-specific repositories. There are several different types of accessible web-based repositories that can be used for plant GO and PO terms browsing, e.g. AmiGO Gene Ontology [<http://amigo.geneontology.org/>] and Plant Ontology Browser [<http://www.plantontology.org/amigo/go.cgi/>]. There are numerous publically accessible databases which have implemented GO or PO annotations in order to improve the classification of their inner repositories, such as microarray data, the QTL directories and mutant germplasm (Table 17.4).

Table 17.4 Representative databases that have implemented the gene ontology (GO) or plant ontology (PO) terms for describing and cataloging their internal resources

Database	Website	Annotated data
AgriGO	[http://bioinfo.cau.edu.cn/agriGO/]	Genes
BarleyBase	[http://www.barleybase.org/]	Microarray
GrainGenes	[http://wheat.pw.usda.gov/GG3/]	Genes, QTLs
Gramene (a resource for comparative grass genomics)	[http://www.gramene.org/]	Genes, QTLs, proteins
Genevestigator (microarray database and analysis toolbox)	[http://www.genevestigator.ethz.ch/]	Microarray
International Rice Information System (IRIS)	[http://irri.org/tools-and-databases/international-rice-information-system/]	Mutants
Maize Genetics and Genomics Database (MaizeGDB)	[http://www.maizegdb.org/]	Genes, mutants
<i>Medicago truncatula</i> Gene Expression Atlas (MtGEA)	[http://mtgea.noble.org/v3/]	Microarray
Nottingham Arabidopsis Stock Centre (NASC)	[http://arabidopsis.info/]	Germplasms, genes
Oryzabase	[http://www.shigen.nig.ac.jp/rice/oryzabase/]	Genes, germplasms, microarray, mutants
Oryza Tag Line	[http://oryzatagline.cirad.fr/]	Mutants
Plant Expression Database (PLEXdb)	[http://www.plexdb.org/]	Microarray
Rice Oligonucleotide Array Project	[http://www.ricearray.org/]	Microarray
Solanaceae Genomics Network (SGN)	[https://solgenomics.net/solanaceae-project/]	Genes
SoyBase	[http://soybase.org/]	Genes, microarray, mutants
The Arabidopsis Information Resource (TAIR)	[http://www.arabidopsis.org/]	Genes, germplasms, microarray

17.2.5 Gene Homology Search

A wide variety of bioinformatics tools enables the analyses of sequence homologies between and within plant genomes. Specific databases dedicated for plant comparative genomics studies group homologous genes into families using clustering algorithms. Different packages are available for an effective verification of the occurrence of possible gene paralogs within genome (Table 17.5).

Table 17.5 Representative databases which can be applied for gene homology search

Database	Website	Function
Ensembl Plants	[http://plants.ensembl.org/]	Gene/protein homology search
GreenPhylDB	[http://www.greenphylo.org/]	
Phytozome	[https://phytozome.jgi.doe.gov/]	Gene/protein phylogenetic data
PLAZA 3.0 database	[http://bioinformatics.psb.ugent.be/plaza/]	Identification of gene families

17.2.6 Selection of Gene Fragments for the Mutational Analysis

The design of an amplicon as a crucial step in TILLING analysis can be facilitated using accessible bioinformatics software packages. The selection of a suitable gene region should be performed to provide a higher probability of the identification of nonsense or beneficial missense mutations within the selected gene region. Bioinformatics tools can be applied to facilitate the choice of gene region most suitable for mutation screening and the prediction of mutation effect on protein stability and function (Table 17.6).

17.2.7 PCR Primer Design for Mutation Screening

There is a wide selection of web-based primer design tools which can be applied for the analysis of predesigned primers or entering nucleotide sequence to design primers which match defined properties (Table 17.7). Designed oligonucleotide primers can be additionally verified in terms of their detailed molecular characteristics and possible secondary structure using appropriate software.

17.3 Methods

17.3.1 Biomedical Text Mining

1. Browse a PubMed database [<http://www.ncbi.nlm.nih.gov/pubmed/>] (*see Note 1*) using either a user-provided query associated with a specific topic or a predefined set of publications for text categorisation.
2. Filter obtained results in terms of article type, text availability, publication date or investigated species.
3. Use a 'Related information' menu within abstract content to link to other related NCBI databases for the selected record (e.g. gene and protein sequence).

Table 17.6 Representative databases and bioinformatics tools which can be applied for the selection of gene fragments for the mutational analysis

Database	Website	Function
CODDLE	[http://blocks.fhcr.org/proweb/coddle/]	Prediction of most suitable gene regions for TILLING analysis
SIFT (Sorting Intolerant From Tolerant)	[http://sift.jcvi.org/]	Prediction of mutation effect on protein stability
PROVEAN	[http://provean.jcvi.org/seq_submit.php/]	
I-Mutant3.0	[http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi/]	
ProtParam	[http://web.expasy.org/protparam/]	Assessment of protein properties
PDBeFold	[http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver]	Modelling of protein secondary structure
SAS	[http://www.ebi.ac.uk/thornton-srv/databases/sas/]	
SOPMA	[http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html/]	
Phyre2	[http://www.sbg.bio.ic.ac.uk/phyre2/]	
SWISS-MODEL	[http://swissmodel.expasy.org/interactive/]	Modelling of protein tertiary structure
DUET	[http://bleoberis.bioc.cam.ac.uk/duet/stability/]	
Conserved Domain Database	[http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/]	Identification of highly conserved protein domains

Table 17.7 Representative tools which can be applied for oligonucleotide designing

Database	Website	Function
Primer3	[http://primer3.ut.ee/]	Primer design
PrimerQuest	[http://eu.idtdna.com/PrimerQuest/]	
Primer-BLAST	[www.ncbi.nlm.nih.gov/tools/primer-blast/]	
OligoAnalyzer	[http://eu.idtdna.com/calc/analyzer/]	Oligonucleotide analysis

PubMed as a part of NCBI Entrez retrieval system provides a linked access to a set of 39 databases.

4. Use a 'Related information' menu for an article record to follow a link to PubMed Central database [<http://www.ncbi.nlm.nih.gov/pmc/>] for retrieving a full-text paper content.
5. The further extraction of biological context of identified text records can be performed using additional resources. The predefined types of information can be automatically extracted in terms of gene ontology annotation using GOPubMed [<http://www.gpubmed.com/>] tool. The entire biological context

for a specific list of records can be clustered using CiteXplore [<http://www.ebi.ac.uk/citexplore/>] tool.

17.3.2 *Retrieving the DNA and Protein Sequences*

1. Browse an Ensembl Plant database (*see Note 2*) by using gene name, symbol or description for all species repositories.
2. Select a most relevant record and explore a ‘Pan-taxonomic Compara’ menu within an Orthologues repository. The information about the number of identified splice variants, orthologs and paralogs is provided in a ‘Gene table’ section. A blue square on a generated graph depicts a speciation event (separating orthologs), whereas a red square stands for a duplication event (separating paralogs).
3. Analyse identified gene orthologs which are presented in results table with specified type of homology, Ensembl identifiers, location and query coverage. The number of species for each type of ortholog class is shown in the Type column in the table. The types of orthologs are assigned for each hit and are as follows: 1-to-1 orthologs indicates that only one copy is found in each species, 1-to-many orthologues indicate that one gene in one species is orthologous to multiple genes in the other species and many-to-many orthologs—multiple orthologs can be found in both species. The identified single-copy (1-to-1) orthologs are the most desirable targets.
4. Check the dN/dS ratio—a ratio of dN (non-synonymous substitutions) to dS (synonymous substitutions) of lower than 1 indicates negative (or purifying) selection, while a ratio of higher than 1 indicates positive selection. The information on the location of the ortholog (either on a chromosome or on a scaffold sequence), and the percentage of identical amino acids in the ortholog compared with the sequence of the target gene (target %ID), is also provided. A low identity for amino acid sequences is below 60 %, whereas high identity is up to 98 % which would be a good characteristic of a true ortholog.
5. Perform a DNA or protein alignment of identified orthologs using a built-in Ensembl Plants tool or directly using a BLAST [<http://blast.ncbi.nlm.nih.gov/blast/>] search.

17.3.3 *Meta-analysis of Gene Expression Data Using Gene Expression Repositories*

1. Browse a gene expression datasets at GEO DataSets toolbox [<http://www.ncbi.nlm.nih.gov/gds/>] (*see Note 3*) using specific search terms of experiment description to locate desired experiments.

2. Select a GEO DataSet results page which contains the information concerning the experiment summary, type of experimental data, experiment variables and the number and characters of samples. A single result description page contains a comprehensive description of experiment, tested variables and all samples. Pay special attention to the experimental conditions and the number of replicates which were tested.
3. Use a reference in GEO Accession Display menu to external data analysis tools. GEO2R [<http://www.ncbi.nlm.nih.gov/geo/geo2r/>] is an implemented interactive web tool that allows to compare two or more groups of samples deposited in a GEO database in order to identify genes that are differentially expressed across different experimental conditions.
4. Perform an analysis of selected data set (characterised by specific accession and platform number) by defining the corresponding groups (Fig. 17.1). Up to ten groups can be defined at once. Define at least two opposite groups in the ‘samples’ panel and select independent analyses within them.
5. Analyse the obtained results which are presented in the browser in a table containing the top 250 genes ranked by *P*-value and characterised by different parameters. Use the select columns option to modify which data and annotation columns are shown in the result table. The data column contains the information on the following parameters: *P.Val*—*P*-value after adjustment for multiple

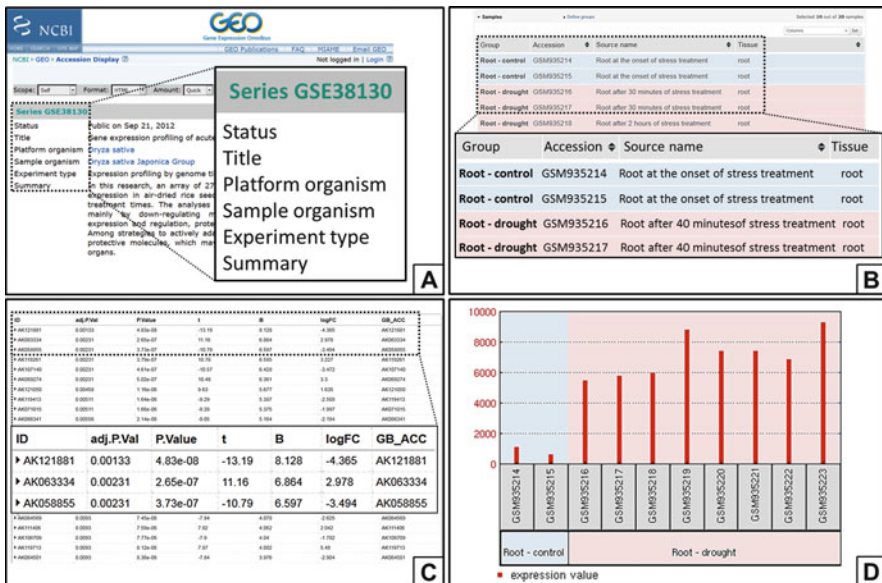


Fig. 17.1 Steps of gene expression data browsing using a Gene Expression Omnibus (GEO) database. The experiment selected for the analysis was ‘Gene expression profiling of acute drought response in leaf and root of rice’, accession: GSE38130 and platform: GPL15594 (a). In the samples panel, two groups were defined consisting of root and shoot tissues at the onset of stress treatment compared with four drought treatments in two replicates (b). Results are presented in the browser as a table of the top 250 genes ranked by *P*-value (c). Gene expression profile graph can be displayed by clicking on a specific row of the gene list (d)

testing, *P*.Value—raw *P*-value, *t*—moderated *t*-statistic, B—B-statistic or log odds that the gene is differentially expressed, logFC—log₂ fold change between two experimental conditions and F—moderated F-statistic.

6. The gene expression profile graphs can be displayed for each row of the gene list.
7. To identify and catalogue the differently expressed genes, the dataset can be exported as a .txt file as a sample-probe matrix with label information.
8. The sorting of differently expressed genes can be accomplished using spreadsheet software (e.g. Microsoft Excel). Genes with the smallest *P*-values will be the most reliable targets. Presort the genes with $p < 0.05$ and fold change of > 1.5 and above.
9. After the identification of specific gene expression profiles, there are several links on the profile records that help to identify additional genes that is of interest, including similarly expressed genes or genes within close proximity on the chromosome. Full information about these links is provided on the About GEO Profiles page.

17.3.4 Gene Ontology (GO) Annotation

1. Search for a specific GO annotation of a gene that is of interest using QuickGO browser for gene ontology [<http://www.ebi.ac.uk/QuickGO/>]. GO search can be conducted using a gene/protein name, symbol, accession number or description word as a query.
2. Obtained GO annotation data consists of three main aspects: molecular process involvement (P), molecular function (F) and localisation in specific component (C).
3. Analyse the QuickGO result table which contains a specific GO terms and identifiers, type of evidence and the affiliation to a database which created the annotation.
4. You can visualise and/or download annotation statistics in .xls format. Statistics reports contain the information on the frequency of covered ontology aspect: molecular process involvement/molecular function/localisation in specific component, the percentage of different types of evidence—experimental (EXP), direct Assay (IDA), physical interaction (IPI), mutant phenotype (IMP), genetic interaction (IGI), expression pattern (IEP) and the frequency of annotation to distinct taxon.
5. Additionally, a created gene list can be classified in terms of specific GO annotation for different ontology categories.
6. Perform a singular enrichment analysis (SEA) using an agriGO [<http://bioinfo.cau.edu.cn/agriGO/>] database.
7. Paste a gene symbols list compatible to an input format as well as the reference (user may choose between the genomic background and customised annotated reference which consist of a second group of GO annotation data).

8. Browse a Detail information table to check the enrichment of input gene targets for different GO terms. GO term can be considered significantly enriched, if the parameter of false discovery rate (FDR) is <0.05 and p -value is <0.01 when compared to all the gene transcripts annotated in the selected genomic background or the set of genes applied as the background.
9. AgriGO tool uses a gene list input to create GO terms abundance chart for the provided accessions (Fig. 17.2).
10. Analyse a provided chart which summarises the gene's affinity to different biological processes in comparison to common genomic reference.
11. If required you may complement the obtained results by the statistical analysis. To test the statistical differences between the enrichments of the input list to the previously computed background or a subset of reference list, select a hypergeometric or a fisher method. If the number of queries is larger than a few, chi-square test is more suitable.

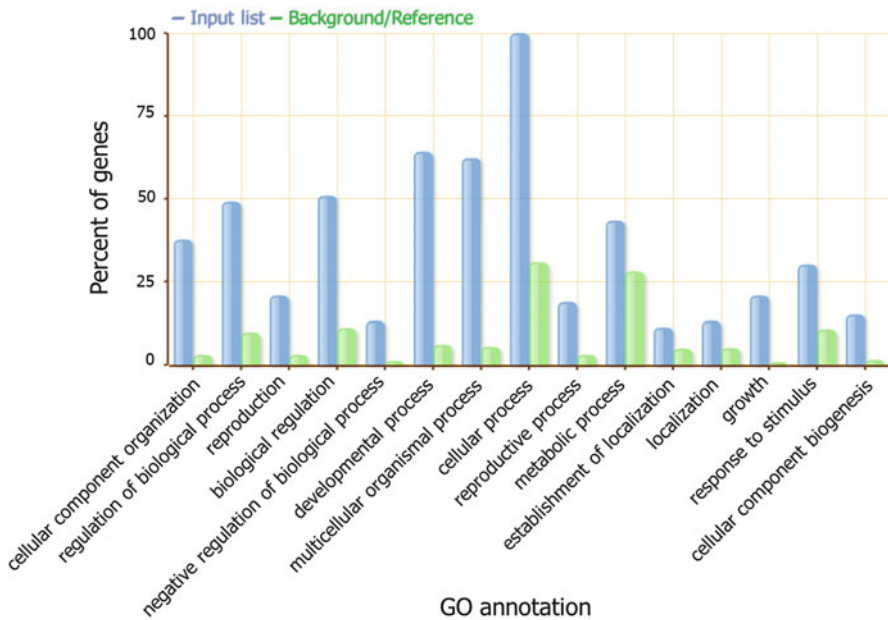


Fig. 17.2 Singular enrichment analysis (SEA) results obtained using agriGO database. Complete preliminary candidate genes list served as a query. *Blue bars* represent the GO term enrichment for the input gene list, whereas *green bars* represent the enrichment for a background genome (*Arabidopsis*)

17.3.5 Identification of Possible Gene Paralogs

1. The identification of the possible paralogs (genes duplicated within a species) of the gene which is of interest can be performed using Ensembl Plants [<http://plants.ensembl.org/>] database.
2. Browse an Ensembl Plants database by using gene name, symbol or description for all species repositories.
3. Select a most relevant record and explore a 'Plant Compara' menu within a paralog repository. The information about the number, ancestral taxonomy, chromosomal localisation and the percentage of identity to the target gene for identified paralogs is provided in a table.
4. Use the 'Region Comparison' function available in Compare tab. The top panel is similar to the chromosome diagram and gene map from the Location tab. The localisation of gene paralogs in the genome of interest can be displayed graphically in the lower panel. This page displays chromosomes, scaffolds and sequence contigs.
5. Use the 'Alignment (protein)' or Alignment (cDNA) function available in Compare tab. Result page contains the information on the gene-paralog alignment details. A multiple sequence alignment (MSA) for the pair of sequences is also provided in CLUSTAL W format.

17.3.6 Selection of a Gene Fragment for Mutation Screening

1. Submit a complete genomic sequence (FASTA format) and an exon/intron position statement (the positions of the start and end of each protein-coding exon in the submitted sequence) compatible with input GenBank format in a CODDLE [<http://blocks.fhrc.org/proweb/coddle/>] (*see Note 4*) tool menu.
2. Specify an optional identifier which will be included in the output.
3. Select a mutagenesis method and scoring system.
4. Perform a computation to a search for a target window that maximises the probability of recovering missense mutations and truncations based on the characteristics of the mutagen and the specified organism.
5. Analyse a graphical display of the search results, indicating the region within the amplicon where mismatch detection is prone to be sensitive for the induction of the mutation (Fig. 17.3).

