

8 Chemical and Irradiation Induced Mutants and TILLING

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8.1 Introduction

Chemical and ionizing radiation mutagenesis have been routinely used to generate genetic variability for breeding research and genetic studies. To-date, through such mutagenesis, 2,428 crop varieties have been released and among them 501 are rice varieties (see <http://www-mvd.iaea.org/MVD>). Because traditional mutagenesis does not use transgenic technology, it has particular appeal to the industry, where prohibitive regulatory costs and the debilitating debate on genetically-modified organisms have restricted many crop-improvement efforts. Mutagen-induced morphological mutations have also provided genetic markers for the development of genetic linkage maps in many plants, including rice.

While *Arabidopsis* has become the paramount model plant system, it is not a crop plant. Thus, the spectrum of its biological traits cannot address fundamental questions of crop plant domestication and agronomic performance. An alternative experimental system based on a crop plant is therefore much needed. At the time of this publication, rice is the only crop for which a complete genome sequence has been made available (Goff et al. 2002; Yu et al. 2002; International Rice Genome Sequencing Project 2005). To realize the potential of rice in the post-sequencing era, however, a complete analysis of function must involve disruption or modification of all of its genes. Several approaches are available for the functional inactivation of genes, including the use of gene-tagging elements, the introduction of new sequences as transgenes, gene silencing (see Chapters 9 to 13 of this book) and the use of chemical mutagens and irradiation. Collectively, sequence-tagged T-DNA insertions amount to over 360,000 for *Arabidopsis* (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and 113,000 for rice (see Chapters 9, 10, 13 and 14). In *Arabidopsis*, even these very high numbers, however, do not provide saturation mutagenesis as thousands of *Arabidopsis* genes, especially those smaller than 1 kb, have no insertions (<http://signal.salk.edu/database/T-DNA/>). Furthermore, although the probability of tagging a gene increases with the number of available tags, it does so asymptotically; hence the efforts to extend the database to cover all the genes may soon approach the point of diminishing returns. Additionally, a tagging approach is more likely to create non-functional proteins rather than multiple alleles with amino acid alterations. On the other

hand, chemical or irradiation mutagenesis can yield base substitution mutant alleles which often play an important role in determining the functional domains of the protein.

Given the above concerns, traditional mutagenesis, coupled with efficient targeting of genes, is an attractive genetic strategy for both *Arabidopsis* and rice. Production of mutants by chemical or irradiation mutagenesis is relatively inexpensive. Any genotype can be mutagenized and the distribution of mutations is probably random in the genome. Because of the high density of mutations, genome-wide saturation mutagenesis can be achieved using a relatively small mutant population (Koornneef et al. 1982; Henikoff and Comai 2003). This also provides a large allelic series as a complement to the knockout mutants produced by insertional mutagenesis or transformation methods (over- and under-expression). Unlike insertional mutagenesis technologies, which require highly-efficient transformation systems, chemical and irradiation mutagenesis do not rely on transformation. Despite these advantages, the use of chemical and irradiation-induced mutants as gene identification tools has been limited. This is mainly because the molecular isolation of mutated gene(s) requires considerable effort as the mutations are not physically tagged. However, advances in high-throughput genotyping have significantly increased the efficiency in detecting point mutations or deletions (Borevitz et al. 2003; Henikoff and Comai 2003; Winzeler et al. 2003). One such example of high-throughput reverse-genetic technique is Targeting Induced Local Lesions in Genomes (TILLING). TILLING is employed to discover point mutations in the mutant libraries created using traditional chemical mutagenesis. Technologies that are developed primarily for discovering single-nucleotide polymorphisms (SNPs) in surveys of human and other populations are being adopted for TILLING (Bentley et al. 2000; McCallum et al. 2000a, 2000b; Comai and Henikoff 2006). Consequently, there has been growing interest in using chemical and irradiation mutagenesis in model organisms for functional genomics research (Liu et al. 1999; Nadeau and Frankel 2000).

In this chapter, we discuss various chemical and irradiation mutagens, mutagenesis strategies and various forward and reverse genetics approaches available with special reference to TILLING and its application as a powerful reverse genetics strategy for plants. We highlight the current status of rice mutant stocks, databases and forward and reverse genetics strategies being employed for rice functional genomics.

8.2 Mutagens and Mutagenesis

Chemical mutagens and ionizing radiation have long been used as plant mutagens in forward-genetic studies (Guenet 2004). They are preferred over insertion mutagenesis considering their ability to (1) generate allelic series, (2) induce mutations at high frequencies, and (3) to be applied to various plant species. Chemicals mainly induce point mutations, and are thus ideal for producing missense and nonsense mutations, which would provide a series of change-of-function mutations. On the other hand, ionizing radiations normally induce chromosomal rearrangements and deletions.