17.3.7 Designing of PCR Primers for Mutation Screening

1. Design oligonucleotide primers for the amplification of selected gene fragments using accessible web-based primer design tools. Primer3 [<http://bioinfo.ut.ee/>

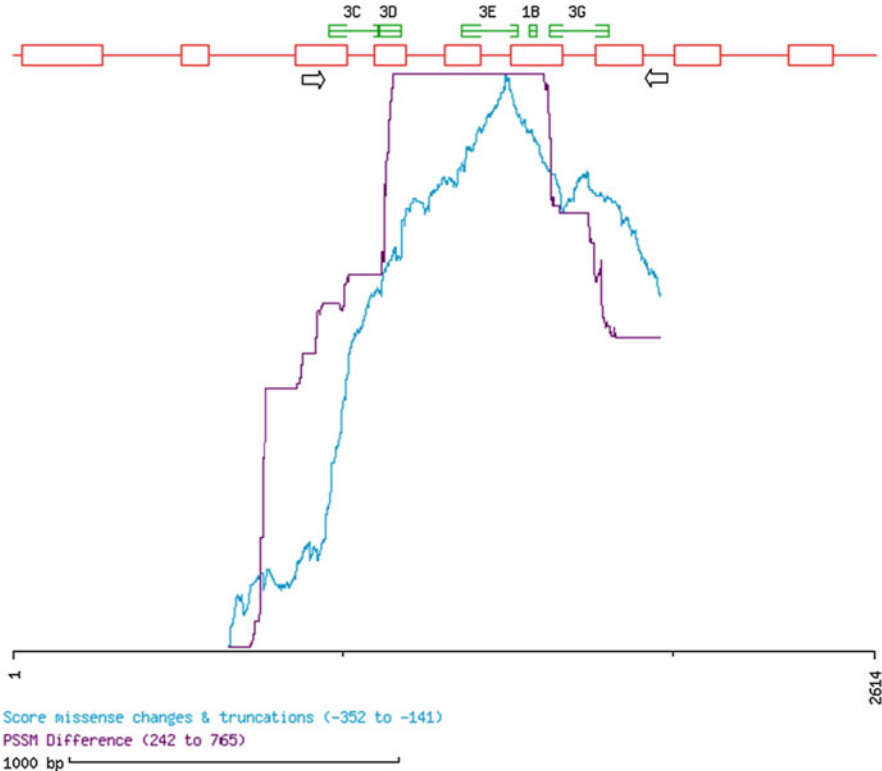


Fig. 17.3 Graphical representation of the results obtained using a CODDLE tool. Exons are indicated as *open boxes* and introns as a *single line*. Nucleotide position numbers are depicted on the *x-axis* at the *bottom* of the plot. The probability to discover a nucleotide change that is disruptive for a gene is indicated by the score on the *y-axis*

[primer3-0.4.0/primer3/](#)] tool can be applied due to a user-friendly interface and numerous primer design properties.

2. Paste a raw nucleotide sequence of a gene that is of interest (5'→3') onto a sequence menu.
3. Predesigned primer sequences can be additionally tested by entering their sequences on appropriate menus.
4. Select a 'General Primer Picking Conditions'. Designed primers for TILLING analysis should match the specified criteria:
 - A length of 18–30 nucleotides.
 - The melting temperature (T_m) of the primers between 65 and 75 °C, and not more than 5 °C difference of each other.
 - The GC content between 40 and 60 %, with the 3' of a primer ending in C or G to promote binding.
 - Lack of regions of secondary structure.

- A balanced distribution of GC-rich and AT-rich domains.
 - Lack of runs of four or more of one base or dinucleotide repeats.
 - Lack of intra-primer homology (more than three bases that complement within the primer) or inter-primer homology (forward and reverse primers having complementary sequences).
5. Choose a 'Pick Primers' command to obtain five pairs of designed primers which best match the selected criteria.
 6. Verify the properties of designed oligonucleotide primers using appropriate software, e.g. OligoAnalyzer [<http://eu.idtdna.com/calc/analyser/>].
 7. Check the sequences of the selected pair of primers using a nucleotide BLAST tool for being unique in the genome of the corresponding species (if possible).

17.4 Notes

1. PubMed database was selected to demonstrate the biomedical text mining process as it contains a most extensive and frequently updated repository of scientific papers on the plant-related studies.
2. The sequence of the gene that is of interest can be easily retrieved from NCBI GenBank repository in case of species of which a full genomic sequence is already known. If the genome of a particular species has not been already sequenced or its genome sequence is not deposited in GenBank or other databases (e.g. species-specific ViroBLAST repository), the homologous sequence of a gene that is of interest can be retrieved by homology search. The identification of homologous gene sequences of particular species can be performed using databases such as Ensembl Plants [<http://plants.ensembl.org/>] for the orthologs search.
3. GEO (Gene Expression Omnibus database) was selected for the meta-analysis of gene expression profiles as it contains a large repository of plant microarray data, which is stored and arranged into a searchable index providing an easy access for further analyses and interpretation.
4. Codons Optimised to Detect Deleterious Lesion (CODDLE) tool is a software which identifies the protein regions that are most likely to contain the largest percentage of deleterious lesions can be generated by G/C to A/T transitions. The software can be downloaded and run locally (<http://blocks.fhrc.org/blocks/uploads/proweb/>).

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Chapter 18

Mutation Detection by Analysis of DNA Heteroduplexes in TILLING Populations of Diploid Species

Miriam Szurman-Zubrzycka, Beata Chmielewska, Patrycja Gajewska, and Iwona Szarejko

Abstract In the beginning of mutation research, mutations could only be detected indirectly through the analysis of the phenotypic alterations that they caused. The detection of mutations at the DNA level became possible with the development of sequencing methods. Nowadays, there are many different methods and strategies that have been created for mutation detection, both in natural and mutagenised populations. The strategies differ in accuracy and sensitivity, as well as in the laboratory facilities, time, costs and efforts that are required. The majority of them involve the pooling of DNA samples and the amplification of a gene (fragment) of interest followed by heteroduplex formation. One of the popular strategies for mutation identification takes advantage of the specific endonuclease (e.g. CEL I) that recognises and cuts heteroduplexes precisely at the 3' position of the mismatch site. The cleaved fragments are usually visualised through electrophoresis in a polyacrylamide gel using LI-COR sequencers, but agarose electrophoresis may also be used for this purpose, although with less sensitivity. A different mutation identification strategy, which is based on the high-resolution melting (HRM) technique, may be the method of choice when working with a short gene or a gene fragment whose length optimally does not exceed 400 bp.

Keywords Heteroduplex • CEL I endonuclease • High-resolution melting • TILLING

M. Szurman-Zubrzycka • B. Chmielewska • P. Gajewska • I. Szarejko (✉)
Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Jagiellonska 28, 40-032 Katowice, Poland
e-mail: iwona.szarejko@us.edu.pl

18.1 Mutation Detection Using Mismatch Cleavage

18.1.1 Introduction

Mutations can occur spontaneously due to errors in DNA replication, or they can be induced by physical or chemical mutagenic agents, such as gamma rays, X-rays, alkylating agents (e.g. EMS, MNU) or sodium azide (for further information, *see* Chaps. 1–3 and 6). Both spontaneous and induced mutations can lead to the development of new alleles that can alter the phenotype of an individual. Only mutations that occur in the germ line cells that give rise to the germ line and production of gametes will be inherited. Some mutations may have a positive influence on the agronomic traits of plants and therefore may be important for breeding. Mutations can also be used in basic research for the analysis of gene function. Recent developments in the sequencing of the genomes of many important crop plants, among them was barley, have greatly facilitated the selection of a gene or its fragment for mutational analysis (IBGSC et al. 2012).

Many different methods for mutation detection in the gene of interest have been developed (reviewed by Shu et al. 2012). Among them are techniques such as single-strand conformation polymorphism (SSCP) (Hestekin and Barron 2006), denaturing high-performance liquid chromatography (DHPLC) (McCallum et al. 2000), temperature/denaturing gradient gel electrophoresis (TGGE/DGGE) (Hestekin and Barron 2006), conformation-sensitive gel/capillary electrophoresis (CSGE/CSCE) (Gady et al. 2009), high-resolution melting (HRM) (Gundry et al. 2003), mismatch cleavage (Colbert et al. 2001; Till et al. 2006a) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) (Chawade et al. 2010). The improvement of new molecular techniques and increased knowledge of gene sequences has led to the invention of Targeting Induced Local Lesions IN Genomes (TILLING)—a reverse genetics method that combines the creation of a mutated population through classical mutagenesis with rapid and high-throughput screening for individuals that carry mutations in the desired genes (McCallum et al. 2000). The application of the TILLING strategy in the functional genomics and breeding programmes of different crops has recently been reviewed by Kurowska et al. (2011) and Wang et al. (2012). The most common method for mutation detection in TILLING is mismatch cleavage using CEL I endonuclease followed by electrophoresis in a polyacrylamide gel. Recently, due to the rapid development of next-generation sequencing technologies, TILLING by sequencing seems to be a modern strategy that will take the lead in mutation detection in the future (*see* Chap. 20).

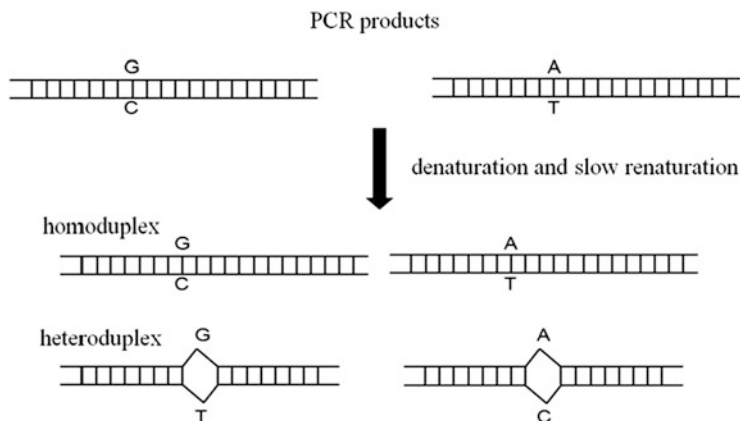


Fig. 18.1 Heteroduplex formation. PCR products are denatured, which leads to separation into single DNA strands, and then slow renaturation leads to the rebanding of single strands into a two-stranded form. Heteroduplexes are formed when a single-stranded fragment with a mutation complements a wild-type single-stranded fragment, thus creating a mismatch in the mutation position

18.1.1.1 Mismatch Cleavage

Mutation detection based on mismatch cleavage takes advantage of the specific endonuclease activity of some enzymes. The most frequently used enzyme is CEL I, which specifically recognises and cuts heteroduplexes.

PCR reactions are usually performed on five- to eightfold pools of the template DNA, and afterwards the amplified samples undergo denaturation and slow renaturation. Heteroduplexes (DNA fragments that have a mismatch) only appear in the pools that contain DNA with a mutation in the fragment that is being analysed (Fig. 18.1).

CEL I is then added to each sample in order to cut the heteroduplexes at the 3' position of the mismatch. As the result of digestion, additional shorter fragments are produced, which indicates the potential presence of a mutation in the DNA from the pool that is being analysed. The products can be visualised in two ways: (1) on polyacrylamide gels in a LI-COR sequencer (when labelled primers are used for PCR reaction) or (2) directly on agarose or polyacrylamide gels (when unlabelled primers are used for PCR reaction). For LI-COR analysis, differently labelled primers for PCR reaction must be used. A forward primer should be labelled with IRDye 700, whereas a reverse primer with IRDye 800. After PCR reaction and CEL I digestion, samples must be purified. Polyacrylamide electrophoresis in a LI-COR sequencer allows very sensitive detection of mutations. The PCR products of the entire length are visible in both channels of the LI-COR (700 and 800 nm), whereas in the case of pools that contain DNA with mutation, one of the cleaved fragments is visible in the 700 nm channel, and the other is visible in the 800 nm channel. The sum of the lengths of the cleaved fragments should equal that of the amplified,

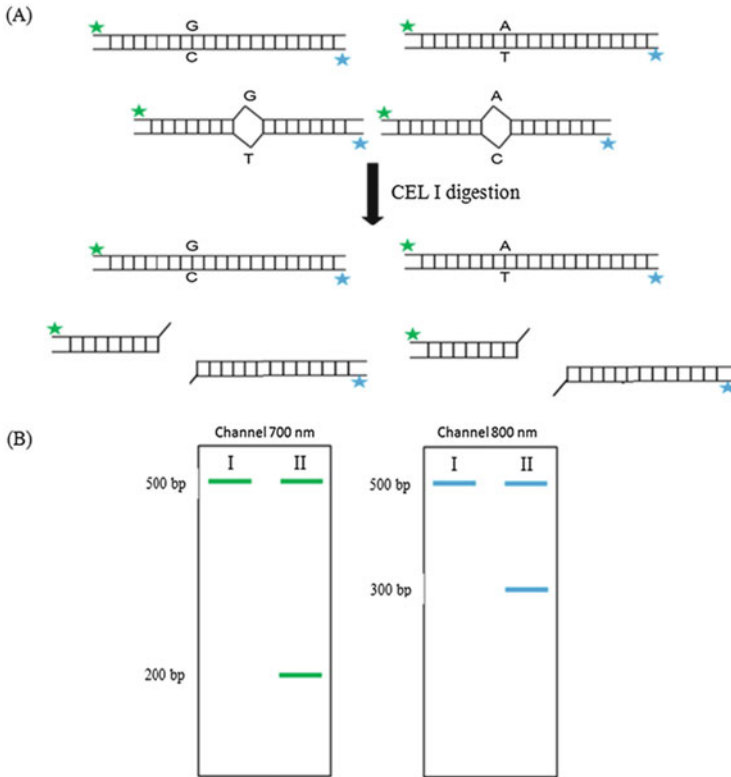


Fig. 18.2 A simplified scheme of mismatch cleavage (a) and the visualisation of products on a polyacrylamide gel using a LI-COR sequencer (b). *Blue asterisk* indicates a forward primer labelled with IRDye 700; *green asterisk* indicates a reverse primer labelled with IRDye 800. I, a pool containing DNA without a mutation in the amplified gene; II, a pool containing DNA with a mutation in the amplified gene

uncleaved PCR product (Fig. 18.2). The visualisation of products through agarose gel electrophoresis is much cheaper, because labelled primers and an expensive LI-COR sequencer are not required, and it is less time consuming as the step of the purification of products is not required. However, the sensitivity of mutation detection using the agarose gel is lower. To obtain better resolution of unlabelled fragments, polyacrylamide gels may be used instead of the agarose gels (Uauy et al. 2009).

CEL I, isolated from celery, is the most common endonuclease used for heteroduplex digestion and mutation detection (Wienholds et al. 2003; Till et al. 2006b; Gottwald et al. 2009). It was found that a high-quality pure enzyme is not required for mismatch cleavage. Till and co-workers (2004) developed a protocol for the isolation of a crude celery juice extract (CJE), which may also be used efficiently for mismatch cleavage. There are also some reports that have shown the usage of other specific endonucleases for mutation detection, for example, ENDO I, which

was isolated from *Arabidopsis thaliana* (Triques et al. 2007, 2008; Minoia et al. 2010) or nucleases isolated from mung bean sprouts and *Aspergillus* (Till et al. 2004). In this part of the chapter, we present the protocols for high-throughput mutation detection in diploid species that is based on mismatch cleavage using celery juice extract. Visualisation of the products is based either on a polyacrylamide gel in a LI-COR sequencer or on a standard agarose gel system.

18.1.2 Materials

18.1.2.1 Mutation Detection Using Mismatch Cleavage and Polyacrylamide Electrophoresis in LI-COR

1. DNA isolated from M₂ plants from a TILLING population

Genomic DNA is extracted from the green leaves of M₂ plants. For DNA isolation the micro-CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle 1987) with modifications (Department of Genetics, University of Silesia) may be used. It is recommended to measure the concentration and quality of DNA using a spectrophotometer (e.g. UV/Vis NanoDrop ND-1000). A value of the 260/280 nm absorbance ratio between 1.8 and 2.0 indicates high-quality DNA. DNA should be diluted to a concentration of 100 ng/μl (see **Note 1**).

2. PCR reagents (see **Note 2**):

- Specific primers that enable the amplification of the gene of interest or its fragment—unlabelled and labelled (a forward primer labelled with IRDye 700, a reverse primer labelled with IRDye 800) (for detailed information about primer design for TILLING see Chap. 17)
- dNTPs
- Taq polymerase
- Reaction buffer for Taq polymerase containing magnesium ions
- Sterile, deionised water

3. CJE (celery juice extract) containing CEL I endonuclease—for an enzyme isolation protocol, see Chap. 15

4. 96 % ethanol with 1 % sodium acetate

5. 70 % ethanol

6. STOP buffer containing 5 % bromophenol blue/xylene cyanol, 40 % formamide and 1 % EDTA

7. Polyacrylamide gel premix containing (for 1 l) 420 g of urea, 200 ml of 30 % acrylamide/bisacrylamide (in ratio 29:1), 100 ml 10×TBE, and sterile deionised water (up to 1 l)

8. 10 % APS (ammonium persulphate)

9. N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)

10. Electrophoresis buffer—1×TBE (tris, boric acid, EDTA)

11. Labelled size marker (e.g. 50–1500 bp sizing standard IRDye 700[®] and IRDye 800[®] from LI-COR Biosciences)
12. Equipment needed:
 - Thermal cycler (e.g. Biometra)
 - Centrifuge for plates (allows spinning at 4000 rpm)
 - LI-COR 4300 S DNA analyser

18.1.2.2 Mutation Detection Using Mismatch Cleavage and Agarose Electrophoresis

1. DNA isolated from the plants that are being tested (as in A2.1 point 1)
2. PCR reagents (*see Note 2*):
 - Specific primers that enable the amplification of the gene of interest or its fragment—unlabelled (for detailed information about primer design for TILLING, *see* Chap. 17)
 - dNTPs
 - Taq polymerase
 - Reaction buffer for Taq polymerase containing magnesium ions
 - Sterile, deionised water
3. CJE (celery juice extract) containing CEL I endonuclease
4. Agarose, DNA stain (e.g. ethidium bromide) and 0.5×TBE (tris, boric acid, EDTA)
5. Size marker for agarose gel
6. Equipment:
 - Thermal cycler (e.g. Biometra)
 - Agarose electrophoresis unit
 - UV lamp (e.g. Vilber Lourmat)

18.1.3 Methods

18.1.3.1 Mutation Detection Using CJE and the Visualisation of Products in LI-COR Sequencer

Sample Pooling and PCR Amplification

1. Prepare eightfold DNA pools that will serve as the template for PCR reactions (Fig. 18.3).

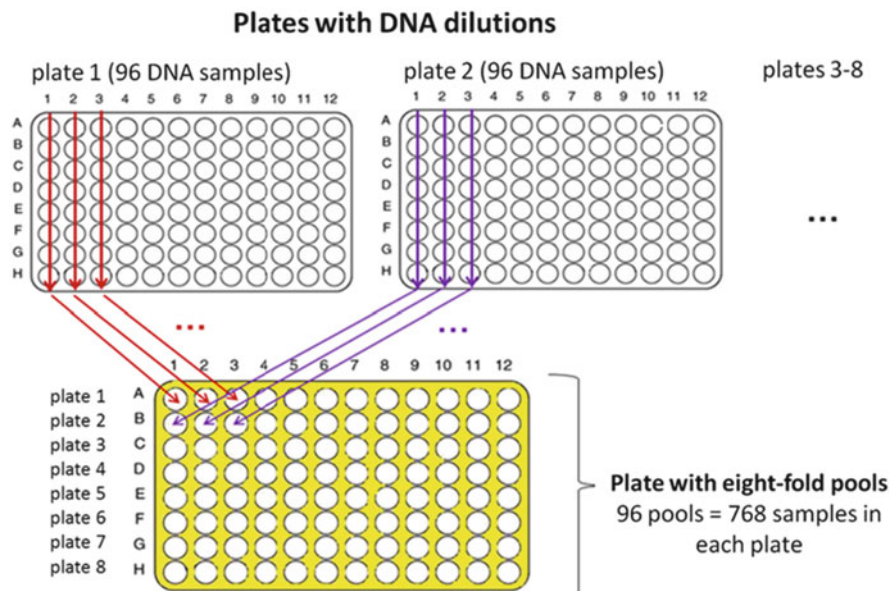


Fig. 18.3 Strategy of DNA pooling in 96-well plates

2. Perform the PCR reaction (in a 20 μl volume) in 96-well plates in a thermal cycler to amplify the gene of interest or its fragment (*see Notes 2, 3 and 4*). Use eightfold DNA pools as the templates (for primer design *see Chap. 17*).