The utility of a comprehensive deletion stocks is best illustrated in yeast. A collection of yeast deletion mutants covering 96% of annotated open reading frames has been proven to be a valuable resource for yeast functional genomics (Giaever et al. 2002). As shown in yeast, achieving a saturated gene-deletion mutant library with a small population is very important. Therefore, selection of a mutagen should be based on its efficiency and specificity to induce mutations, such that the resulting mutant library is of manageable size. At the same time, the mutagenesis procedure should be as simple as possible. It is also important to know the major type of mutation induced by a particular mutagen as the screening strategy to be used will depend on the predominant type of mutation it creates (Koorneef et al. 1982).

8.2.1 Chemical Mutagens

Ethylmethane Sulfonate (EMS)

Alkylating agents were the first class of chemical mutagens to be discovered when Auerbach and Robson (1946) found the mutagenic effects of mustard gas and related compounds during World War II. Alkylating agents such as mustard gas, methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS) and nitrosoguanidine have several effects on DNA. Because of its potency and ease with which it can be used, EMS is the most commonly-used chemical mutagen in plants. EMS alkylates guanine bases and leads to mispairing - alkylated G pairs with T instead of C resulting in primarily G/C-to-A/T transitions (Sega 1984; Vogel and Natarajan 1995). EMS mutagenesis in rice involves soaking the seeds in an aqueous solution at a chosen concentration (from 0.2–2.0%) for 10-20 h (based on the sensitivity or kill curve of the genotype used). Since EMS

produces a large number (genome-wide) of non-lethal point mutations a relatively small mutant population (~10,000) is sufficient to saturate the genome with mutations. In *Arabidopsis*, point mutation density can be as high as four mutations per Mb (Comai and Henikoff 2003, 2006; Till et al. 2003b).

An important advantage of using a common mutagen, such as EMS, is that a substantial body of literature has accumulated that confirms its utility in forward genetic screens in a variety of organisms. These include the favorite model animal and model plant for mutagenesis studies, *Drosophila melanogaster* and *Arabidopsis thaliana*, respectively. EMS is remarkably consistent, in that apparently similar levels of mutagenesis have been achieved in these organisms, despite the ~1 billion years of divergence between them. For example, recessive lethal mutations are estimated to occur at similar rates in both cases with EMS doses causing acceptable levels of sterility and lethality (Koornneef et al. 1982; Ashburner 1990). In addition, direct estimates confirm that base substitution rates are comparable for *Arabidopsis* seeds soaked in EMS (McCallum et al. 2000a; McCallum et al. 2000b) and *Drosophila* males fed EMS (Bentley et al. 2000), and approximately similar rates were found in a reverse-genetic screen of zebrafish progeny exposed to N-ethyl-N-nitrosourea (ENU) (Wienholds et al. 2002). Thus, chemical mutagenesis causes a high frequency of nucleotide substitutions in a variety of organisms. Genome size does not appear to be an important factor in EMS mutagenesis because estimates of per gene mutational density found for *Arabidopsis* appear to be similar for maize (Goll and Bestor 2002), which has a 20-fold larger genome size. Therefore, EMS may likely be the mutagen of choice for TILLING in plants (see the subsequent section in this chapter). However, the toxicity of EMS may vary depending on the species, and other mutagens or post-treatments with antitoxicants may be worth considering (Henikoff and Comai 2003).

Over the last few years, several new projects have been initiated with the aim of producing EMS-induced rice mutant populations in USA (Crops Pathology/Genetics Research Unit of UC Davis, USDA-ARS), China (The Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences), Taiwan (Taiwan Agricultural Research Institute), Japan (Institute of Genetic Resources, Kyushu University, Japan) and the Philippines (International Rice Research Institute).

Diepoxybutane (DEB)

Diepoxybutane (DEB) is a potent chemical mutagen used in a variety of biological systems (Ehrenberg and Hussain 1981) and is capable of producing alkali-labile sites in DNA and forming inter- and intra-strand cross-links (bi-functional alkylating agent). The exact mode of action and the precise end results of its pre-mutagenic lesions are not well understood, but it was shown to be mutagenic in *Drosophila melanogaster* (Graf et al. 1984), an efficient chromosome breaker (Watson 1966; Zimmering 1983) and an efficient inducer of multi-locus deletions in *Drosophila* (Shukla and Auerbach 1980; Olsen and Green 1982). DEB has been shown to induce mutations at the *rosy* locus in *Drosophila*, 43% of them being deletions ranging from 50 bp to 8 kb (Reardon et al. 1987). DEB mutagenesis in rice involves soaking seeds in an aqueous solution of 0.004% or 0.006% DEB with gentle shaking at 30°C for 13 h, and has been successfully used for forward genetics (Wu et al. 2005).

N-methyl-N-nitrosourea (MNU)

N-methyl-N-nitrosourea (MNU) is a mono-functional alkylating agent causing single-strand DNA breaks during interphase stages. The pronounced clustering of chromosomal aberrations in heterochromatic regions (as shown by *in situ* hybridization studies) after treatment with MNU is thought to be mainly due to an error-prone interference of recombinative repair and replication in damaged basic repeats of large tandem repeat arrays (Vogel and Natarajan 1995).