An example of the PCR reaction mix (should be optimised individually for each pair of primers and the type of polymerase used):

ddH ₂ O	14.4 μl
10 \times buffer B for colour Taq polymerase	2 μl
dNTPs (5 mM, Promega)	0.8 μl
Primer F (10 μM , labelled) (<i>see Note 3</i>)	0.4 μl
Primer R (10 μM , labelled) (<i>see Note 3</i>)	0.4 μl
Color Taq polymerase (2 U/ μl , EURx)	0.5 μl
Eightfold DNA pool (100 ng/ μl)	1.5 μl
	20 μl

An example of PCR conditions (should be optimised individually for each pair of primers and the type of polymerase used):

Initial denaturation	Temp. 95 °C 5 min
Denaturation	Temp. 95 °C 45 s
Annealing	Temp. 65 °C 30 s
Elongation	Temp. 72 °C 45 s
Final elongation	Temp. 72 °C 5 min
Pause	Temp. 15 °C ∞

}
× 37

Endonuclease Digestion and Ethanol Purification of Products

1. Perform homo- and heteroduplex formation using a thermal cycler:

Temp. 95 °C	3 min
Temp. 70 °C	20 s—repeat this step 70 times—each time decrease temperature 0.3 °C
Temp. 8 °C	∞

- Add 20 µl of 0.1×CJE (*see Note 5*) to each sample in a plate, vortex.
- Incubate the plate at 45 °C for 15 min.
- Add 60 µl of 96 % ethanol with 1 % sodium acetate to each sample, vortex.
- Centrifuge the plate at 4000 rpm in 4 °C for 20 min.
- Remove ethanol by turning the plate upside down on a paper towel.
- Add 30 µl of 70 % ethanol to the remaining pellets.
- Centrifuge plate at 4000 rpm at 4 °C for 10 min.
- Remove ethanol by turning the plate upside down on a paper towel.
- Incubate the plate (uncovered) in an open thermal cycler at 80 °C until the samples are completely dry (~15 min).
- Add 3 µl of STOP buffer to each sample.
Samples should be stored at 4 °C (for a longer period of time at -20 °C) and protected from light.

Electrophoresis in a 6 % Denaturing Polyacrylamide Gel Using a LI-COR Sequencer

- Add 15 µl of TEMED and 150 µl of 10 % APS to 20 ml of polyacrylamide gel premix.
- Pour the gel between thoroughly cleaned glasses that are separated by 0.2 mm spacers and insert the comb in order to set a brow of the gel—polymerisation of the gel takes around 1 h.

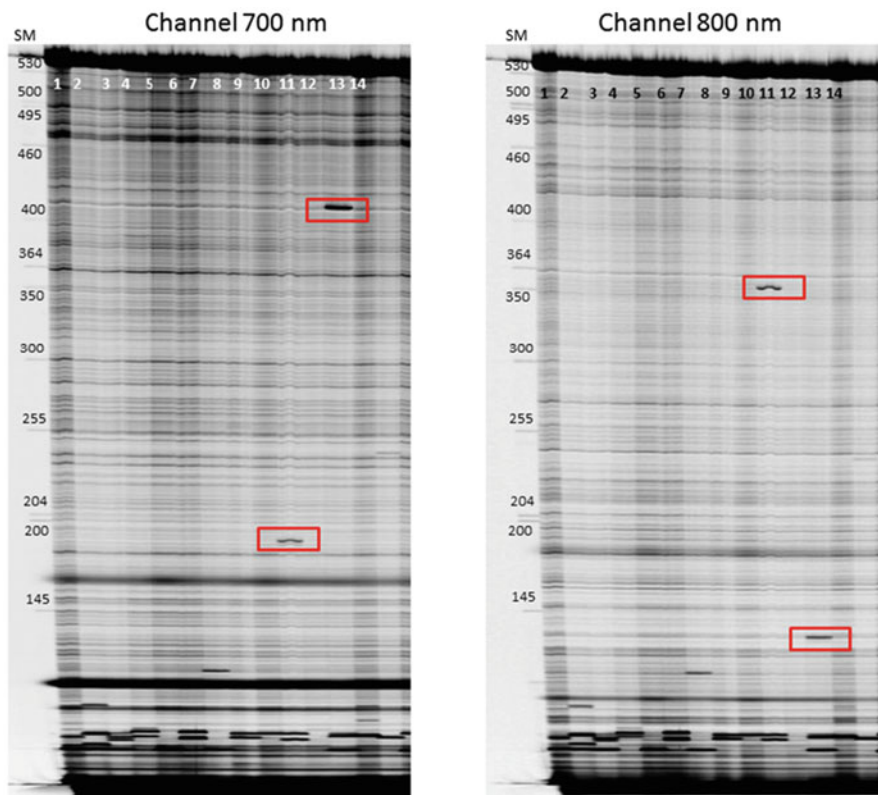


Fig. 18.4 An example of polyacrylamide gel analysis, indicating two positive bulks—lane 10 and lane 12. *SM* size marker

3. Remove the comb and clean the brow of the gel and the outside part of the glasses from the rest of the polyacrylamide (*see Note 6*). Put the comb into the gel in order to create pockets.
4. Put the gel in holders into the LI-COR sequencer in a $1\times$ TBE running buffer.
5. Run pre-electrophoresis under the conditions of 1300 V, 30 W, 30 mA and 45°C for 15 min.
6. Load 1–1.5 μl of each sample onto the gel. Samples need to be denatured in 95°C for 5 min prior to loading. Remember to load a labelled size marker.
7. Run electrophoresis under the conditions: 1300 V, 30 W, and 30 mA with a medium speed of laser scanning in two channels (700 and 800 nm). Depending on the length of the product, electrophoresis can take 3–5 h.
8. When the electrophoresis is finished, analyse the images from the LI-COR 700 nm and 800 nm channels in order to choose the pools (positive bulks) with the DNA that carries a potential mutation, which would be indicated by additional bands (Fig. 18.4).

Identification of the Particular M_2 Individual that Is Carrying a Mutation

1. Prepare DNA templates by mixing the DNA of individual M_2 plant from the positive bulk with the wild-type DNA. For simplification, this template is hereby referred to as twofold DNA pool in the protocol.
2. Perform PCR amplification as in A3.1.1 point 2, but using twofold DNA pools as templates.
3. Perform all steps from A3.1.2 and A3.1.3.

The analysis of image of the gel enables the positive twofold pools to be identified (indicated by additional bands) and therefore enables the particular plants that carry a potential mutation to be identified.

4. Confirm any potential mutation by sequencing the analysed fragment from the M_2 individuals that were identified.

18.1.3.2 Mutation Detection Using CJE and Visualisation of Products on Agarose Gel

Sample Pooling and PCR Amplification

1. Pool samples as indicated in A3.1.1 step 1 (or use pools containing DNA of a lower number of individuals—*see Note 7*).
2. Perform PCR reaction as indicated in A3.1.1 step 2, but only using unlabelled primers.

Endonuclease Digestion and Visualisation of Products

1. Perform homo- and heteroduplex formation as indicated in A3.1.2 step 1.
2. Add 20 μl of $5\times\text{CJE}$ (*see Note 7*) to each sample, vortex.
3. Immediately after endonuclease digestion, load samples (total volume—40 μl) on 2–3 % agarose gel and run electrophoresis under 100 V. Remember to add DNA stain, e.g. ethidium bromide, to the agarose gel before sample loading and to load the size marker. The duration of electrophoresis depends on the length of the amplicons.
4. Analyse the picture of the gel taken under a UV lamp in order to identify positive bulks (Fig. 18.5).

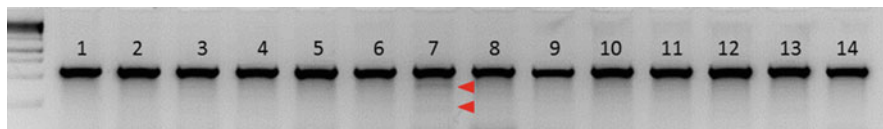


Fig. 18.5 An example of agarose gel analysis. A positive bulk is shown in lane 7

Identification of a Particular M_2 Individual that Carries a Mutation

1. Prepare twofold DNA pools containing the DNA of each M_2 plant from the positive bulk individually mixed with the wild-type DNA.
2. Perform PCR amplification as in A3.1.1 step 2, but using twofold DNA pools as the templates and unlabelled primers.
3. Perform all of the steps from A3.2.2.

The analysis of the image of the gel enables positive twofold bulks to be identified (indicated by additional bands) and therefore enables particular plants that carry a potential mutation to be identified.

4. Confirm the potential mutation by sequencing the analysed fragment from the M_2 individuals that were identified.

18.1.4 Notes

1. The concentration of DNA required for good PCR amplification may vary depending on many factors, such as the genome size of the species of interests or type of polymerase used. Optimisation of the DNA concentration for PCR may be needed.
2. To increase PCR performance, additional reagents may be added, such as bovine serum albumin (BSA) or dimethyl sulfoxide (DMSO).
3. The length of the PCR amplicon should not exceed 1500 bp. The elongation time during PCR depends on the length of the amplicon (for 1000 bp ~1 min).
4. In order to improve the PCR performance, a mixture of labelled and unlabelled primers can be used at a ratio of 3*:2:4*:1 (*labelled forward primer, unlabelled forward primer, *labelled reverse primer, and unlabelled reverse primer, respectively).
5. The concentration $1 \times \text{CJE}$ is the concentration of the celery juice extract that is required in order to detect a mutation in a twofold DNA pool on an agarose gel. The optimal CJE concentration used for mutation detection through LI-COR system (usually $0.1 \times \text{CJE}$) and duration of digest should be optimised under particular laboratory conditions.
6. Glasses need to be absolutely clean so that the laser can easily read the signal of fluorescence through them. Use deionised water to clean them.

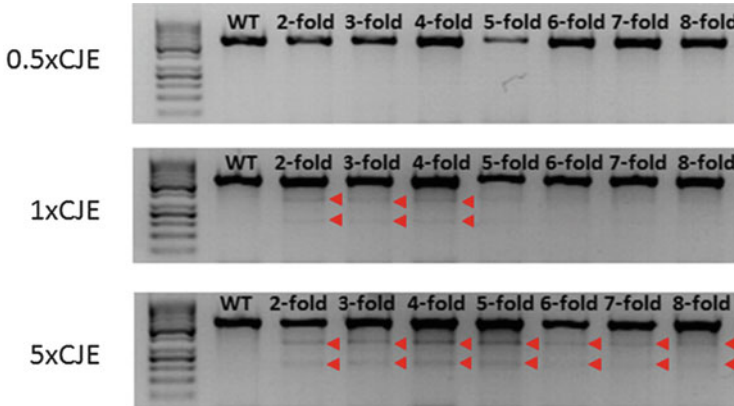


Fig. 18.6 Mutation detection on agarose gel. The different concentrations of CJE that were used indicated that 5×CJE is sufficient for analysing eightfold DNA pools

- Mutation detection using agarose gels is less sensitive. The 0.1× concentration of CJE (used for detection with a LI-COR sequencer) is not sufficient to detect mutations using agarose gels. Instead, 1×CJE should be used to detect a mutation in twofold DNA pools, whereas 5×CJE may enable the detection of mutations in eightfold DNA pools (Fig. 18.6). In the event that fragments are difficult to see, the number of M_2 individuals used in a pool may need to be reduced to increase the strength of additional bands that indicate potential mutations (Fig. 18.6).

18.2 Mutation Detection Using High-Resolution Melting

18.2.1 Introduction

High-resolution melting (HRM) is a non-enzymatic technique that was applied for the first time to analyse polymorphisms in three human genes: *HTR2A*, β -globin and cystic fibrosis (Gundry et al. 2003). HRM has many advantages over other methods for mutation detection. It is “a closed-tube” system that requires standard PCR reagents that are supplemented only with new generation of saturating fluorescent dsDNA dyes, e.g. EvaGreen (Biotium Inc., Hayward, CA, USA), LightCycler[®] 480 ResoLight Dye (Roche Diagnostics, GmbH, Mannheim, Germany) or LCGreen Plus (BioFire Defense, Salt Lake City, UT, USA). These dyes only interact with dsDNA, and therefore, 100 % of fluorescence is observed only in this case. Because the fluorescent dye is present during PCR and high-resolution melting, both an increasing amount of the PCR product and changes of the fluorescence during melting can be observed. In the HRM method, there is no need for the further

processing of PCR products (e.g. cleavage with CEL I enzyme, purification, gel electrophoresis and visual analysis) which is time consuming and increases the risk of contamination. The melting process is fast, and an analysis can be made in a few minutes (Li et al. 2011). These advantages have made HRM a powerful tool for many applications—in the diagnosis of human diseases (Ezgu et al. 2014) and mutation scanning in humans (e.g. *BRCA1* and *BRCA2* genes, Hondow et al. 2011). This method has also been used to detect mutations (single-nucleotide polymorphisms, SNPs and small insertions/deletions, InDels) in plant genetic studies, including TILLING in *Lycopersicon esculentum* (Gady et al. 2009), *Oryzias latipes* (Ishikawa et al. 2010), *Triticum aestivum* (Botticella et al. 2011), *Brassica rapa* (Lochlainn et al. 2011) and genetic mapping in *Hordeum vulgare* (Lehmensiek et al. 2008), *Zea mays* (Li et al. 2010) and *Oryza sativa* (Li et al. 2011). HRM has also been used for the identification of transgenic plants (Milner et al. 2014) and the detection of DNA methylation (Wojdacz and Dobrovic 2007).

In the HRM method, the amplification of a selected sequence by PCR in the presence of fluorescent dsDNA dyes, the formation of homo- and heteroduplexes and the monitoring of fluorescence changes are performed using one HRM instrument (e.g. Light Cycler[®] 480 II). After PCR (Fig. 18.7a), a brief denaturation followed by the rapid reannealing of the amplicons takes place (Fig. 18.7b). In the case of homozygous samples, only homoduplex DNA fragments are present, whereas in the case of heterozygous samples or pools containing a mutation (in the homo- or heterozygous stage), four DNA duplexes are formed—two homoduplexes and two heteroduplexes. An increasing temperature causes DNA melting and the release of the fluorescent dye (Fig. 18.7c). The change in the fluorescence of the saturating fluorescent dye during temperature increasing is monitored using an HRM instrument in real time (e.g. Light Cycler[®] 480 II), so the entire process can be observed as a characteristic melting profile (Gady et al. 2009; Li et al. 2011) (Fig. 18.7d).

A change in the DNA sequence, e.g. a SNP, is easily detected because of the formation of the heteroduplexes for which an altered melting profile is observed. Because heteroduplexes are less stable than homoduplexes, a loss of fluorescence is observed at lower temperatures than for homoduplexes (Ishikawa et al. 2010; Hondow et al. 2011; Martino et al. 2010). Differences between homozygous samples can be observed as a shift in melting temperature, whereas changes in the shape of the melting curves (which are composites of the homo- and heteroduplex) demonstrate different heterozygous samples (Gundry et al. 2003). Although it is possible to distinguish homozygous mutations from the wild type, sample pooling facilitates the detection of homozygous changes due to the mismatch production between the homozygous mutant and wild-type DNA.

The standard TILLING method with CEL I cleavage is more sensitive for detecting a heterozygous mutation in pooled samples (eightfold) than HRM. Usually up to fivefold pools are used in HRM (Gady et al. 2009; Li et al. 2010), although up to 12-fold pools were used for SNP detection in peach cultivars (Chen and Wilde 2011). The differences in the pooling sizes for which the correct mutation detection is possible are probably due to the different instruments that

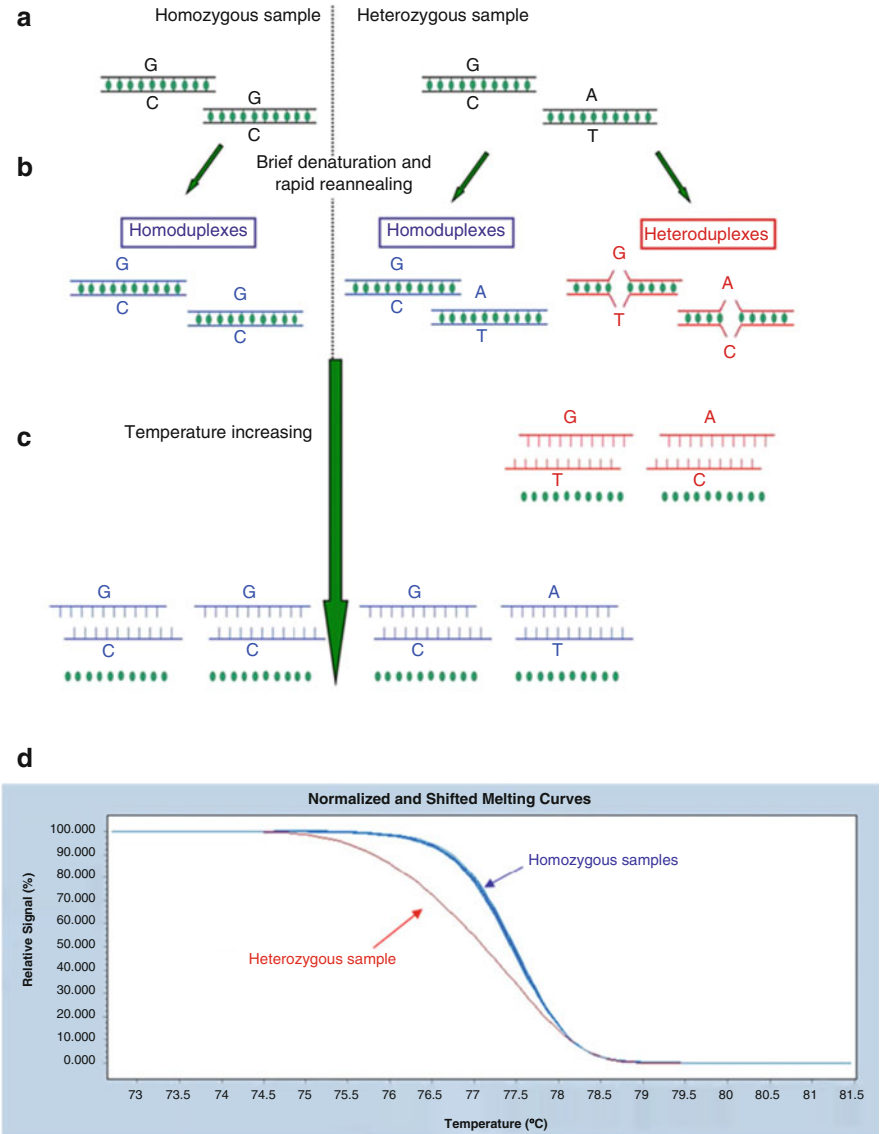


Fig. 18.7 Scheme of the HRM method. (a) Products after PCR, saturated with intercalating fluorescent dye—green oval. (b) Formation of homo- and heteroduplexes. (c) Increasing temperature causes DNA melting and release of the fluorescent dye. (d) Normalised melting curves for all of the samples that were analysed. In this example, 40 samples were analysed, i.e. 39 homozygous (blue) and one heterozygous (purple)

are used in different studies or in the genome size of the species that is being studied (Chen and Wilde 2011).

In contrast to traditional TILLING with CEL I cleavage in which larger amplicons (up to 1500 bp) can be analysed, HRM is the most sensitive for smaller amplicons (100–400 bp), although PCR products up to 1000 bp have also been analysed. A change of one nucleotide in a 100 bp product affects the melting behaviour more than in 400 bp. In the case of longer (>500 bp) amplicons, there is a possibility that such a product will contain more than one melting domain, and as a result, complex melting curves are produced, and therefore the analyses are much more difficult (Reed and Wittwer 2004). However, melting curves of such a long PCR product can be predicted using uMelt, a web-based application (<http://www.dna.utah.edu/umelt/umelt.html>).

In this part of the chapter, we present the protocol for high-throughput mutation detection in diploid species using HRM.

18.2.2 Materials

18.2.2.1 Mutation Detection Using the HRM Technique

1. DNA isolated from the plants that were tested (as in A2.1 step 1), which were diluted to a 30 ng/μl concentration (*see Note 1*)
2. Reagent:
 - A ready-made component kit containing High-Resolution Melting Master Mix Version 06 (Roche Cat. No. 04909631001)
 - Primers (for the appropriate designing of primers *see Chap. 17* and **Notes 2–5**)
3. Equipment
 - LightCycler[®] 480 II instrument connected to a computer with LightCycler[®] 480 Gene Scanning Software Version 1.5.0 SP4 software installed (Roche Diagnostics, GmbH, Mannheim, Germany) (*see Note 6*)

18.2.3 Methods

18.2.3.1 Mutation Identification Using the HRM Technique

Reaction Preparation and Temperature Cycling

1. Prepare fourfold DNA pools, which will serve as the template for the PCR reactions. DNA from each of four M₂ individuals should be mixed in equal amounts.

2. Prepare samples for PCR on a 96-well plate.

PCR components per one sample (20 μ l)

Master mix, 2 \times conc.	10 μ l
Magnesium chloride solution (25 mM)	1.6 μ l
Forward/reverse primer mix (4 μ M) (<i>see Note 3</i>)	2.0 μ l
Water, PCR-grade	4.4 μ l
Fourfold DNA pool (30 ng/ μ l)	2.0 μ l

3. Secure samples against evaporation using a foil seal. Avoid putting fingerprints on the foil seal.
4. Before placing the plate into the instrument, spin the plate at 2129 g at 4 °C for 2 min.
5. Place the plate into the LightCycler[®].
6. Conduct the reaction using the cycling parameters (optimised individually for each pair of primers):
 - Pre-incubation (initial denaturation):
95 °C for 10 min
 - Amplification (number of steps *see Note 7*):
 - Denaturation: 95 °C for 10 s
 - Annealing temperature specific to the primer pair for 15 s (*see Note 8*)
 - Elongation: 72 °C for 10–20 s (*see Note 9*) with single fluorescence acquisition (*see Note 10*)
 - High-resolution melting:
 - Formation of homo- and heteroduplexes:
95 °C for 1 min
40 °C for 1 min
 - Melting interval (increasing temperature and release of fluorescent dye):
65–95 °C, with continuous fluorescence acquisition (*see Note 11*)

Analysis of the Results by Gene Scanning

Data analysis can be performed using the LightCycler[®] 480 Gene Scanning Software Version 1.5.0 SP4 (Roche Diagnostics, GmbH, Mannheim, Germany), which allows the analysis of any differences between the homo- and heterozygous probes. Prior to an analysis, the crossing points for all of the curves should be checked (*see Note 12*).

1. Mark the stable pre-melt (initial fluorescence, when all DNA samples are double stranded and have 100 % fluorescence) and post-melt (final fluorescence, when all of the amplicons are single stranded and fluorescence is 0 %) regions of all of the samples (Fig. 18.8a). This process permits the elimination of differences in

the background fluorescence and the detection of minor differences in the melting profile (*see Note 13*). In this step, the software algorithm groups the melting curves into clusters according to their similarity (Fig. 18.8b). After the calculation by the software, each group (i.e. genotype) is indicated as a different colour (Fig. 18.8c).

2. Select a reference curve. A sample with a known genotype should be chosen as a reference (usually a sample containing homozygous DNA). This step allows the

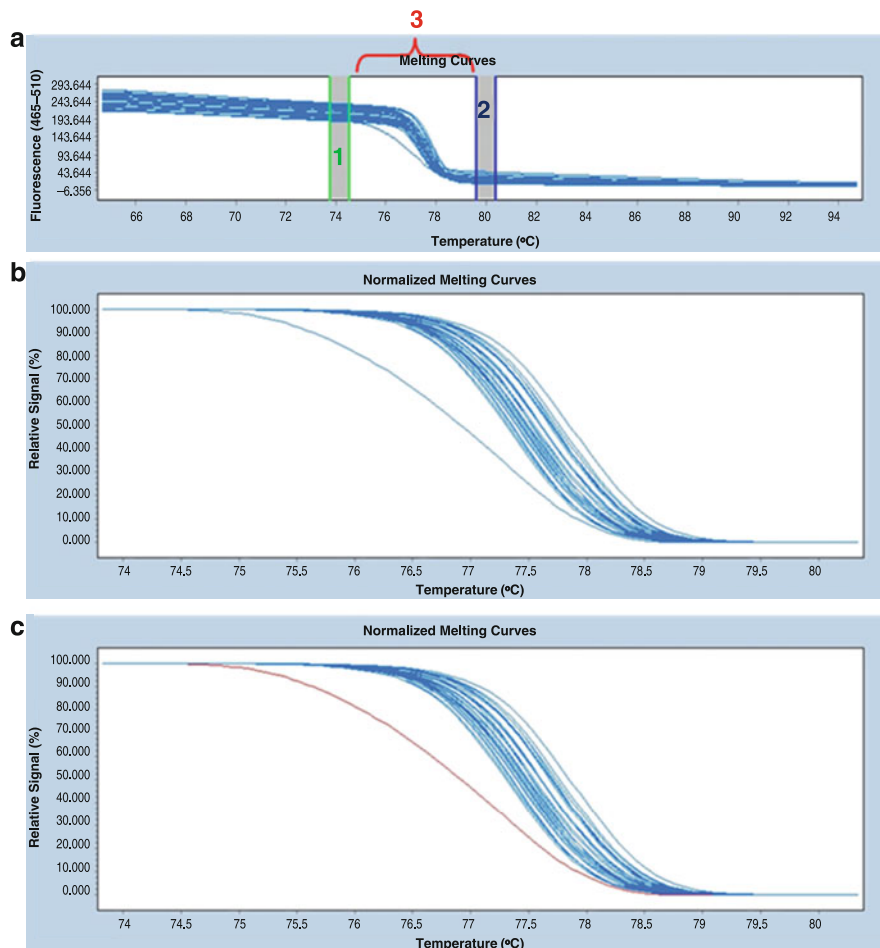


Fig. 18.8 Normalisation of the raw melting curves. (a) The graph presents the pre- and post-melting regions. The area between the *vertical green lines* defines the pre-melt region (1), whereas the *blue lines* define the post-melt region (2). The region that was designated for analysis (3) is located between the pre-melt and post-melt areas. (b) The graph presents the correlation between fluorescence change and the temperature. Differences between the genotypes are visible. (c) After the calculation two groups as indicated by different colours (*blue and purple*) can be seen

normalised curves to be subtracted from the base curve and a plot of similar curves that represent the same genotypes to be obtained (Fig. 18.9). In this figure, the differences in the relative fluorescence signal are seen for two groups of curves—the purple curve represents the pool containing DNA that has a mutation, and the blue curves represent the DNA pools that have no mutation.

3. In the next step, create the difference plots for single DNA pools. A difference plot that shows a slight relative difference of fluorescence between a reference sample (the horizontal blue line) and a DNA pool (a blue curve) is presented in Fig. 18.10a. The slight difference in fluorescence (value of ~ 4.4) indicates that the DNA pool does not contain any mutation. In Fig. 18.10b, the difference in fluorescence between the reference sample (the horizontal blue line) and the fourfold DNA pool (represented by a purple curve) is significant (value of ~ 29), which indicates that the pool contains DNA with a mutation. In Fig. 18.10c, two curves are seen—the purple one represents a pool that contains a mutation, and the blue one represents a DNA pool without mutation. The differences between these two curves are clearly visible.

Identification of a Particular M_2 Individual Carrying a Mutation

1. Prepare twofold DNA pools containing the DNA of each plant from the positive pool (pool with mutation) mixed with the wild-type DNA.
2. Perform PCR amplification as in B3.3.2, but using twofold DNA pools as the templates.
3. In order to indicate potential mutants, perform an analysis of the results according to the instructions that are attached to the LightCycler[®] 480 Gene Scanning Software.
4. Confirm a potential mutation by sequencing the fragment being analysed for the identified M_2 individuals.

18.2.4 Notes

1. In order to avoid reaction-to-reaction variability, use the same procedure to extract the DNA from every sample. Moreover, the variability can be also reduced by using a nested (two-round) PCR for amplicon production. This is often an essential step in polyploid species as there may be insufficient polymorphism between homoeologues to generate specific primers (Botticella et al. 2011).
2. Use a relatively low primer concentration to avoid dimerisation of the primers.
3. The annealing temperature of the designed primers should be around 60 °C. The melting temperature is calculated according to the Bolton and McCarthy formula (Sambrook et al. 1989). It is important to note that the melting

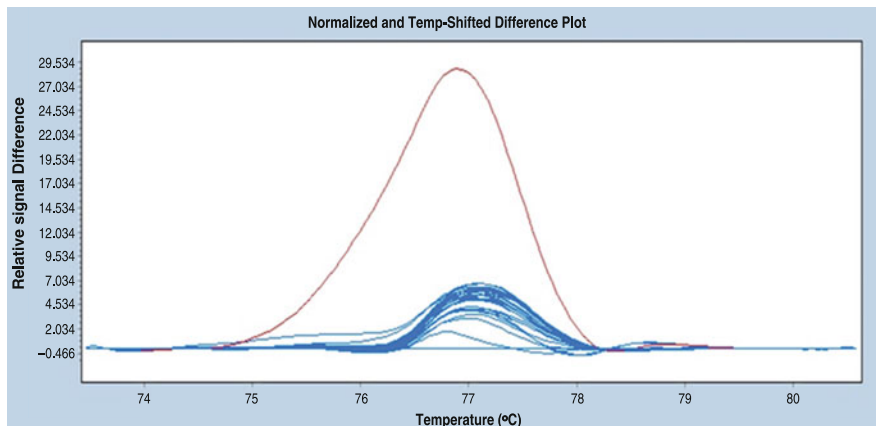


Fig. 18.9 An example of a difference plot. Normalised difference plot for 40 (in this case all of the samples that were analysed) fourfold pools, the wild types are indicated in *blue*, and the pool containing a mutation is indicated in *purple*. The wild type was selected as the base curve (*blue horizontal line*)

temperature depends on the type of applied polymerase (e.g. Phusion or Phire DNA polymerase, Taq-based DNA polymerase) and the use of an appropriate T_M algorithm.

4. Blast your primers in order to check their specificity to the sequence that is being analysed.
5. Use only highly purified primers (e.g. HPLC purified).
6. Other HRM-compatible qPCR instruments can be used, e.g. Rotor-Gene Q with HRM (Qiagen) or LightScanner (Idaho Technology). Moreover, it is possible to perform the PCR reaction on routine thermal cyclers and then use the special HRM equipment for analysis of melting curves.
7. For most analyses, 45 cycles are suitable.
8. If you do not know the exact melting temperatures of your primers, use the touchdown PCR protocol, for which a wide spectrum of annealing temperatures is used. For detailed information, see the supplier's protocol.
9. The elongation time (in seconds) should be calculated as follows—amplicon length divided by 25.
10. Fluorescence data are acquired at the end of this step.
11. Fluorescence data are acquired continuously (characteristic setting for melting analysis).
12. Check crossing point (C_p)—for all of the samples that are being analysed. It should be below 30 cycles. Curves that represent all of the samples should reach a similar plateau height (the phase in which the amount of product does not change because of the depletion of the primers and nucleotides). You can also calculate the efficiency of the reaction for all of the samples that are being tested.

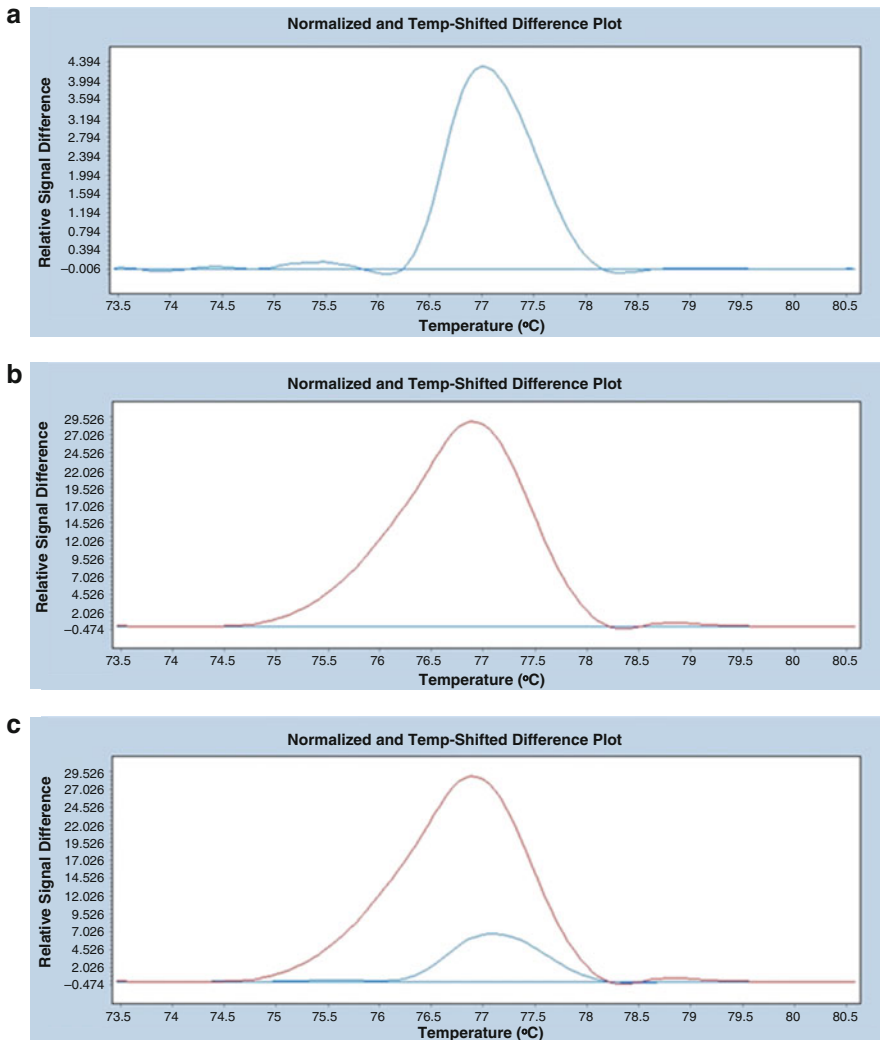


Fig. 18.10 An example of a difference plot. In each case, the reference sample is indicated as a horizontal blue line. (a) A normalised difference plot for a single fourfold DNA pool without a mutation (blue). (b) A normalised difference plot for a fourfold DNA pool that contains DNA with a mutation (purple). (c) A normalised difference plot for two fourfold pools—a pool without a mutation (blue) and a DNA pool containing a mutation (purple)

13. The pre- and post-melt regions that are selected should cover about a 1 °C interval before melting (after fluorescence begins to drop) and after melting is completed for all of the samples.

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Chapter 19

Determining Mutation Density Using Restriction Enzyme Sequence Comparative Analysis (RESCAN)

Diana Burkart-Waco, Isabelle M. Henry, Kathie Ngo, Luca Comai, and Thomas H. Tai

Abstract The average mutation density of a mutant population is a major consideration when developing resources for the efficient, cost-effective implementation of reverse genetics methods such as Targeting Induced Local Lesions in Genomes (TILLING). Reliable estimates of mutation density can be achieved by the analysis of several selected loci from hundreds of individuals via mismatch cleavage of heteroduplexes or sequencing. A more rapid and less expensive alternative involves reduced representation sequencing of the genomes of a few individuals. Here we present a detailed protocol for the construction of restriction enzyme sequence comparative analysis (RESCAN) sequencing libraries using a combination of single restriction enzyme digestion and solid phase reversible immobilization-based size selection of restriction fragments. Indexing of the libraries using barcoded adapters enables cost-saving multiplexing prior to sequencing on an Illumina platform. Mutation density can be determined from the resulting sequence data with or without a reference genome.