A large collection of MNU-induced mutants has been produced in Japan (<http://www.shigen.nig.ac.jp/rice/oryzabase/nbrpStrains/kyushuGrc.jsp>). Twelve classes of visible phenotypes, including 49 easily identifiable phenotypes, have been used to classify these mutant lines. Phenotypic classification of the MNU-induced mutants is identical to that used for *Tos17*-induced mutant lines. These mutants represent a promising resource for characterizing mutant genes using reverse-genetic tools such as TILLING (Kurata and Yamazaki 2006).

Sodium Azide

Mutagenicity of sodium azide, an inhibitor of catalase and peroxidase enzymes, has been demonstrated in barley, maize soybean, pea, *Brachypodium* and rice. There have been some reports of synergistic increase in the frequency of chromosomal aberrations when gamma-ray irradiation was followed by sodium azide treatment although there was no apparent affect

on chlorophyll mutation frequency. Synergism with respect to chlorophyll mutation frequency has been observed when used following MNU treatment. Mutation frequency as well as biological damage showed a linear response to an increase in the concentration of sodium azide from 5×10^{-4} M to 2×10^{-3} M (in 0.1 M phosphate buffer, pH 3). Pre-soaking of seeds in water for 4-12 h induced highest chlorophyll mutation frequency with reduction in the frequency with longer pre-soaking treatments (Sarma et al. 1979).

Researchers at the Taiwan Agricultural Research Institute have undertaken sodium azide mutagenesis of rice cultivar Tainung 67 (TNG67). No data is available on the type of genetic lesions produced by sodium azide in rice. However, in barley it has been shown to induce substitutions comprising transitions and transversions (Olsen et al. 1993).

8.2.2 Irradiation Mutagens

Ionizing radiation has been widely used to induce mutations for plant breeding and classical genetic analysis, but in-depth analyses at the molecular level have been done in only a few organisms. In plant genomes, ionizing radiation normally induces rearrangements and deletions, (Shirley et al. 1992; Bruggemann et al. 1996; Cecchini et al. 1998; Shikazono et al. 1998, 2001). Mutants in crop species produced by ionizing radiation have proved to be valuable in the fields of genetics and mutational breeding. The International Atomic Energy Agency (IAEA) has been a strong advocate of applying irradiation mutagenesis for crop improvement, and continues to provide gamma ray irradiation as a public service, and organize regional research and training networks to apply mutation methods for crop breeding (R. Afza, IAEA, personal communication; <http://www-naweb.iaea.org/nafa/pbg/index.html>).

Fast Neutron

Fast neutron has been shown to be a very effective mutagen in plants. An *Arabidopsis* line treated with fast neutrons at a dose of 60 Gy would have ~10 genes deleted on average (Koornneef et al. 1982), and thus, ~2,500 lines would be sufficient to represent deletions in each of the expected 25,000 genes (The Arabidopsis Genome Initiative 2000). In another study, Bruggemann et al. (1996) found that most (13 out of 18) fast neutron-induced *hy4* mutations in *Arabidopsis* were deletions larger than 5 kb. Molecular characterization of *Arabidopsis gal-3* (Sun et al. 1992) and tomato

prf-3 (Salmeron et al. 1996) further demonstrated that fast neutron bombardment induces relatively-large deletion mutations.

Recently, a reverse genetics system based on fast-neutron induced deletions was developed to identify and isolate targeted plant genes (Li et al. 2001; Li et al. 2002; Li and Zhang 2002; Wu et al. 2005). According to Li et al. (2001), the reverse genetics system using fast-neutron-generated deletions is highly efficient and that fast neutrons could produce mutant lines with complete coverage much easier than that by T-DNA insertional mutagenesis. In rice, fast neutron mutagenesis has been used to produce about 8,000 M₄ lines in an *indica* variety IR64 (Wu et al. 2005). Although the size of induced deletions (in several kb range) in a few characterized mutants appears to be suitable for PCR screen, the usefulness of this collection in a reverse-genetic screen as described by Li et al (2001) has not been thoroughly tested.

Gamma Irradiation and X-rays

Irradiation with gamma-(γ) and x-rays is also known to produce deletions and other chromosomal rearrangements, but a only few of them have been characterized at the molecular level (Oppenheimer et al. 1991; Wilkinson and Crawford 1991; Shirley et al. 1992; Kieber et al. 1993; Nambara et al. 1994). In *Arabidopsis*, all eight gamma radiation-induced mutations of a negatively-selectable suicide marker (*tms2*), which was integrated into the genome, had deletions larger than 5 kb (Cecchini et al. 1998). Gamma- and X-rays have been used to produce mutants in *Arabidopsis* (Rédei and Koncz 1992) and rice (Cheema and Atta 2003; R. Bhat, unpublished data). Wu et al. (2005) used two doses of gamma irradiation (250 and 500 Gy) to produce a large collection of mutants in IR64. The size of the genetic lesions (in the kb range) appeared amenable to detection by chip-based techniques.

Accelerated Ions

High linear energy transfer (LET) radiation, such as ion particles, causes more localized, dense ionization within cells than low-LET radiation (Smith 1972; Kraft et al. 1992; Blakely and Kronenberg 1998; Shikazono et al. 2003). On the basis of microdosimetric and radiobiological considerations, it is assumed that high-LET radiation could produce double-strand breaks with damaged end groups and consequent low frequency of repair (Hagen 1994; Goodhead 1995; Blakely and Kronenberg 1998; Nikjoo et al. 1998). High-LET radiation would therefore generate mutations more frequently (more closely-positioned) than low-LET radiation. Most

likely, large structural alterations are also induced by the high-LET radiation more frequently than those by low-LET radiation.

Using accelerated carbon ions (C ions), several novel *Arabidopsis* mutants (*ast*, *frll*, *uvi1*, *suvi1*, *tt18*, and *tt19*) have been isolated (Tanaka et al. 1997; Hase et al. 2000; Sakamoto et al. 2003; Shikazono et al. 2003; Kitamura et al. 2004). Analyses of these mutants at the nucleotide sequence level revealed inversions, translocations, and short deletions at comparable frequencies (Shikazono et al. 1998, 2001, 2003, 2005; Sakamoto et al. 2003; Kitamura et al. 2004). From the analysis of rearrangements, deletions were found to be generated at a frequency of 6.1×10^{-5} , which was comparable to that induced by fast neutrons (Li et al. 2001). These results imply that mutagenesis by accelerated ions could be used for both forward and reverse genetics in plants.

Abe et al (2005, 2006) have studied the mutation frequency in rice cv. Nipponbare with accelerated C and Neon (Ne) ions. Seeds soaked for 3 days in water at 30°C without light were exposed to ions accelerated to 135 MeV/u by the RIKEN Ring Cyclotron (RRC, The RIKEN Accelerator Research Facility, Japan) within a dose range of 10 to 40 Gy. The LET values of the C and Ne ion corresponded to 22.6 and 63.0 keV/μm at the surface of the seeds. Treated seeds were grown and progeny raised to measure the indicator mutation frequency (chlorophyll-deficient mutant or CDM). Half seed fertility doses were 40-80 Gy and 20 Gy for C and Ne ions, respectively. This result shows that biological effects depend strongly on their LETs. The optimum irradiation dose to induce CDM was 20-40 Gy with C ion. They also adjusted the LET values of the C ions at a dose of 20 Gy using the absorbers from 22.6 to 60.3 keV/μm at the surface of the seeds. They observed reduction in seed fertility with high LET irradiation but no difference in mutation rates at LET values of 22.6, 37.4, 48.0 keV/μm. A tall mutant and a lesion-mimic mutant segregated in M₂ generation, showed homozygous lines in the M₃ generation (Abe et al. personal communication).

8.2.3 Raising Mutant Populations

After treatment with an appropriate mutagen, the treated seeds are washed free of the mutagen and sown to produce M₁ generation plants. Because each cell of the embryo is mutagenized independently of the other cells, M₁ individuals are chimeric in the sense that they have mutated tissue sectors that descend from a single embryonic cell. In addition, each mutated sector is heterozygous for any mutation. Mutations present in the cells that

form the reproductive tissues are inherited by the selfed progeny, the M_2 generation. M_2 plants are used to prepare pooled DNA samples for reverse genetics screening (see section 8.5), while their seeds are inventoried. Forward genetics screening (phenotypic analysis) is normally performed on M_3 plants. For assaying quantitative traits, it is particularly important to advance the lines to M_4 or beyond because of the need to evaluate phenotypes in replicated trials. Bulk seeds from advanced generations are also more useful for the purpose of distributing the materials for examining different phenotypes.

For the purpose of identifying mutated genes, it is better to aim for a moderate to high mutation density in the genome so that fewer mutants are needed to achieve genome coverage. However, too high a dose presents practical problems. At high doses, lethality and sterility of M_1 plants make it difficult to produce an appropriately-large population in a single attempt (Wu et al 2005).

From the oligo-hybridization experiments using several DEB- and gamma ray-induced rice mutants, it has been estimated that over 100 mutations could be present in each genome of the chemical or irradiation-induced mutants (H. Leung, unpublished data). Theoretically, it would take many generations to eliminate all the background mutations. However, with one or two backcrossings, one can quickly establish the inheritance pattern and at the same time remove a significant portion of the background mutations. Producing a useful mutant population therefore is often a trade off between the need to produce high-density mutations and the practicality of keeping a vigorous population without too many deleterious effects and background mutations (Wu et al. 2005).