Keywords Mutation density • Reverse genetics • Reduced representation sequencing • TILLING

D. Burkart-Waco • T.H. Tai (✉)
Crops Pathology and Genetics Research Unit, USDA-ARS, Davis, CA 95616, USA
Department of Plant Sciences, University of California, Davis, CA 95616, USA
e-mail: thomas.tai@ars.usda.gov

I.M. Henry • K. Ngo • L. Comai
Genome Center, University of California, Davis, CA 95616, USA
Department of Plant Biology, University of California, Davis, CA 95616, USA

19.1 Introduction

Traditional mutagenesis has a rich history with regard to the generation of useful genetic variation for plant breeding (Micke et al. 1987; Ahloowalia et al. 2004). More recently, advances in genomic technologies have elevated the efficiency of mutation discovery, thus allowing populations generated by chemical mutagens or irradiation to be utilized for reverse genetics. Traditional mutagenesis holds several advantages over insertional approaches such as transposon and T-DNA tagging. With relative ease and broad applicability of chemical or irradiation mutagenesis, functional genomics can be pursued in plant species that are recalcitrant to tissue culture or genetic transformation. Mutagens such as ethyl methanesulfonate and gamma rays also produce a wider array of genetic lesions (i.e. point mutations, deletions and translocations) than is typically observed with insertional mutagenesis. Point mutations are of particular interest as missense mutations which result in varying degrees of functionality can provide significant insight into gene function in addition to the utility of nonsense mutations that abolish gene function (i.e. knockout mutations). Another advantage of traditional mutagenesis is the ability to produce high densities of randomly distributed mutations, thus enabling saturation mutagenesis to be achieved with much smaller populations than insertional mutagenesis would require (Henikoff and Comai 2003). While high mutation densities may complicate forward genetics screens and mutation breeding efforts, reverse genetics methods such as Targeting Induced Local Lesions in Genomes (TILLING) are predicated on the development of populations with sufficient numbers of mutations to enable cost-effective screening of DNA pools to identify those rare events (Comai and Henikoff 2006; Till et al. 2007).

The first step in establishing a reverse genetics pipeline based on traditional mutagenesis involves the identification or generation of populations with optimal mutation densities. Here a balance is struck between a sufficient mutation density to permit identifying rare mutations through screening of a reasonable number of M_2 individuals (i.e. 2–4000) and adequate fertility of those individuals to permit the recovery of the mutations in the M_3 generation. While mutation densities can be determined by screening amplicons of selected loci using sequencing or mismatch cleavage detection assays, hundreds of individuals must be examined to establish a reliable estimate for a given population (Comai and Henikoff 2006; Till et al. 2007; Monson-Miller et al. 2012). Such an approach is costly, time-consuming and impractical in the case where multiple populations are being evaluated or if a protocol for mutagenesis needs to be established. An alternative approach is to directly sequence a small subset of individuals from the population(s) of interest (Monson-Miller et al. 2012). This is now feasible given the advances in DNA sequencing which have greatly increased throughput while reducing costs.

Although whole-genome sequencing is becoming a more common approach in model and major economic species, the cost remains prohibitive for assessment of mutation densities in the context of developing reverse genetics resources in non-model species and specialty crops. A powerful alternative involves the

reduction of genomic complexity. This can be achieved by targeting a subset of the genome prior to sequencing either by selective hybridization using specially designed oligonucleotide probes (e.g. exome capture) or through a combination of restriction enzyme digestion and fragment size selection (Monson-Miller et al. 2012). The selected DNA is then used to construct a reduced representation sequencing (RRS) library. This sampling approach increases the likelihood that a particular locus will be sequenced multiple times; sufficient sequencing coverage is necessary to reliably call mutations. During library construction, barcoded adapter sequences are used so that multiplexing of several libraries can be performed, thus increasing the numbers of individuals that can be sequenced concurrently.

The original RRS method was described by Altshuler et al. (2000) well over a decade ago and involved pooling of a modest number of human DNA samples that had been digested with various restriction enzymes followed by size selection using agarose gel fractionation. Size-selected fragments were then cloned and subjected to Sanger sequencing to identify single nucleotide polymorphisms. A number of variations on this approach have been developed for ultrahigh-throughput sequencing platforms (Monson-Miller et al. 2012; Baird et al. 2008; van Orsouw et al. 2007; Van Tassel et al. 2008; Andolfatto et al. 2011; Elshire et al. 2011), and comparisons of these methods have been reviewed recently (Davey et al. 2011; Poland and Rife 2012). Restriction enzyme sequence comparative analysis (RESCAN), our version of RRS, involves the simple construction of barcoded Illumina sequencing libraries from DNA samples that have been digested using a restriction enzyme with a 4 bp recognition site (e.g. *Nla*III, *Mse*I) and subjected to solid phase reversible immobilization (DeAngelis et al. 1995) bead-based size selection. Libraries can be constructed from as little as 100 ng of DNA and barcoding facilitates multiplexing of up to 96 libraries/individuals per sequencing lane. The RESCAN method has been successfully employed to assess mutation density in rice mutants derived from sodium azide mutagenesis with and without the use of the Nipponbare reference genome sequence (Monson-Miller et al. 2012). RESCAN has also been used in various genotyping applications including QTL mapping (Seymour et al. 2012), evaluation of genetic diversity (Kim and Tai 2013a) and identifying introgressed regions in advanced backcross lines (Kim and Tai 2013b).

Since the original report of the RESCAN method (Monson-Miller et al. 2012), minor modifications have been made in the library construction protocol to improve the efficiency of adapter ligation and the subsequent library enrichment step. In our current version of RESCAN, restriction enzyme-digested DNAs are subjected to end repair and A-tailing prior to adapter ligation rather than directly ligating digested DNAs to adapters with compatible ends. As a result, commercially available next-generation sequencing (NGS) library kits may be used for ease, efficiency and robustness. Integration of the standard NGS library construction procedures within the RESCAN protocol has improved ligation efficiency, leading to more consistent and better enrichment of libraries (i.e. fewer cycles of PCR amplification) prior to sequencing. In addition to a detailed protocol for construction of RESCAN libraries, considerations for sequencing and an overview of the

computational pipeline for identifying mutations from RESCAN sequence data are described.

19.2 Materials

19.2.1 DNA Restriction Digestion

1. Genomic DNA (*see Note 1*).
2. NlaIII restriction enzyme (New England Biolabs). Store at -80°C (*see Note 2*).
3. 10x NlaII digestion buffer (New England Biolabs) supplied with NlaIII. Store at -80°C .
4. Thermocycler or other incubator set at 37°C .

19.2.2 Digest Clean-Up and Size Fractionation

1. $1\times$ Tris-acetate EDTA (TAE) buffer and 1 % agarose/ $1\times$ TAE gel (*see Note 3*).
2. DNA size standards such 1 kb ladder or λ DNA-HindIII. Store at 4°C or -20°C depending on frequency of use (*see Note 4*).
3. DNA stain (e.g. ethidium bromide) and gel documentation equipment.
4. Agencourt AMPure XP beads (Beckman Coulter). Store at 4°C (*see Note 5*).
5. Magnet stand (*see Note 6*).

19.2.3 RESCAN Library Preparation

1. Agencourt AMPure XP beads.
2. Indexed adapters (NEXTflex DNA Barcodes, Bioo Scientific) for multiplexing of DNA sequencing libraries (*see Note 7*).
3. Thermocycler.
4. Magnet stand.
5. NGS library preparation kit (KAPA Biosystems). Store at -20°C (*see Note 8*).

19.2.4 PCR Enrichment

1. Phusion High-Fidelity DNA polymerase (New England Biolabs). Stored at -20°C .
2. $5\times$ Phusion HF Buffer (New England Biolabs) supplied with Phusion polymerase. Store at -20°C .

3. Adapter primers (5 μ M), complementary to indexed adapters, for library amplification (*see Note 9*). Store at -20°C .
4. 2 % agarose/1 \times TAE gel and 1 \times TAE electrophoresis buffer.
5. 100 bp DNA ladder size standard (New England Biolabs). Store at -20°C .

19.3 Methods

19.3.1 *NlaIII* Restriction Digestion

1. Turn on thermocycler or incubator and set to 37°C (hold).
2. Measure DNA concentration of genomic DNA and calculate the volume containing 500 ng (*see Note 10*).
3. On ice combine the following in a 0.2 ml tube:
 - 44.5 μ l DNA (500 ng; add ultrapure water to final volume as needed)
 - 5.0 μ l 10 \times *NlaIII* digestion buffer
 - 0.5 μ l *NlaIII* enzyme (10 units/ μ l)
4. Mix well by gently pipetting up and down and spin down contents for a few seconds. Incubate restriction digest(s) at 37°C for >6 h (*see Note 11*).

19.3.2 *NlaIII* Digest Check and Clean-Up

1. Check that DNA digest has proceeded to completion by evaluating 2 μ l (out of 50 μ l) of the digest on a 1 % agarose/1 \times TAE gel. Include appropriate DNA size standards (e.g. 0.5–1 μ l of 1 kb DNA ladder). DNA should appear as a smear with no distinct bands.
2. During the gel electrophoresis, place AMPure XP beads at room temperature for 20 min to allow for equilibration.
3. Following confirmation of complete digestion of the DNA, prepare a fresh solution of 80 % ethanol.
4. Thoroughly mix remaining digest with 50 μ l of equilibrated AMPure XP beads. Let stand at room temperature 5–15 min to allow the DNA to bind to beads.
5. Place beads on magnet and wait until liquid has cleared (approximately 2 min). Discard supernatant.
6. With the tube still on the magnet, wash beads two times with 200 μ l ethanol (80 %). Work quickly so that the AMPure beads do not dry. Discard final ethanol wash (pipetting off all excess) and let beads dry on the magnet (\sim 2–10 min; *see Note 12*).
7. Remove dry beads from magnet and resuspend in 51 μ l elution buffer (EB; 10 mM Tris-HCl, pH = 8.0) by pipetting up and down at least ten times. Let beads stand at room temperature for 5 min to elute DNA from beads.

8. Return sample to the magnet and once clear, carefully pipette 50 μl cleared liquid (no beads!) into a new tube. Discard the tube with the beads. Sample is now ready for end repair or storage in the freezer (*see Note 13*).

19.3.3 *RESCAN Library Preparation (End Repair, A-Tailing, Adapter Ligation and Size Selection)*

1. Place AMPure XP beads and PEG/NaCl SPRI solution (supplied with KAPA NGS library kit) at room temperature for 20 min (*see Note 14*).
2. Programme a thermocycler with the following temperature profiles:
 - 20 °C, 30 min
 - 30 °C, 30 min
 - 20 °C, 15 min
3. On ice, prepare the KAPA end repair master mix as follows (*see Note 15*):
 - Water, 8 μl
 - 10 \times end repair buffer, 7 μl
 - End repair enzyme mix, 5 μl
4. For end repair, combine 20 μl master mix with 50 μl NlaIII-digested DNA in a 0.2 ml PCR tube, mix well, place in thermocycler, and incubate at 20 °C for 30 min. Proceed immediately to next step (end repair clean-up).
5. To each end repair reaction, add 120 μl AMPure XP beads and pipette up and down to thoroughly mix. Let stand at room temperature 5–15 min to allow the DNA to bind to beads.
6. During binding period, prepare A-tailing master mix as follows (*see Note 15*):
 - Water, 42 μl
 - A-tailing buffer, 5 μl
 - A-tailing enzyme mix, 3 μl
7. Place beads on magnet and wait until liquid has cleared (approximately 2 min). Remove liquid by pipetting and discard (do not remove tube from magnet).
8. With the tube still on the magnet, wash two times with 200 μl ethanol (80 %). Work quickly so that the AMPure XP beads do not dry. Discard final ethanol wash (pipetting off all excess) and let beads dry on the magnet stand (~2–10 min; *see Note 12*).
9. Remove beads from magnet and resuspend beads in 50 μl of master mix by pipetting up and down.
10. Incubate at 30 °C for 30 min and then proceed immediately to clean-up.
11. To each A-tailing reaction with beads, add 90 μl PEG/NaCl SPRI solution and thoroughly mix by pipetting. Total volume is now 140 μl . Let stand at room temperature for 5–15 min to allow the DNA to bind to beads.

12. During the binding period, prepare adapter ligation master mix as follows (*see Note 15*):
 - Water, 34 μl
 - 5 \times KAPA ligation buffer, 10 μl
 - KAPA T4 DNA ligase, 5 μl
13. Place beads on magnet stand and wait until liquid has cleared (approximately 2 min). With tube still on the magnet, remove liquid by pipetting and discard. Wash beads two times with 200 μl of 80 % ethanol. Work quickly so that the AMPure XP beads do not dry between washes. Discard final ethanol wash (pipetting off all excess) and let beads dry on the magnet (~2–10 min; *see Note 12*). *Immediately proceed to next step (i.e. adapter ligation)*.
14. Remove beads from magnet and suspend thoroughly in 49 μl adapter ligation master mix by pipetting up and down. Carefully pipette 1 μl of 0.5–5 μM premixed adapter (Table 19.1), mix well, and incubate at 20 $^{\circ}\text{C}$ for 15 min (*see Note 16*).
15. Following incubation, immediately add 50 μl of the PEG/NaCl SPRI solution to the ligation and thoroughly mix by pipetting. Reaction volume should be 100 μl . Incubate at room temperature for 5–15 min to allow DNA to bind to the beads.
16. Place beads on magnet and wait until liquid has cleared (approximately 2 min). Remove liquid by pipetting and discard (do not remove tube from magnet).
17. With the tube still on the magnet stand, wash two times with 200 μl ethanol (80 %). Work quickly so that the AMPure XP beads do not dry. Discard final ethanol wash (pipetting off all excess) and let beads dry on the magnet (~2–10 min; *see Note 12*).
18. Remove beads from magnet and suspend in 100 μl elution buffer (10 mM Tris–HCl, pH = 8.0). Incubate at room temperature for 2 min to elute the DNA. Proceed to the next step (i.e. size selection) or store sample at 4 $^{\circ}\text{C}$ (*see Note 17*).
19. For size selection, add 60 μl PEG/NaCl SPRI solution to the 100 μl of eluted DNA (buffer and beads) and mix thoroughly by pipetting. Total volume is now 160 μl . Incubate at room temperature for 5–15 min to allow DNA fragments larger than 450 bp to bind to the beads.
20. Place beads on magnet stand and wait until liquid has cleared *completely*. Do not remove tube from the magnet stand. Transfer 155 μl of the supernatant to a clean tube. The supernatant should contain DNA fragments ≤ 450 bp (*see Note 18*).
21. Add 20 μl of room temperature-equilibrated AMPure XP beads and mix thoroughly by pipetting. Incubate at room temperature for 5–15 min to bind DNA fragments greater than 250 bp to the beads.
22. Place beads on magnet and wait until liquid has cleared (approximately 2 min). Remove liquid by pipetting and discard (do not remove tube from magnet).
23. With the tube still on the magnet stand, wash two times with 200 μl freshly prepared 80 % ethanol. Work quickly so that the AMPure XP beads do not dry

Table 19.1 Illumina-compatible adapter indexes

Name	Index ^a	Name	Index	Name	Index	Name	Index
iADB96_1	AACGTGAT	iADB96_26	AGCAGGAA	iADB96_51	GCGAGTAA	iADB96_79	AGGCTAAC
iADB96_2	AAACATCG	iADB96_27	AGTCACTA	iADB96_52	GCTAACGA	iADB96_80	ATAGCGAC
iADB96_3	ATGCCTAA	iADB96_28	ATCCTGTA	iADB96_53	GCTCGGTA	iADB96_81	ATCATTCC
iADB96_4	AGTGGTCA	iADB96_29	ATTGAGGA	iADB96_54	GGAGAACA	iADB96_82	ATTGGCTC
iADB96_5	ACCACTGT	iADB96_30	CAACCACA	iADB96_55	GGTGGCAA	iADB96_83	CAAGGAGC
iADB96_6	ACATTGGC	iADB96_31	CAAGACTA	iADB96_56	GTACGCAA	iADB96_84	CACCTTAC
iADB96_7	CAGATCTG	iADB96_32	CAATTGAA	iADB96_57	GTCCTAGA	iADB96_85	CCATCCTC
iADB96_8	CATCAAGT	iADB96_33	CACITCGA	iADB96_58	GTCCTGTA	iADB96_86	CCGACAAAC
iADB96_9	CGCTGATC	iADB96_34	CAGCGTTA	iADB96_59	GTTGTTCTA	iADB96_87	CCTAATCC
iADB96_10	ACAAGCTA	iADB96_35	CATACCAA	iADB96_60	TAGGATGA	iADB96_88	CCTCTATC
iADB96_11	CTGTAGCC	iADB96_36	CCAGTTCA	iADB96_61	TATCAGCA	iADB96_89	CGACACAC
iADB96_12	AGTACAAG	iADB96_37	CCGAAAGTA	iADB96_65	TGGAAACA	iADB96_90	CGGATTGC
iADB96_13	AACAACCA	iADB96_38	CCGTGAGA	iADB96_66	TGGCTTCA	iADB96_91	CTAAGGTC
iADB96_14	AACCGAGA	iADB96_39	CCTCCTGA	iADB96_67	TGGTGGTA	iADB96_92	GAACAGGC
iADB96_15	AACGCTTA	iADB96_40	CGAACTTA	iADB96_68	TTCACGCA	iADB96_93	GACAGTGC
iADB96_16	AAGACGGA	iADB96_41	CGACTGGA	iADB96_69	AACTCAC	iADB96_94	GAGTTAGC
iADB96_17	AAGGTACA	iADB96_42	CGCATACA	iADB96_70	AAGAGATC	iADB96_95	GATGAATC
iADB96_18	ACACAGAA	iADB96_43	CTCAATGA	iADB96_71	AAGGACAC	iADB96_96	GCCAAGAC
iADB96_19	ACAGCAGA	iADB96_44	CTGAGCCA	iADB96_72	AATCCGTC		
iADB96_20	ACCTCCAA	iADB96_45	CTGGCATA	iADB96_73	AATGTTGC	iADA ^b	N/A
iADB96_21	ACGCTCGA	iADB96_46	GAATCTGA	iADB96_74	ACACGACC		
iADB96_22	ACGTATCA	iADB96_47	GACTAGTA	iADB96_75	ACAGATTG		
iADB96_23	ACTATGCA	iADB96_48	GAGCTGAA	iADB96_76	AGATGTAC		
iADB96_24	AGAGTCAA	iADB96_49	GATAGACA	iADB96_77	AGCACCTC		
iADB96_25	AGATCGCA	iADB96_50	GCCACATA	iADB96_78	AGCCATGC		

^a'B' adapters (ADB) have the following eight-base pair index sequences

^b'Universal 'A' adapter (ADA). Contains no index. Mix with each 'B' adapter to obtain the desired concentration for ligation

between washes. Discard final wash and make sure to remove all traces of ethanol. Let beads dry on the magnet for 2–10 min (*see Note 12*).

24. Remove beads from magnet and resuspend in 25 μL elution buffer (10 mM Tris–HCl, pH = 8.0). Incubate beads at room temperature for 2 min to elute DNA. Place back on magnet stand to clear beads from the liquid and transfer the supernatant to a new tube. Sample is now ready for library enrichment (*see Note 19*).