8.3 Rice Mutant Stocks and Databases

The FAO/IAEA Mutant Variety Database or MVD provides information on induced mutations suitable for breeding programs and genetic analyses (<http://www-mvd.iaea.org/Refs/MutBree-Rev-1.pdf>). MVD collects information on crop mutant varieties, mutagens used and characters improved and a good number of rice entries are included in this database. Various chemical and irradiation mutagens used in rice by different laboratories are summarized in Table 8.1.

Table 8.1. Summary of various chemical and irradiation mutagenesis attempts made in rice

Mutagen	Cultivar	Nature of mutation	Method for detection	Group used
Ethylmethane sulfonate (EMS)	IR64 (<i>indica</i>) M202 (<i>japonica</i>) Nipponbare	Point mutations	TILLING	IRRI UC Davis IPPE
Diepoxybutane (DEB)	IR64	Point mutations, deletions	PCR, TILLING	IRRI
N-methyl-N-nitrosourea (MNU)	Kinmaze Taichung 65	Single-strand DNA breaks	TILLING	IGRKU, Japan
Fast neutron	IR64 M202	Large deletions, translocations	PCR	IRRI; USA
Gamma irradiation and X-rays	IR64	Large deletions, point mutations	PCR	IRRI
Accelerated carbon ions	Nipponbare	Double-strand breaks, large structural alterations	CDM ^a frequency, PCR	RIKEN, Japan
Sodium azide	China-45 Tainung 67	Data not available	CDM frequency	Sarma et al. (1779); TARI, Taiwan

^aChlorophyll-deficient mutant

USA Mutant Stocks

A fast neutron-induced population was originally developed by Pamela Ronald at the University of California-Davis using *japonica* variety M202 and was subsequently acquired by a private company. The collection consists of 24,660 M₂ lines that have been used for high-throughput PCR screening (Li et al. 2001). However, these are proprietary stocks and are not available publicly.

The Crops Pathology/Genetics Research Unit of UC Davis (USDA-ARS) is developing a sizable stock (~10,000) of chemical (EMS)-induced rice mutants in cultivar Nipponbare as a public resource (Dr. Tom Tai personal communication, http://www.ars.usda.gov/research/projects/projects.htm?ACCN_NO=408015).

IRRI Mutant Stocks and Database

The International Rice Research Institute (IRRI) in the Philippines is maintaining a mutant collection derived from the *indica* cultivar IR64. These were produced using four mutagenic agents- fast neutron, gamma ray, DEB, and EMS in order to have different sizes of genetic lesions in the population. IR64 is the most widely-grown rice variety in the tropics and it has many valuable agronomic traits related to yield, plant architecture, grain quality, and tolerance to biotic and abiotic stresses. For many traits, IR64 has intermediate phenotypes, thus enabling screening for gain- and loss-of-function mutations. Producing mutations in this elite genetic background can maximize the detection of phenotypic changes in important agronomic traits (Leung et al. 2001). Currently, the population consists of approximately 45,000 lines at the M₄ stage.

‘Mutant View’, a database of these mutants, has been developed at the International Rice Information System (IRIS, <http://www.iris.irri.org>). This database, in addition to providing descriptions of the mutants, also serves as a portal for users to request materials.

To consistently describe the mutant phenotypes through different seasons of field phenotyping, and to recognize synonymous mutations observed in different mutant studies, a set of controlled vocabulary (CV) descriptors documenting the observed mutant phenotypes was established. The set of vocabulary in use from the agronomic observations were compiled and curated, and 86 distinct agronomic mutant phenotypes were listed. In 2003, in collaboration with the *Tos17* mutant group at NIAS (A. Miyao and H. Hirochika), the list of controlled vocabulary descriptors from the *Tos17* mutant phenotypes was merged with the IR64 mutant phenotype controlled vocabulary. Fifty-six distinct phenotype observations from *Tos17* mutants and the 86 IR64 CV terms were rationalized and a composite CV set of 91 terms was developed.

In its current release, the phenotype CV is posted as a table in the IRIS-mutant website; it has mapped most of the CVs to public term ontology databases. Its purpose is not to create a new ontology but to utilize existing ontological classifications. In the case of novel CVs discovered through additional mutant screenings, these would be deposited in the appropriate ontology databases (e.g., Trait Ontology database) for incorporation into publicly-accessible resources. This may serve as an inter-database resource enabling clear and unambiguous queries of mutant traits across the various existing rice mutant stock resources.

The use of CVs in describing agronomic phenotypes, and the ability to accurately map this description into other mutant resource databases, is an important first step in gene discovery experiments. Using CV to cross-reference databases, it is possible to query mutants with either enhancement or knockout of the same or a correlated trait. Since one or more of these mutants could be sequence-indexed, it will enable quick identification of allelic series across different mutant collections. This approach could be particularly useful for finding allelic series in the non-transgenic *indica* mutants using the sequenced-indexed databases of the transgenic mutants.