19.3.4 PCR Enrichment of RESCAN Libraries

1. Programme thermocycler with the following amplification profile for enrichment (*see Note 20*):

- 98 °C for 45 s (initial denaturation)

10 cycles of	98 °C for 15 s
	60 °C for 30 s
	72 °C for 30 s

- 72 °C for 1 min (final extension)
 - 4 °C hold
2. Thaw 2 \times KAPA mix and primers
 3. For each sample (i.e. library) combine the following in a 0.2 ml PCR tube:
 - Size-selected DNA, 20 μl
 - 2 \times KAPA mix, 25 μl
 - Primers (10 μM), 5 μl
 4. Load samples in thermocycler and run enrichment profile.
 5. Verify enrichment by removing 4 μl from the 50 μl PCR reaction and running this sample on a 2 % agarose/1 \times TAE gel along with a 100 bp DNA ladder (*see Note 21*). Store remainder of the reaction at 4 °C.
 6. If enrichment is not observed, return the remaining 46 μl of the PCR reaction to the thermocycler and perform another two cycles of amplification using an abbreviated profile. Run another 4 μl on an agarose gel to check enrichment (*see Note 20*).
 7. To each PCR reaction, add 50 μl AMPure XP beads and mix thoroughly by pipetting. Let stand at room temperature 5–15 min to allow the DNA to bind to beads.
 8. Place beads on magnet stand and wait until liquid has cleared (approximately 2 min).

9. Discard liquid and with the tube still on the magnet stand, wash beads two times with 200 μ l of 80 % ethanol. *Work quickly so that the AMPure XP beads do not dry between washes.* Discard final ethanol wash (pipetting off all excess) and let beads dry on the magnet (~2–10 min; *see Note 12*).
10. Remove beads from magnet stand and suspend beads in 21 μ l EB (10 mM Tris–HCl, pH = 8.0). Incubate at room temperature for 2 min to elute DNA.
11. Return sample to magnet stand and incubate beads until the liquid is clear. With the sample still on the magnet stand, carefully remove 20 μ l of eluted DNA into clean 1.5 ml microfuge tube. Do not disturb the beads.
12. Measure the concentration of 1 μ l of eluted DNA. Run another 1–2 μ l of the DNA on a 2 % agarose/1 \times TAE gel to ensure adapter dimers are not present prior to sequencing. Store sample(s) at –20 °C.
13. Prior to sequencing, prepare multiplexed sample by combining an equal amount of DNA from each library to be sequence. We recommend pooling a maximum of 20 libraries per lane (*see Note 22*).

19.3.5 Sequencing

1. Sequencing of RESCAN libraries is typically performed by dedicated sequencing core facilities or commercial sequencing labs. The Illumina sequencing platforms available (e.g. HiSeq 2500, MiSeq) and the protocols for submission, handling and analysis of samples are dependent on the facility. For RESCAN, we currently employ single-read 50 bp (SR50) or paired end read 100 bp (PE100) read lengths using a HiSeq 2500 (or higher) platform. Sequencing facilities will provide an option of determining the DNA concentration of the sample you submit using either qPCR or a bioanalyser. It is recommended that this quality control step is performed.
2. At the time RESCAN libraries are submitted for sequencing, it should be specified that the samples are indexed and the size of the barcode (e.g. 8 bp) should be indicated.

19.3.6 Computational Analysis of Sequence Data

1. Illumina raw sequence reads are provided as compressed fastq file (*see Note 23*), containing both reads that passed quality filters and those that did not. Reads are often divided across multiple files (e.g. each file might contain four million reads) for more efficient acquisition. Because of large file size, it is recommended that processing be performed on a computer cluster as memory requirements may exceed file size, especially during reference-dependent alignment and pile-up generation (*see Note 24*).
2. Sort sequence reads by their barcodes and quality trim using ‘Allprep’, a custom python script available for download at <https://github.com/Comai-Lab/allprep>.

The following parameters are used for both 50 and 100 bp runs: mean PHRED quality score over a sliding window of 5 bp ≥ 20 and sequence length ≥ 35 bp. Reads containing N bases can be discarded and index sequences are trimmed.

3. Align processed reads reference genome using BWA version 0.7.5a (Li and Durbin 2009) and default mismatch parameters. Sam/bam files are automatically generated using Samtools (Li et al. 2009) and an additional python script (<http://comailab.genomecenter.ucdavis.edu/index.php/Bwa-doall>; see **Note 25**).
4. Create BWA Samtools-derived mpileup (mapping quality ≥ 20) and parsed mpileup files, using scripts available here: (<http://comailab.genomecenter.ucdavis.edu/index.php/Mpileup>).
5. Perform mutation detection using the mutations and polymorphisms surveyor (MAPS) bioinformatics pipeline (Henry et al. 2014) in mutation mode (available at <http://comailab.genomecenter.ucdavis.edu/index.php/MAPS>). In short, the MAPS pipeline is based on the assumption that mutations occur randomly and should not be shared by multiple individuals. It is therefore designed to detect mutations in single samples, using all other samples as controls. The minimum number of samples that can be analysed is three although we strongly recommend using higher number of samples (see **Note 24**). The size of the genome that is assayed for the presence of mutations is also important (see **Note 24**). Although reads are initially mapped to a reference sequence, wild-type alleles are not assumed to be the reference allele at that location. Instead, the wild-type allele is determined from the data as the allele shared by all samples except from the mutant sample. Most of the time, the wild-type and reference sequence alleles are identical but not always, especially when the samples originate from a different variety or species of rice than the reference sequence used. The MAPS pipeline is divided into two steps. Thresholds for mutation detection vary based on each experiment, but guidelines can be found in Henry et al. (2014). In our experience, the mutant allele coverage (MACov), i.e. the number of times that a mutant allele is observed in the mutant sample, is the most important criteria for robust mutation detection. For homozygous mutations, a MACov >3 can be sufficient. For heterozygous mutations, a MACov >5 is usually required. For EMS populations, the percentage of false positive mutations can be assessed by calculating the percentage of canonical mutations observed (CG $>$ TA) (Henry et al. 2014).

19.4 Notes

1. Any extraction protocol that yields DNA of high molecular weight (≥ 15 kb) and good purity (A_{260}/A_{280} ratio of ≥ 1.8). We have used both home-made reagents (e.g. potassium acetate-sodium dodecyl sulphate method) and commercial kits (e.g. Qiagen DNeasy). Total genomic DNA (including chloroplast and mitochondrial genomes) is routinely used.

2. Restriction enzymes with 4 bp recognition sites are typically used. We have had success with *NlaIII* and *MseI* although others, including those with longer recognition motifs, may work well. For species with high-quality reference genome sequence information, *in silico* restriction digests may be performed to estimate the number of restriction fragments in a given size range (Monson-Miller et al. 2012).
3. TAE buffer is typically made at 50× (1 L contains 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 mM ethylenediaminetetraacetic acid, pH 8.0). Dilute 1:50 with deionized water to make 1× TAE electrophoresis buffer. For 1 % agarose/1× TAE gel, add 1 g agarose (electrophoresis grade) per 100 ml of 1× TAE buffer and heat to boiling to completely dissolve agarose (stir as needed). Cool to 50–55 °C before pouring into gel mould.
4. DNA size standards may be purchased ready to use or require the addition of standard gel loading dye solution. Comparable size standards (i.e. covering similar size ranges) can be purchased from several commercial sources. Home-made size standards may also be used.
5. Solid phase reversible immobilization (SPRI) beads are sold commercially as AMPure XP beads. SPRI beads are paramagnetic (i.e. only magnetic in the presence of a magnetic field) and consist of a polystyrene core surrounded by a layer of magnetite which is coated with carboxyl molecules. In the presence of polyethylene glycol (PEG) and salt, DNA will reversibly bind to these carboxyl molecules. Depending on the concentration of the PEG/salt, different size DNA will preferentially bind to the carboxyl molecules, thus permitting size fractionation. Currently sold in quantities of 5 ml, 60 ml and 450 ml, AMPure beads should be well mixed and equilibrated to room temperature before use. We typically transfer small ‘working’ aliquots of the AMPure beads (0.5–1 ml) to 1.5 ml tubes. It is advised that the instructions provided by the manufacturer on the handling and storage of the beads are carefully reviewed. The greatest source of error (and loss of DNA) during the RESCAN library construction is due to the mishandling of the AMPure beads.
6. Use of paramagnetic bead separation technology has become a ubiquitous tool in molecular biology. There are numerous manufacturers and suppliers of magnetic stands for various tube sizes and formats. In this protocol, we use a magnetic stand that can hold single 0.2 ml tubes as well as 0.2 ml tube strips (8, 12, and) or 96 well PCR-style plates for flexible throughput. We recommend stands in which the magnets are located in such a way that the beads collect along the side of the tubes so that pipette tips can touch the bottom of the tubes without disturbing the pellets.
7. Indexed adapters that are compatible with Illumina sequencing platforms can be purchased with unique six or eight nucleotide barcodes.
8. Next-generation sequencing (NGS) library kits are available from many commercial sources. Home-made kits consisting of components obtained from various sources may also be used. We have found the convenience and reliability of commercial kits to outweigh the possible cost savings of assembling individual reagents.

9. Adapter-ligated DNA is usually very low in concentration; PCR amplification is necessary for proper cluster formation on the Illumina analyser. Primers for amplifying libraries are complementary to sequences in the indexed adapters. Both primers and indexed adapters can be purchased from Illumina or may be purchased separately from suppliers of DNA oligonucleotides.
10. Any standard method for quantifying DNA is acceptable. Concentration of DNA should be at least 12 ng/ μ l, preferably much higher (100–200 ng/ μ l) to minimize the amount of possible contaminants added to the reaction mixture that might inhibit the restriction enzyme. Total library input can be as little as 100 ng, but higher (500 ng to 1 μ g) inputs yield more consistent library preparation.
11. It is important that the DNA is completely digested prior to size selection and ligation. It may be necessary to adjust enzyme amount based on concentration of input DNA. This can be determined empirically, but a good metric is 1 U of enzyme per 1 μ g of DNA. Overnight incubation is preferred for complete digestion. Most thermocyclers have heated lids, which should be set to prevent evaporation of the reaction mixture. When performing digests in an incubator, place tubes in a sealed plastic bag with a wet paper towel covering the caps.
12. Be careful not to overdry the AMPure beads. Overdrying can negatively impact yields. The surface of the beads (i.e. the pellet, Fig. 19.1) should not appear cracked. Optimal drying time is 3–5 min, but additional time (up to 10 min) may be needed if ethanol wash is not completely removed.
13. This is a safe stopping point in the protocol. DNA can be stored in the elution buffer at -20°C for 2 weeks.
14. The PEG/NaCl solution is light sensitive so keep covered with aluminium foil.
15. Volumes shown are for a single reaction. To process additional samples, prepare a master mix with 5 % excess to account for losses during pipetting. New reagent volumes = volumes shown \times number of samples \times 1.05.
16. Each indexed forward ‘B’ adapter should be mixed with the universal ‘A’ adapter to the desired concentration prior to ligation. Recommendations for the adapter concentration can be found in the KAPA LTP Library Preparation Kit Technical Data Sheet, p. 5, KAPA Biosystems.

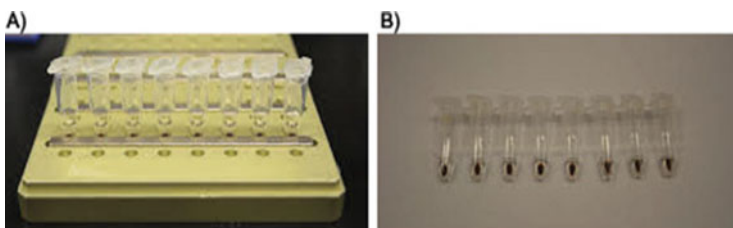


Fig. 19.1 Size selection of restriction fragments using solid phase reversible immobilization (SPRI) bead technology. (a) AMPure XP beads in 8-tube strip on a 96-well magnet stand. (b) Pellets of AMPure beads. The position of the magnet results in the formation of a pellet on the side wall, allowing complete removal of the supernatant from the bottom of the tubes

17. This is a safe stopping point. Eluted DNA and beads can be stored for 24 h at 4 °C.
18. Be careful not to transfer any beads when the supernatant is removed as DNA fragments greater than 450 bp are bound to them at this stage.
19. This is a safe stopping point. Ligations can be kept at 4 °C for 24 h or –20 °C for up to 72 h.
20. This amplification profile takes a total of about 30 min to complete. Amplification for more than 12 cycles is not recommended as this will significantly increase the percentage of clonal reads.
21. A strong smear of DNA between 300 and 600 bp should be visible if good enrichment has been achieved. This is a safe stopping point. Enrichment can be kept at 4 °C for 24 h or –20 °C for up to 72 h.
22. The throughput of the Illumina HiSeq platform depends on the read lengths, run mode and the number of lanes on which your samples will be run. These details are provided by the sequencing facility you use (additional information can be found at http://www.illumina.com/systems/hiseq_2500_1500/performance_specifications.html).
23. In addition to sequence data, each file contains basic information such as Illumina instrument ID, run and lane number, read quality scores and index information.
24. Mutation rate varies from population to population and within populations. It is therefore advised to use multiple individuals when assessing mutation rate. We recommend assessing a mutation rate in at least ten randomly selected individuals to obtain a mean rate for the population. Next, a sufficient number of bps should be assayed in order to reliably assess mutation rate. This will depend on the number of RESCAN fragments selected and the average number of reads obtained from each library (Table 19.2). How much of the genome is covered depends on what restriction enzyme is used and the size fraction selected (Table 19.3). The conditions used in this protocol result in the majority of the selected fragments being in the range of 250–450 bp. In species such as rice, the availability of a reference genome sequence enables *in silico* restriction digests to be performed to estimate the number of fragments generated by a restriction

Table 19.2 Predicted number of base pairs for RESCAN mutation discovery

Cut ^a	# Fragments ^b	Coverage ^c	Individuals	Base pairs ^d
200–400 bp	800,000	10	8	64,000,000
400–600 bp	284,000	10	8	22,720,000
600–800 bp	114,000	10	8	9,120,000

^aSelected fragment size range (including adaptor sequence)

^bApproximate number of RESCAN fragments predicted by *in silico* digest of rice genome with *NlaIII*. This number will vary depending on enzyme used in fragmentation and is described in Monson-Miller et al. (2012)

^cDesired average coverage across each base pair position per individual

^dBase pairs needed to obtain desired coverage for selected fragments. This number will vary depending on the type of sequencing read

enzyme which are within a given size range (Monson-Miller et al. 2012) (Tables 19.2 and 19.3). Although the number of larger fragments generated by restriction enzymes with 4 bp recognition sites is smaller (in theory allowing for better coverage and greater multiplexing), in practice, size selection of larger fragments (>600 or 700 bp) is not very efficient; thus library construction is similarly inefficient (Monson-Miller et al. 2012) (Table 19.2).

Table 19.3 Observed output of RESCAN sequencing

Coverage	Control	Mutant 1	Mutant 2	Mutant 3
1	6510854	6942998	2008388	2115612
2	7277450	7919905	2774391	2791511
3	7313837	8214305	3107639	2998225
4	7238022	8260580	3254888	3088785
5	6965402	8143343	3386032	3150122
6	6624534	7753662	3455459	3167802
7	6149250	7117397	3523409	3208508
8	5652154	6302374	3589064	3270266
9	5037951	5391161	3710559	3314511
10	4432331	4432398	3740468	3350680
11	3838628	3556563	3774913	3422762
12	3261074	2785463	3769033	3468882
13	2757780	2097776	3771291	3490467
14	2286167	1572730	3712628	3492411
15	1874226	1158630	3601172	3441210
16	1529713	849899	3439371	3382565
17	1243158	617623	3255083	3267024
18	990138	439349	3065568	3144214
19	792794	314932	2850866	2983811
20	624292	228160	2597803	2790071
105	0	0	0	22
Total	63571627	61596560	76783350	76768420

One control *O. sativa* ssp. *Japonica* (cv. Kitaake) and three sodium azide mutants were subjected to RESCAN library preparation (*MseI* fragmentation), >250 bp size selection, and sequenced on Illumina GAI (15–30 million reads per individual) (Monson-Miller et al. 2012). After alignment to the Nipponbare reference genome (release 6.1 of the MSU Rice Genome Annotation Project), mapped positions were counted based on coverage, which ranged from 1 read to 105 reads. Due to space constraints, data shown here are only for coverage values between 1 and 20 and the number of reads with 105 times coverage. A threshold of four coverage values was set (based on MAPs parameters), and the total number of base pairs covered at least four times up to 105 was summed (‘Total’) to determine the genomic space assayable for mutation detection. This number (~55 Mb–61 Mb) is only slightly lower than predicted number for 200–400 bp cut (~65 Mb, Table 19.2)

25. By default, Bwa-DoAll performs a step that filters reads for clonal reads, i.e. reads that are the result of PCR amplification post-library construction, by identifying read pairs for which both the forward and reverse reads map at the exact same positions. Because of the repetitive nature of RESCAN libraries, i.e. reads are located adjacent to *Nla*III cut sites and therefore have the same start positions, this step is omitted. Instead, the reads are filtered for chimeric sequences by searching read sequences for internal restriction site (ATGC). Assuming full digestion of the input DNA, restriction sites should only be found at the start and end of the DNA fragment but not in the middle. If an internal restriction site is found, the read is trimmed at the beginning of the internal site.

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Chapter 20

Next-Generation Sequencing for Targeted Discovery of Rare Mutations in Rice

Diana Burkart-Waco, Helen Tsai, Kathie Ngo, Isabelle M. Henry, Luca Comai, and Thomas H. Tai

Abstract Advances in DNA sequencing (i.e., next-generation sequencing, NGS) have greatly increased the power and efficiency of detecting rare mutations in large mutant populations. Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetics approach for identifying gene mutations resulting from chemical mutagenesis. In traditional TILLING, mutation discovery is accomplished through mismatch cleavage of mutant and wild-type DNA heteroduplexes using endonucleases. This is followed by Sanger sequencing to determine the specific sequence changes. TILLING by sequencing (TBS) uses NGS to facilitate the concurrent detection and sequence characterization of mutations, which allows researchers to prioritize mutants for further analyses. NGS increases the sensitivity of mutation detection and thus improves screening efficiency by allowing the pooling of more DNAs. Here we describe a protocol for TBS using rice as an example. First, DNA from a mutant population is quantified and combined in an overlapping pool design. Then, target genes are amplified from DNA pools and amplicons are combined to maximize throughput and increase likelihood of mutation detection during sequencing. Once sequence data is obtained, mutations are called using statistical approaches that weigh likelihood of rare mutations versus the probability of PCR and sequencing error.

Keywords Mutation discovery • Reverse genetics • TILLING (Targeting Induced Local Lesions in Genomes) by sequencing

D. Burkart-Waco • T.H. Tai (✉)
Crops Pathology and Genetics Research Unit, USDA-ARS, Davis, CA 95616, USA
Department of Plant Sciences, University of California, Davis, CA 95616, USA
e-mail: thomas.tai@ars.usda.gov

H. Tsai • K. Ngo • I.M. Henry • L. Comai
Genome Center, University of California, Davis, CA 95616, USA
Department of Plant Biology, University of California, Davis, CA 95616, USA

20.1 Introduction

Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetics method that combines the generation of large mutant populations through traditional mutagenesis with the moderate- to high-throughput discovery of rare mutations. Originally described over a decade ago in *Arabidopsis* (McCallum et al. 2000) and *Drosophila* (Bentley et al. 2000), TILLING is a particularly attractive method for those working with species for which there are few, if any, genomics resources available (Comai and Henikoff 2006). The relative ease, low cost, and broad applicability of chemical mutagenesis enable rapid development of the requisite mutant populations in most species, in contrast to the generation of insertional mutants.

Traditional TILLING involves mutation detection based on enzymatic cleavage of mismatched sites in heteroduplexes formed after the annealing of PCR amplicons derived from genes of interest. This approach has been adapted for use by laboratories with limited resources (Jankowicz-Cieslak et al. 2012). However, discovery of mutations in pools of more than eight individuals is challenging, and once a putative mutation has been detected, Sanger sequencing of all individuals in the pool is needed to verify the mutation (McCallum et al. 2000; Bentley et al. 2000; Comai and Henikoff 2006; Jankowicz-Cieslak et al. 2012; Tsai et al. 2011). Because Sanger sequencing requires a large amount of template and is prone to sequencing errors, traditional TILLING can be slow and cost prohibitive. The advent of next-generation DNA sequencing (NGS), which is cheaper and faster than the classic Sanger method, provides new opportunities for mutant identification in TILLING populations (Tsai et al. 2011).

While traditional TILLING has been employed to great effect, the most direct approach for identifying mutations is to sequence putative mutant alleles and compare them to a reference sequence. Recent advances in ultrahigh-throughput sequencing and bioinformatics analyses have enabled the discovery of rare mutations based on massive parallel sequencing. Such an approach requires sufficient sequencing coverage to allow the discrimination of real mutations from changes introduced through sequencing errors. While the technology exists to detect single-nucleotide polymorphisms by whole-genome resequencing (Ossowski et al. 2010), the application of this approach to large mutant populations is still too costly for most species (Tsai et al. 2011). Instead, mutation discovery using massive parallel sequencing approaches have been focused on the gene space, where mutations are most likely to affect function. This can be accomplished by 3D DNA multiplexing followed by pooling of amplicons derived from genes of interest (Tsai et al. 2011, 2013; Rigola et al. 2009) or via the use of sequence capture methods (Gnirke et al. 2009; Fisher et al. 2011) to select for gene coding sequences (Porreca et al. 2007; Ng et al. 2009; Nijman et al. 2010). The latter of the two strategies represents a global or genome-wide approach to mutation discovery by focusing on all or most of the gene coding regions within a genome of interest (i.e., the exome). Indeed, exome sequencing has been successfully employed in animals

(Ng et al. 2010; Ramos et al. 2012; Sun et al. 2012) and plants (Bolon et al. 2011; Henry et al. 2014) to discover rare mutations and could be extended to characterize large mutant populations such as those currently employed for TILLING, although the investment required likely limits this approach in plants to model and major economic species.

Sequencing of PCR amplicons represents a natural extension of the traditional TILLING method (Tsai et al. 2011; Rigola et al. 2009). An advantage of mutation detection using next-generation sequencing platforms is increased sensitivity, which enables discovery in more complex DNA pools (64× deep) than traditional TILLING (8× deep). This translates to more flexibility with regard to the pooling strategy, which in turn facilitates concurrent identification of mutations and the corresponding individuals. This approach increases the throughput of mutation discovery and speeds the identification and prioritization of putative mutants for in-depth functional studies. Here we describe a protocol for the TILLING by sequencing (TBS) of diploid individuals, which has been successfully employed in rice (Tsai et al. 2011) and tested in *Arabidopsis*, tomato, and *Camelina* (Tsai et al. 2011; unpublished data). In this version of TBS, populations of 512 M₂ individuals are tridimensionally pooled and candidate genes are amplified and pooled, indexed and pooled, and sequenced (Fig. 20.1). Resulting sequencing reads are then processed and aligned to a reference genome for mutation discovery. By taking advantage of the high yield of next-generation sequencing platforms, it is possible to rapidly screen individuals with a higher degree of sensitivity. In addition, the availability of an array of sequencing platforms and bar coding allows users to employ multiple pooling schemes and flexibility with regard to the number of candidate genes that are screened. TBS can be easily adapted for mutation detection in other plant and animal species at a relatively low cost.

20.2 Materials

20.2.1 Genomic DNA Quantification

1. Genomic DNA (*see Note 1*).
2. TE buffer (10 mM Tris, 1 mM EDTA; pH 7.4).
3. λ DNA (100 ng/ μ l).
4. NanoDrop (Thermo Fisher Scientific) or similar instrument.
5. SYBR Green I Nucleic Acid Stain (Lonza Cat. No. 50512). Store at $-20\text{ }^{\circ}\text{C}$ (*see Note 2*).
6. PCR plates (standard 96-well).
7. Adhesive plate seals for PCR (Applied Biosystems Cat. No. 4306311).
8. Micropipettes (single and multichannel) and tips.
9. 1.7 ml microcentrifuge tubes.
10. Costar[®] 96-well plates, flat bottom, black (Corning Cat. No. 3915).

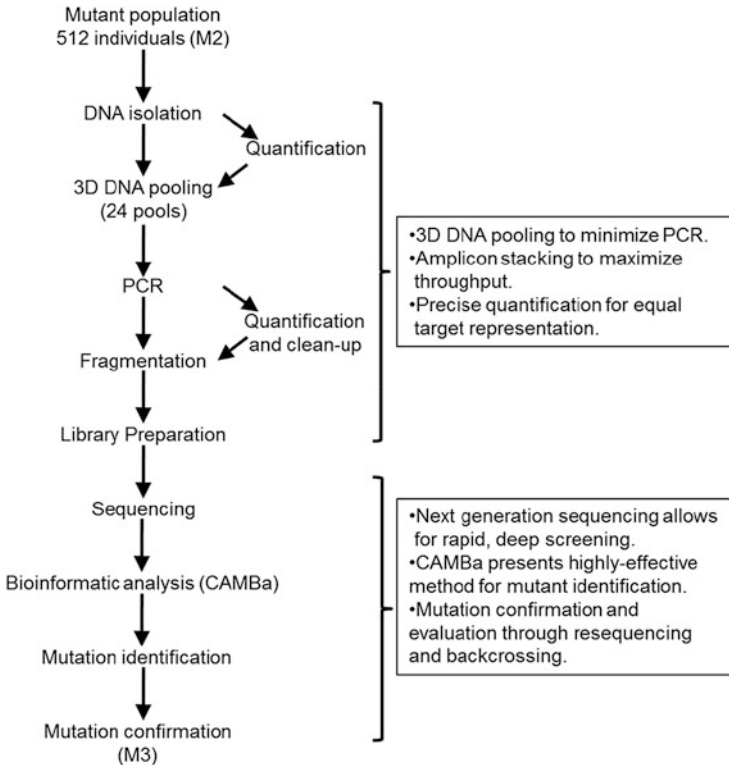


Fig. 20.1 Targeted discovery of rare mutations in a rice mutant population via TILLING by sequencing. Diagram depicting TILLING by sequencing workflow starting with a mutant population and ending with mutant identification and confirmation. DNA is isolated, carefully quantified, and collapsed into pools, which are then amplified using PCR. Illumina libraries are prepared from fragmented PCR products and then sequenced. Putative mutations are confirmed through resequencing of deconvoluted pools

11. 0.2 ml 8-strip PCR tubes with caps.
12. Adhesive plate seals for storing samples in 96-well plates (Thermo Fisher Scientific Cat. No. AB-0580).
13. Aluminum foil.
14. Microplate reader (fluorescence).

20.2.2 DNA Pooling for PCR Preparation

1. Diluted and normalized genomic DNA.
2. PCR plate (standard 96-well).
3. Multichannel micropipette and tips.

20.2.3 *PCR, Quantification, and Cleanup of Target Amplicons*

1. *Ex Taq* DNA polymerase, Hot Start Version (Takara). Store at $-20\text{ }^{\circ}\text{C}$ (*see Note 3*).
2. $10\times$ *Ex Taq* PCR buffer (supplied with *Ex Taq*). Store at $-20\text{ }^{\circ}\text{C}$.
3. Premixed 2.5 mM dNTPs (supplied with *Ex Taq*). Store at $-20\text{ }^{\circ}\text{C}$.
4. TE: 10 mM Tris-HCl 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4.
5. Left primer, T_m 67–73 $^{\circ}\text{C}$, 100 μM in TE (*see Note 4*). Store at $-80\text{ }^{\circ}\text{C}$.
6. Right primer, T_m 67–73 $^{\circ}\text{C}$, 100 μM in TE (*see Note 4*). Store at $-80\text{ }^{\circ}\text{C}$.
7. PCR plates (standard 96-well).
8. Adhesive plate seals (Applied Biosystems Cat. No. 4306311).
9. Thermocycler.
10. 1 % agarose/ $1\times$ TAE gel and $1\times$ TAE electrophoresis buffer.
11. Standard agarose gel electrophoresis equipment.
12. Agencourt AMPure XP beads (Beckman Coulter Scientific). Store at $4\text{ }^{\circ}\text{C}$.
13. 80 % ethanol (made fresh from 95 % stock solution).
14. Elution buffer (10 mM Tris-HCl, pH 8.0).
15. Magnetic separation device.
16. SYBR Green I Nucleic Acid Stain (*see Note 2*).
17. Costar[®] 96-well plates, flat bottom, black (Corning Cat. No. 3915).
18. λ DNA (100 ng/ μl).
19. TE buffer (10 mM Tris, 1 mM EDTA; pH 7.4).
20. 0.2 ml 8-strip PCR tubes with caps.

20.2.4 *Fragmentation of PCR Products*

1. NEBNext[®] dsDNA Fragmentase (New England Biolabs). Store at $-20\text{ }^{\circ}\text{C}$.
2. $10\times$ Fragmentase Buffer (New England Biolabs) supplied with NEBNext[®] dsDNA Fragmentase. Store at $-20\text{ }^{\circ}\text{C}$.
3. $100\times$ BSA (New England Biolabs) supplied with NEBNext[®] dsDNA Fragmentase. Store at $-20\text{ }^{\circ}\text{C}$.
4. 0.5 M EDTA (pH 8.0).
5. Agencourt AMPure XP beads.
6. 80 % ethanol (prepared fresh from 95 % stock solution).
7. Elution buffer (EB; 10 mM Tris-HCl, pH 8.0).

20.2.5 *NGS Library Preparation and Pooling*

1. NGS Library Preparation Kit (KAPA Biosystems). Store at $-20\text{ }^{\circ}\text{C}$ (*see Note 5*).

2. NanoDrop (Thermo Fisher Scientific) or similar instrument.
3. Agencourt AMPure XP beads.
4. 80 % ethanol (prepared fresh).
5. Elution buffer (10 mM Tris-HCl, pH 8.0).
6. Indexed adapters (100 μ M in TE). Stored at -20°C (*see Note 6*).
7. Adapter mixture: 5 μ M premixed adapters (universal adapter + indexed adapter), diluted from 100 μ M stocks with distilled deionized water. Stored at 4°C (short term) or -20°C (long term).
8. Left primer for adapter amplification, 100 μ M in TE. 5'-AATGATACGGCGACCACCGAGATCTACAC-3'. Stored at -20°C .
9. Right primer for adapter amplification, 100 μ M in TE. 5'-CAAGCAGAAGACGGCATACGAGAT-3'. Stored at -20°C .
10. Library enrichment primer mixture: 5 μ M left primer + right primer (diluted from 100 μ M stocks with distilled deionized water).

20.2.6 Library Sequencing

1. Pooled, enriched libraries.
2. Qubit fluorometer (Thermo Fisher Scientific).
3. Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific).
4. Clear fluorometry tubes for DNA quantification.
5. Bioanalyzer (Agilent) for library size estimates.

20.2.7 Bioinformatics Analysis

1. 1 TB hard drive for compressed raw read storage.
2. 2 TB storage space for read processing and data analysis.
3. Minimal memory required = ~ 8 Gb.

20.2.8 Mutation Confirmation

1. Plant growth supplies.
2. Genomic DNA from mutant plants.

20.3 Methods

20.3.1 Genomic DNA Quantification

DNA quantification is perhaps the most important part of TILLING method. If the input the DNA is variable, the ability to detect mutations will be diminished. Fluorescence-based methods for DNA quantification are recommended because they scale well to hundreds of individuals, but alternate methods are available (*see Note 7*).

1. Prepare λ DNA standard by diluting 100 ng stock with TE in serial dilution.
 - (a) Vortex the 100 ng/ μ l λ DNA and spin down. Then, identify the concentration by recording the average of three NanoDrop (*see Note 8*) readings.
Concentration (ng/ μ l) = _____
If stock is not 100 ng, adjust standard concentrations accordingly.
 - (b) Prepare the λ DNA standard in 0.2 ml PCR tubes (8 or 12-tube strips may be used for convenience).
 - (c) Pipette Std.1 into first tube.
 - (d) Transfer 30 μ l to the next well and add 20 μ l 1XTE and mix well. This is the first dilution step.
 - (e) Prior to starting subsequent dilution, vortex and spin down.

Std.	Conc.	Dilution
1	100	50 μ l starting concentration
2	60	30 μ l Std.1 + 20 μ l 1XTE
3	36	30 μ l Std.2 + 20 μ l 1XTE
4	21.6	30 μ l Std.3 + 20 μ l 1XTE
5	12.96	30 μ l Std.4 + 20 μ l 1XTE
6	7.78	30 μ l Std.5 + 20 μ l 1XTE
7	4.67	30 μ l Std.6 + 20 μ l 1XTE
8	2.8	30 μ l Std.7 + 20 μ l 1XTE

2. In a light-protected tube or reagent reservoir, prepare SYBR Green I master mix by combining 200 μ l TE with 0.08 μ l SYBR Green I for each reaction, and mix well (*see Note 9*). Protect mixture from light to prevent loss of fluorescence signal.
3. Pipette 200 μ l 1 \times SYBR Green I master mix into each well of a black Costar plate (*see Note 10*).
4. Transfer 2 μ l λ DNA standard or 2 μ l unknown DNA (*see Note 11*) to wells containing 1 \times SYBR Green I master mix. Mix well by pipetting up and down.
5. Cover plates with sealing film and aluminum foil.
6. Shake for 2.5 min at 55 rpm on a plate shaker (or vortex with platform attachment) and then spin down. Store the plates in the dark at 4 $^{\circ}$ C prior to quantification.

7. Measure fluorescence with a microplate fluorescence reader according to manufacturer's protocol to determine DNA concentration.
8. Once DNA is quantified with a plate reader, calculate DNA concentration and appropriate normalization. The final concentration should be equal to the most dilute DNA sample and consistent across plates (if at all possible). Final concentration should not be <2.0 ng/ μ l (see **Note 12**).

20.3.2 Three-Dimensional Pooling of Genomic DNAs

1. Layout eight "pre-pool" 96-well PCR plates, which will contain diluted DNA (see Sect. 20.3.1, **Step 8**) to be used for constructing an 8×8 pooled array.
2. Transfer at least 35 μ l of diluted DNA per sample to wells in columns 1–8 and rows A–H of the pre-pool plates (see **Note 13**). Label two sets of 24 microfuge tubes (1.7 ml), which will contain the master pools. Master pools are labeled by row (R1–R8), column (C1–C8), and plate (P1–P8). Collapse pre-pool plates into master pools (see Fig. 20.2 and **Note 14**). Using multichannel pipette is probably the most efficient way to collapse pre-pool plates. It also reduces probability of sample swaps. To do this, collapse pre-pool plates in another PCR plate and then transfer to a microfuge tube. All pooling should be done in duplicate. Master

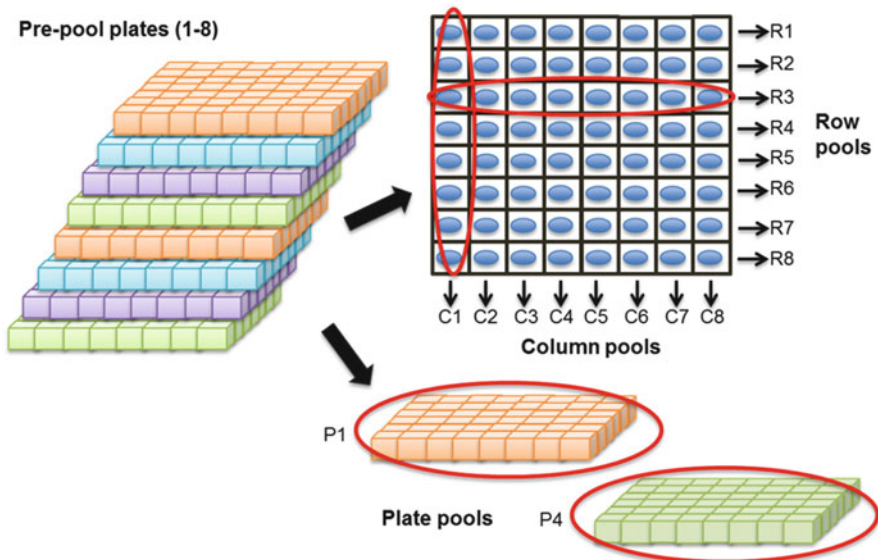


Fig. 20.2 Three-dimensional pooling facilitates simultaneous mutation detection and identification of individual mutants harboring the mutations. Mutant individuals are arrayed on eight pre-pool plates with one individual in each well. Plates are then pooled in three dimensions. Each individual is present in three pools (a row pool, R; a column pool, C; and a plate pool, P) and each pool contains 64 individuals

pools can be kept at 4 °C for short-term and –20 °C/–80 °C for long-term storage.). For example:

(a) R3 master pool (horizontal pool):

- Combine 5 µl DNA from row 3 (wells C1–C8) from pre-pool plates #1–8. This will yield a pool of 64 individuals.

(b) C1 master pool (vertical pool):

- Combine 5 µl DNA from column 1 (wells A1–H1) from pre-pool plates #1–8. This will yield a pool of 64 individuals.

(c) P4 master pool (plate pool):

- Combine 5 µl DNA from all individuals on pre-pool plate #4 (row wells A–H and column wells 1–8). This will yield a pool of 64 individuals.