China Mutant Stocks

Researchers at the Institute of Plant Physiology and Ecology (IPPE) of Shanghai Institutes for Biological Sciences (SIBS), in collaboration with Plant Research International (Netherlands), have produced ~60,000 rice mutant lines via EMS mutagenesis (<http://202.127.18.254/research/field3.htm>). These populations were obtained with seed treatment of 20mM, 40mM and 60mM EMS. This group is poised to set up a mutant database (~6,000 entries) for forward genetics and DNA pools for large-scale reverse genetics.

Taiwan Mutant Stock

The sodium azide-induced mutant stock developed and maintained at Taiwan Agricultural Research Institute (TARI), Taiwan (<http://www.agnet.org/library/article/rh2003009b.html>) contains more than 2,000 mutants (M_{12}) with diverse variations including pathogen (*Pyricularia oryzae*, *Xanthomonas campestris* pv. *oryzae*) and insect (brown planthopper, white-back hopper) resistance, herbicide (bentazone and glyphosate), stress (UV, chilling) tolerance, chemical composition (starch, storage proteins, and aroma), taste quality, pericarp color of grain, and hundreds of agronomic variations in the growth stage, grain development, morphology, plant type, and yield capabilities.

Japan Mutant Stock and Database

The Institute of Genetic Resources at Kyushu University has produced more than 6,000 rice mutants by N-methyl-N-nitrosourea treatment in cultivars Kinmaze and Taichung 65. Information relating to this collection can be viewed at <http://www.shigen.nig.ac.jp/rice/oryzabase/nbrpStrains/kyushuGrc.jsp>. The list of mutant strains presented here contains the link

to characteristic data, the request form and the MTA form for seed distribution.

Preliminary results on TILLING indicate a mutation frequency of 0.8% per 1 kb region and so one can expect about eight different mutations for every 1 kb of genome sequence in 1,000 mutant lines (Suzuki et al. 2005). The mutant population with this high mutation ratio should serve as a promising TILLING resource and for reverse-genetic studies in rice. At present 1,000 mutant lines of each cultivar are available for public distribution and it is expected that by the end of 2007, all mutant lines will be ready for seed distribution.

8.4 Forward Genetics with Mutants

In the forward genetics strategy, one starts with a phenotype and its inheritance, followed by genetic mapping to locate the target gene on a chromosomal region. With the help of genetic markers it is possible to “walk down” the chromosome and eventually the DNA sequence responsible for the trait can be identified. The availability of the entire rice genome sequence will hasten gene identification considerably.

8.4.1 Phenotyping

Mutant populations harbor a large amount of genetic variability that can be revealed when the mutants are subjected to appropriate phenotypic screening. Morphological mutants can be identified based on phenotypic categories. But it is more difficult to estimate the variation for conditional traits because of the differences in experimental conditions. As mentioned above, the availability of seeds from advanced generations is most important in screening for quantitative phenotypes. In fact, except for simple qualitative traits, it is not possible to identify mutant phenotypes in early generations. With the screening of replicated lines, mutants with altered response to diseases (blast, bacterial blight, tungro virus) and brown plant hopper have been recovered from the IRRI collection. More recently, mutants with quantitative changes in salinity tolerance have also been isolated (B. Nakhoda and A. Ismail, IRRI, unpublished data). In many cases, both gain- and loss-of-resistance mutants were found. Overall, the rate of mutant detection in the population is ~0.1% for a broad category of traits such as altered disease resistance. However, the rate is an order of magnitude

lower (~0.01%) for a highly specific trait, such as a change in response to tungro viruses (P. Cabauatan and I. Choi, IRRI, unpublished data).

8.4.2 Map-Based Cloning

Map-based cloning is a forward genetics approach to identify the function of a gene by delimiting the chromosomal region conferring the phenotype of interest with markers linked to the mutated gene. Until recently, map-based cloning approach has been rather tedious and time consuming. Although initial progress is easy to achieve, the fine mapping of a candidate gene is increasingly difficult. Thus, defining gene function by reverse genetics approaches has offered an attractive alternative. However, reverse genetics can be limited by a lack of phenotypes in reverse screenings, mainly because of gene function redundancy. Also, the choice of phenotypes to be evaluated can be biased by preconceptions about the possible function of a chosen gene. The advances in sequencing projects, improvements in map-based cloning approaches, the wealth of available marker systems and the progress made in methods to detect DNA polymorphisms, have brought map-based cloning back into the limelight (for a review see Peters et al. 2003).

Map-based cloning strategies are based on the fact that as distances between the target gene and the analyzed markers decrease, so does the frequency of recombination. Therefore, the availability of increasingly-denser genetic maps, culminating in physical maps is a key factor determining the speed with which map-based cloning can be achieved. Although sizable numbers of polymorphic isozyme and DNA-based markers such as RFLP, RAPD, SSR, AFLP and SSCP, are available for many crops, their detection is not as straightforward and inexpensive as that of SNPs and insertion/deletions (indels). Recent developments and improvements in high-throughput sequencing technology and the availability of large sets of ESTs are enabling easy detection of SNPs and indel polymorphisms.