3. Quantify master pools with a NanoDrop or with a similar method and proceed to PCR amplification.

20.3.3 PCR and Pooling of TILLING Targets

1. Program a thermocycler with the following amplification profile (*see Note 15*):

- 95 °C for 2 min (initial denaturation)

Eight cycles of:	73 °C for 30 s. (Increments of –1 °C/cycle, ramp to 72 °C at 0.5 °C/s)
	72° for 1 min
25 cycles of:	94 °C for 20 s 65 °C for 30 s (ramp to 72 °C at 0.5 °C/s).
	72 °C for 1 min

2. Set up the following PCR reaction *on ice* (*see Note 16*):

DNA (1.5 ng) and water	22.65 µl
10× <i>Ex Taq</i> buffer	3.00 µl
2.5 mM dNTPs	2.40 µl
5 µM premixed primers	1.80 µl
<i>Ex Taq</i> HS enzyme	0.15 µl
Final volume	30.0 µl

3. To check reaction efficiency, run 1 µl of the 30 µl PCR on 1 % agarose/1× TAE gel (*see Note 17*).

4. Follow DNA quantification protocol described above to quantify PCR products (*see* subheading 20.3.1, and **Note 18**), and calculate PCR sample normalization (*see* **Note 19**).
5. Normalize the PCR product.
6. Calculate the average amount (in ng) of each of the PCR products needed to obtain 500 ng of pooled PCR DNA for sequencing library preparation by dividing 500 ng by the number of PCR products (*see* **Note 20**).
7. For each target, calculate the size factor by dividing the PCR fragment length by the mean fragment length (*see* **Note 21**).
8. For each target, calculate the input amount (in ng) needed by multiplying the average amount (from step 6) by the size factor (from step 7). *This is the amount you will add as input DNA.*
9. For each target, calculate input volume by dividing the final volume (from step 8) by the normalized concentration.
10. Pool PCR products according to input volume. Clean PCR pool with an equal volume of AMPure XP beads (1:1) and elute in 52 μl water or elution buffer (*see* **Note 22**).

20.3.4 Fragmentation of PCR Products

1. Set up fragmentation reactions *on ice* (*see* **Note 23**).

DNA and water	51.4 μl
10 \times Fragmentase buffer	6.0 μl
100 \times BSA	0.6 μl
dsDNA fragmentase	2.0 μl
Final volume	60.0 μl

2. Incubate at 37 $^{\circ}\text{C}$ for ~20 min (*see* **Note 24**).
3. Remove 2 μl of the 60 μl reaction and store the remainder at -20°C . Check reaction efficiency by running the 2 μl sample on a 1 % agarose/1 \times TAE gel with a 1 kb DNA ladder.
4. If most DNA fragments are between 300 and 600 bp, then proceed to step 5. If there are many large fragments (>800 bp), place the frozen reaction back at 37 $^{\circ}\text{C}$ for another 5–10 min and check again on gel.
5. Add 5 μl 0.5 M EDTA and mix well to stop the reaction.
6. Clean DNA by mixing 60 μl AMPure XP beads (1:1 v/v) with the fragmentation reaction and follow the manufacturer's protocol for AMPure cleanup of PCR reactions (*see* **Note 22**).
7. Elute DNA with 50 μl elution buffer.

20.3.5 NGS Library Preparation and Pooling

1. Follow protocol outlined in the *KAPA Technical Data Sheet* (see **Note 25**). All reactions are performed according to the manufacturer's specifications except 1 μ l premixed 5 μ M adapters, and 4 μ l of water is added to ligation reaction (see **Note 26**).
2. Determine concentration of each of the enriched libraries by analyzing 1 μ l using a NanoDrop or Qubit. If some libraries are a lot more concentrated than the mean across all other samples, dilute accordingly, and then re-quantify the dilution.
3. Assuming relatively similar fragment size distribution, pool samples 1:1 based on the DNA amount which is derived from the sample concentration obtained from SYBR Green 1 quantification (see **Note 27**).

20.3.6 Sequencing of NGS Libraries

The NGS libraries constructed from the PCR pools in this protocol are for Illumina NGS platforms (e.g., HiSeq 3000 or 2500) and are typically submitted to sequencing core facilities at universities or research institutions or to commercial sequencing labs. The protocols for submission, handling, and analysis of samples are dependent on the facility. A general outline for sample submission can be found below.

1. Determine purity of pooled libraries through fluorometry, for example, Qubit quantification (see **Note 28**).
2. Assess quality of libraries through qPCR, such as the KAPA Library Quantification Kit (see **Note 29**). This will yield an estimate of cluster forming capacity and thus predicted read output from Illumina sequencing.
3. Confirm library size with Bioanalyzer (Agilent), which can quantify the size of small samples of DNA (see **Note 30**).
4. Select desired number of reads/sequencing lanes. This is dependent on (1) the number of TILLING targets, (2) size of TILLING targets (before library preparation), (3) read length, and (4) predicted number of sequencing reads (see **Note 31**).

20.3.7 Computational Analysis of Sequence Data

A bioinformatics pipeline is required to process the large volume of sequence data that is generated and to effectively discriminate between real mutations and background "noise." This is complicated by the fact that the intensity of the mutation "signal" and the background noise may vary greatly as a result of the sequence coverage and the location of the mutation (see **Note 32**). The specifics of the bioinformatics analysis are

beyond the scope of this chapter and have been described in detail elsewhere (*see Note 32*). The general steps for mutation discovery are as follows:

1. Obtain Illumina raw sequence reads (*see Note 33*).
2. Quality filter the sequence reads and trim to remove adapter sequences.
3. Identify samples according to index and de-multiplex (*see Note 34*).
4. Align sequences to reference using Burrows-Wheeler Aligner (BWA) software (Li and Durbin 2009).
5. Identify and parse sequence changes from BWA output.
6. Compare frequency of the sequence changes across libraries.
7. Derive a probability score for each sequence change based on quality scores and other parameters (*see Note 35*).

20.3.8 *Confirmation of Mutations and Initial Characterization of Mutants*

Putative mutations identified through the bioinformatics pipeline are prioritized for further analysis based on their nature (e.g., nonsense, missense) and location (e.g., exon, intron, splice site, untranslated regions).

1. Obtain the M₃ seeds corresponding to the M₂ individual identified as harboring a mutation of interest. Evaluate seed and plant phenotypes as warranted.
2. Harvest tissue and prepare DNA from M₃ plants.
3. Identify M₃ individuals harboring the mutation of interest by Sanger sequencing or, if possible, restriction digestion (*see Note 36*).
4. Cross M₃ individuals with wild-type and other mutants to produce materials for further analysis (*see Note 37*).

20.4 Notes

1. Any extraction protocol that yields DNA of high molecular weight (≥ 15 kb) and good purity (A_{260}/A_{280} ratio of ≥ 1.8). We have used both homemade reagents (e.g., potassium acetate–sodium dodecyl sulfate method) and commercial kits (e.g., Qiagen DNeasy). Total genomic DNA (including chloroplast and mitochondrial genomes) is routinely used.
2. SYBR Green I Stain can be obtained from many suppliers. Aliquots should be made to avoid freeze thawing. Because of light-sensitive and fluorescence decay, the stock solution as well as any working stocks should be kept covered with foil at all times. It should be thawed to room temperature for 30 min before use. As with any reagent that binds to nucleic acids, it should be handled with care and disposed of according to your institution's hazardous waste policy. Additional safety information can be obtained from the Material Safety Data Sheet.

3. Takara *Ex Taq* HS DNA Polymerase works well for most TILLING applications. However, for difficult templates, alternate polymerases may be considered. To reduce false positives due to polymerase error, an enzyme with a low error rate and proofreading ability is advisable. Phusion[®] High-Fidelity DNA Polymerase (New England Biolabs) is a likely candidate because of its robust performance. When considering alternatives to *Ex Taq*, additional time may be needed for protocol optimization.
4. Primers should be designed with Primer3 or similar software and ideally sit in intergenic regions flanking exons. Optimal primer length is 27 bp (range of 20–30 bp) and primer temperature of 67 °C–73 °C (Till et al. 2007). Product size should be between 1000 bp and 2000 bp, with an optimal size of 1500 bp. Larger amplicons are possible, but it will be necessary to perform additional fragmentation trials.
5. Next-generation sequencing (NGS) library kits are available from many commercial sources. Homemade kits consisting of components obtained from various sources may also be used. We have found the convenience and reliability of commercial kits to outweigh the possible cost savings of assembling individual reagents. While it is possible to prepare PCR fragments for sequencing with a homemade DNA-seq kit, it is more efficient with a kit. There are many library preparation kits available on the market which contain all enzymes required for library construction, and reactions are performed in the presence of magnetic beads, thus cutting down on the cost of multiple AMPure reactions.
6. These adapters serve multiple functions. They are used for amplification of the libraries, they included specific barcodes (usually 6 or 8 bp long) that identified the samples, and they bind to the sequencing flow cell. Premade indexed adapters can be ordered from many companies including Illumina (San Diego, CA, USA), Bioo Scientific (Austin, TX, USA), and New England Biolabs (Ipswich, MA, USA), or homemade adapters can be designed. The “TruSeq” Illumina single index adapter system has a universal adapter and a 6- or 8-bp indexed adapter. The adapters are complementary in a 12-base region; binding in this region creates the “Y-shaped” adaptor—a short region of complementarity with long mismatched arms. Barcodes should be unique to each pool and differ at a minimum of 2 bps (for 6-bp barcodes) or 3 bps (for 8-bp barcodes), to easily distinguish between pools at the bioinformatic analysis stage.
7. If a microplate reader capable of fluorescence detection is not available, gel electrophoresis with λ DNA standard has been effectively used to quantify DNA for TILLING (Till et al. 2003; Cooper et al. 2008).
8. Any method for quantifying the λ DNA can be used here.
9. SYBR Green I dye master mix is prepared fresh prior to quantification. Work quickly and keep all reagents in the dark! Make 1 \times SYBR Green I dye master mix by combining the following:
TE (ml) = $0.2 \times (\# \text{ reactions}) = \underline{\hspace{2cm}}$
SYBR Green I (μ l) = $0.08 \times (\# \text{ reactions}) = \underline{\hspace{2cm}}$

Make sure to include at least 24 standard reactions, eight blank reactions, extra for pipetting error, etc.

10. Corning Costar[®] black plates are used for fluorescence-based assays as the absorbed light, thus reducing background and cross talk. Pipette 200 μ l 1 \times SYBR Green I dye mixture into each well of labeled quantification plate using a P200 or P1000 multichannel pipette. Cover with aluminum foil until ready for use.
11. Add 2 μ l of DNA standard to SYBR Green I mixture. Pipette up and down a few times to make sure no liquid is retained in tip. Standards should be done in triplicates and are usually placed in A1–H1, A2–H2, and A3–H3. Leave wells A4–H4 blank. Add 2 μ l unknown DNA to SYBR Green I mixture. Unknowns can be should be aliquoted as singlets. Pipette up and down a few times to make sure no liquid is retained in tip. If quantifying a large number of samples, plates should be covered with foil to protect from light and placed at 4 °C while the remaining 1 \times SYBR Green I mix is distributed.
12. DNA samples which have an initial concentration below 2 ng/ μ l should be excluded, or more DNA should be extracted. Very concentrated templates are also not ideal. Calculating appropriate initial concentration is crucial to the success of this experiment. Take extra care when pipetting and consider repeating quantification if any of the following occur: (1) Standard curve has low R-value (less than 0.95). If this is the case, discard experiment. (2) DNA concentration is not within range of curve. If initial concentration is >100 ng, dilute DNA and re-quantify. If initial concentration is <2 ng, either (a) DNA was not added or (b) DNA extraction needs to be redone. (3) Initial concentration calculated for standards is far off from the expected values. If this is observed, quantify (e.g., NanoDrop) all standards and run on gel (λ DNA could be degraded).
13. This amount depends on starting concentration of dilutions. DNA from all samples must be the *same concentration*. If it is not standardized, dilute accordingly. A sufficient number of input DNA molecules are needed for accurate mutation calling. Studies in diploid rice estimate that 24 copies of DNA per allele ensure coverage of the mutant allele and reduce copy number variants (Tsai et al. 2011). Having fewer copies of DNA in pools increases the probability of having no coverage of an individual allele and increases likelihood of false mutation calls due to polymerase-based error (Tsai et al. 2011; Akbari et al. 2005). For a 64-individual pool, approximately 1.5 ng template is needed for PCR. Diploid rice has 2C value of approximately 1.0 pg (Johnston et al. 1999), therefore 0.5 ng total for each dimensional pool (Tsai et al. 2011). After preparing master pools, pre-pool plates can be heat sealed and stored at –20 °C.
14. It is good to include control(s) to ensure pooling has been done correctly. Include at least one DNA sample with a known mutation in a gene that you are amplifying on a pre-pool plate. Pooling is perhaps the most difficult/error-prone step of TBS pipeline. Use caution when pipetting (i.e., make sure you are always transferring the same amount of DNA).

15. Because TILLING primers are likely to have high annealing temperatures (due to length and GC content), the use of a touchdown thermocycling program is employed to prevent non-specific annealing.
16. It is very important to have specific amplification for mutation detection. The use of ice and a hot start taq polymerase significantly reduces non-specific priming. To increase efficiency, PCR reactions are performed in a 96-well assay. PCR master mix for a 96-well plate (including 5 % extra of each reagent and not including template DNA): 303 μ l 10 \times *Ex Taq* buffer, 242.4 μ l of 2.5 mM dNTP mixture, 181.8 μ l of 10 μ M primer mix, and 15.2 μ l of Takara *Ex Taq* HS enzyme (0.75 unit/reaction).
17. Failed reactions should be set up again in duplicate or triplicate.
18. Do not use a NanoDrop to determine sample concentration of PCR reactions. The presence of dNTPs, polymerase, etc., will skew results.
19. While it is necessary for all PCR from a particular primer pair to be of the same concentration, PCR products from different genes do not need to be standardized. As with DNA normalization, the final dilution will most likely be determined by the concentration of the most dilute sample on the plate. Dilute PCR products by adding appropriate volume of TE.
20. Larger fragments need more input DNA because mutation detection requires an even representation of each base in the sequencing space. For rice TILLING, 500 ng–1 μ g of pooled PCR product is sufficient for library preparation and mutation discovery. The current protocol is optimized for 500 ng of pooled PCR product with an average size of ~1.5 kb.
21. For example, to sequence five TILLING fragments of the same size, you would need 100 ng DNA for each fragment. This amount should be adjusted according to fragment size, i.e., larger fragments require a proportionally greater amount of input DNA.
22. Agencourt AMPure XP beads can be stored at 4 $^{\circ}$ C for approximately 1 year. Beads settle over time, so it is necessary to fully resuspend beads prior to use. Observe all of the manufacturer's guidelines for handling beads.
23. NEBNext[®] Fragmentase enzyme will digest up to 3 μ g DNA. However, enzyme amount and incubation time will need to be adjusted. Take special care when working with Fragmentase. The enzyme needs to be vortexed prior to reaction setup. It is important to keep all samples on ice (or at –20 $^{\circ}$ C for longer periods), thus reducing enzymatic activity and avoiding undesirable digestion. In addition, it appears that digestion can be highly variable depending on template or Fragmentase batch. Optimize enzyme amount (try between 1 and 3 μ l) and digestion time (20–45 min) on a few samples prior to scaling to all pools. DNA can also be mechanically fragmented by sonication, for example, with a Covaris Focused-ultrasonicator. This yields consistent shearing with reduced template bias and can be preferable to chemical fragmentation because it does not depend on template concentration and yields a more uniform fragment distribution.
24. This time may be shorter or longer and is determined by visualizing fragment size on a gel.

25. The KAPA data sheet has a lot of good information regarding handling of reagents. For example, AMPure beads and PEG/SPRI solution must be equilibrated to room temperature prior to use. It is strongly suggested that it is read in entirety.
26. Approximately eight cycles should be sufficient for enrichment although new kits may need only 2-4 cycles or can be used PCR-free. Run 5 μ l of 50 μ l of enrichment PCR on an agarose gel to confirm (while sample is stored at 4 °C). Reaction can be returned to thermocycler for another 2–4 cycles if needed. Do not exceed 12 cycles of enrichment. After final PCR cleanup, run 2 μ l of 20 μ l on 2 % gel to make sure that library is primer and adapter-free. This can also be confirmed with a Bioanalyzer trace.
27. DNA concentration can be obtained through SYBR Green 1 (*see* Methods Sect. 20.3.1). NanoDrop may also be used but does not yield as reliable results. Most sequencing facilities will not need very much input DNA (e.g., 10 μ l of 10 nM). Therefore do not pool all of your samples. Start with 1–2 μ l of most concentrated sample and adjust amount of others accordingly.
28. Input DNA purity should have an approximate 260/230 >2.0 and 260/230 between 1.8 and 2.0.
29. Illumina sequencing facilities often offer library quantification services as part of sequencing. Therefore, it is usually not necessary to perform library quantification prior to library submission.
30. If 150 bp paired-end reads are obtained, the average insert size for sequencing should be approximately 350 bp (not including adaptors).
31. On the HiSeq 2500, assuming 150PE reads, approximately 1024 individuals, with 20 (1500 bp) targets can be screened in a single HiSeq lane.
32. Details of the bioinformatics pipeline can be found in Tsai et al. (2011, 2013) and Missirian et al. (2011). Like sequencing, most labs may not have the necessary personnel and resources to conduct the bioinformatics analyses completely in-house. Sequencing core facilities are typically associated with bioinformatics analysis core groups that can provide support and some infrastructure to facilitate more efficient processing and analysis of sequence data.
33. Sequence reads are provided as compressed FASTQ format files that contain both the reads that passed quality filters and those that did not. It is recommended that alignment be performed on a computer cluster as memory requirements for alignment and mutation identification exceed that which can be handled on a personal computer.
34. Each quality trimmed read file (sequenced libraries from row, column, and plate pools) is placed in its own directory. For example, reads from first-row pool would be placed in directory labeled “T1R1.”
35. Probability scores are assigned using Coverage Aware Mutation Calling Using Bayesian Analysis (CAMBa; available at <http://web.cs.ucdavis.edu/~filkov/CAMBa/>), a software program that estimates the probability that a mutation is falsely identified with a F_t score, the mean-centered log of the posterior probability of mutation (Tsai et al. 2011; Missirian et al. 2011). CAMBa uses the overlaps resulting from the tridimensional library pooling strategy to identify the mutations and the corresponding mutant individuals simultaneously.

36. Use original TILLING primers or newly designed primers closer to the location of the mutation to amplify and sequence to confirm mutation and identify individuals that are heterozygous and homozygous at the mutation. If the mutation is predicted to create or remove a restriction enzyme recognition site, it may be more efficient and cost effective to perform restriction digest of PCR products.
37. Generally backcrossing of mutants to the wild-type cultivar from which the mutant population was derived is performed to eliminate mutations that may confound characterization. It may be necessary to backcross two or more times. It may also be possible and useful to evaluate the M₃ plants directly depending on the nature of the mutant phenotype. It is recommended that approximately 20–25 M₃ plants should be screened for stable mutation recovery.

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