A genome-wide rice DNA polymorphism database (<http://shenghuan.shnu.edu.cn/ricemarker>) has been constructed using the genomes of Nipponbare, a *japonica* cultivar, and 93-11, an *indica* cultivar (Shen et al. 2004). This database contains 1,703,176 single SNPs and 479,406 indels, approximately one SNP every 268 bp and one indel every 953 bp in the rice genome. Both SNPs and indels in the database have been experimentally validated. Of 109 randomly-selected SNPs, 107 SNPs (98.2%) are accurate. PCR analysis indicated that 90% (97 of 108) of indels in the database could be used as molecular markers, and 68% to 89%

of the 97 indel markers have polymorphisms between other *indica* cultivars (Guang-lu-ai 4 and Long-te-pu B) and *japonica* cultivars (Zhonghua 11 and 9522). By validating indel polymorphisms in the database, sets of indel markers for all chromosomes have been developed. These markers are inexpensive and easy to use, and can be used for any combination of *japonica* and *indica* cultivars. This rice DNA polymorphism database will be a valuable resource and an important tool for map-based cloning of rice gene. Recently, indel polymorphism is being exploited for genetic mapping using a low-cost microarray platform (David Galbraith, University of Arizona, personal communication).

Besides their use as DNA markers, SNPs can also be used for allele discrimination in the analysis of allele-sharing status among distant or related rice strains. An 'allele-sharing map' has been proposed as an effective strategy to convert huge amounts of complicated SNP data into a compact but informative map for various study purposes (Monna et al. 2006).

8.4.3 Detecting Genomic Changes Using Genome-wide Chips

Single-feature polymorphisms (SFP) have been detected successfully in *Arabidopsis* ecotypes using oligonucleotide (oligo) chips (Borevitz et al. 2003). Chang et al. (2003) reported preliminary results on using the Syngenta GeneChip, which contains 24-mer oligos representing 24,000 rice genes, to detect deletions. Genes/probes that generate hybridization signals below those of the wild-type cultivar (based on significant t-test) were considered as candidate genes. The gene chip approach was first tested with mutant alleles of two known genes: A γ -ray-induced dwarf mutant having a deletion in *d1* (AB028602) encoding a heterotrimeric G protein (Ashikari et al. 1999); diepoxybutane- and fast neutron-induced deletion mutants at the *Xa21* locus conditioning bacterial blight resistance in rice cultivar IRBB21 (Wang et al. 2004). DNA from the mutants and wild-type lines were hybridized separately to the Syngenta Rice GeneChip genome arrays. The GeneChip arrays successfully detected deletions spanning the single copy *d1* gene. Detection of the *Xa21* deletions was ambiguous because of the presence of multiple members of the gene family. Although the chip detection technique may not always pinpoint the target gene, it enables rapid localization of the approximate position of candidate regions.

There are limitations to the chip-based detection technique. It depends on genome coverage of the oligoarray chip and the size and the position of deletions relative to the oligos represented in the chip. It would be difficult to detect large deletions or multiple mutations across the genome. Back-

crossings are often required to remove background mutations. To overcome these problems, one may use multiple alleles, if available, to narrow the search for candidate mutations. Pooling of DNA from segregants with common phenotypes is another way to 'mask' irrelevant mutations (Gong et al. 2004). Finally, availability of the newer versions of oligoarrays such as the 44K Agilent oligoarray and rice genome chips such as the 51K Affymetric GeneChip[®] (see Chapter 4 in this book) will greatly improve the utility of deletion mutants for gene discovery.

8.5 Reverse Genetics with Mutants

With the availability of near-complete genome sequences for rice, identifying specific functions of each of the predicted 40,000 rice genes is a huge and challenging task for biologists. For genes which show detectable phenotypes when mutated, forward genetics approaches are the most feasible. However, for genes showing no detectable phenotypes, forward genetics is not feasible. Reverse genetics strategies are becoming increasingly useful, especially with the expanding collection of insertion-tagged lines and the advancement in RNAi-based gene silencing technology. Yet, production and curation of large libraries of insertional mutants with recoverable tags (such as T-DNA or transposon) for each gene will be very difficult to achieve because of "cold spots" in the genome (regions apparently inaccessible for insertion). Chemical and radiation mutagens on the other hand allow saturation mutagenesis to be achieved using relatively few individuals with multiple lesions in the genome. The use of such mutant populations for reverse genetics is becoming a reality with the development of high-throughput PCR-based detection (Li et al. 2001; Li and Zhang 2002) and TILLING technologies (McCallum et al. 2000a, 2000b).

8.5.1 PCR Screening

Small to medium-sized deletions in genomes (such as the ones produced by fast neutron mutagenesis) can be detected through PCR analysis. A method that identifies smaller than expected amplicons due to the presence of a deletion was first described by Jansen et al. (1997). In this method, primers flanking a genomic region containing a target gene are designed in such a way that the product generated by the wild-type allele is difficult to PCR amplify because of its large size. When a deletion reduces the length of the region flanked by the primers, the fragment with such deletion can often be amplified with higher efficiency. As a result, such smaller product

can be detected even if the DNA from the individual allele carrying the deletion is mixed with DNA from many wild-type individuals. Li and colleagues exploited this strategy in rice to develop a reverse genetics resource (Li et al. 2001; Li and Zhang 2002). Applying PCR-screening to 5,000 IR64 mutant lines has yielded one deletion mutation in a defense gene but the experiment required stringent optimization of PCR conditions, making the method less robust (P. Manosalva and J. Leach, Colorado State University, USA, unpublished data).

Nonetheless, the deletion detection strategy has advantages not found in other methods. Most notably, it can yield mutations in which tandemly-arranged genes (paralogous or not) can be simultaneously deleted. While this approach is potentially quite powerful, the mutants identified have been limited. The inefficiency of this approach can be attributed to several factors. Firstly, deletions can have severe consequences on the affected genes and mutagenic treatments that produce a high density of these lesions are lethal because essential genes are knocked out. Secondly, the severity of deletions makes it impossible to study the function of essential genes. Thirdly, the production of an appropriately-mutagenized population is difficult in most organisms because of the conflict between mutation density and survival. Fourthly, the strategy to detect mutant alleles is constrained: deletions that are too small to change the amplification efficiency of the target or those that are in the primer-binding sites cannot be identified. Notwithstanding the drawbacks, it would be useful to have such a resource in rice. Unfortunately, the mutagenized population described in Li et al (2001) is proprietary and unavailable as a public resource. No public population has been described and, therefore, this approach appears to be presently not easily available to rice researchers.

8.5.2 TILLING

The TILLING approach makes use of DNA strand mismatches formed between mutant and wild-type DNA. DNA from individual M_2 plants is isolated, pooled, and arrayed in 96-well plates. Primers are designed (e.g., using CODDLE, www.proweb.org/input/) to bracket a 1-kb region that most likely contains a deleterious mutation in a target gene. The primers are then used to amplify the gene of interest followed by denaturing and re-annealing of DNA to allow formation of homo- and heteroduplexes in the DNA pool. Originally, denatured HPLC was being used to detect the presence of a DNA mismatch, but now it is detected by enzymatic cleavage of PCR-amplified heteroduplexed DNA and band visualization using fluorescent end-labeling and denaturing polyacrylamide gel electrophoresis

(Henikoff and Comai 2003; Henikoff et al. 2004). The TILLING approach is working well in *Arabidopsis*, where a relatively high mutation frequency was induced and over 5000 mutations have been identified in more than 400 targeted genes (Cooper et al. 2005). To date, mutants/alleles identified by TILLING have resulted in the identification of six *Arabidopsis* genes, namely *DAWDLE* (Morris et al. 2006), *REVERSION-TO-ETHYLENE SENSITIVITY1* (Resnick et al. 2006), *AtISA3* (Delatte et al. 2006), *Arabidopsis* carotenoid beta-ring hydroxylase (Kim and DellaPenna 2006), *AtWEX* (Li et al. 2005), *ARABIDOPSIS CRINKLY4* (Gifford et al. 2005).

The generality of the mutagenesis and the mutation discovery methods allow application of this approach to most organisms. TILLING can be used to identify allelic series of mutations, including knockouts. Indeed, TILLING can be applied to selected target genes even if genomic sequencing is limited. The high density of chemically-induced point mutations makes TILLING suitable for targeting small genes, and it allows an investigator to focus on single protein domains when targeting larger genes. In contrast to insertional mutagenesis, TILLING is widely applicable, as chemical mutagenesis has been successfully applied to most taxa. Indeed, TILLING results have been reported for a variety of plant and animals (McCallum et al. 2000a, 2000b; Perry et al. 2003; Wienholds et al. 2003; Till et al. 2004; Gilchrist and Haughn 2005; Gilchrist et al. 2005; Slade et al. 2005; Winkler et al. 2005; Wu et al. 2005). Because it is broadly applicable and non-transgenic, TILLING has the potential to become a standard reverse-genetic strategy for plant functional genomics.

8.6 TILLING in Rice

8.6.1 Seattle TILLING Project

The Seattle TILLING Project (<http://tilling.fhrc.org:9366/>) in collaboration with the International Rice Research Institute (IRRI) and the Agricultural Research Station of the US Department of Agriculture at Davis, has been applying the TILLING method to rice. A critical requirement for TILLING is the availability of a mutagenized population with a sufficient density of induced mutations. The estimate of mutation density per Mb of DNA is the single most important determinant of the feasibility of TILLING as an effective reverse-genetic strategy. Although it is possible to TILL (i.e., to find suitable mutations in) a population that has one mutation per Mb of diploid DNA, efficient TILLING requires at least two mu-

tation per Mb. Thus, an important step in TILLING is determining the best dosage of mutagen. Too severe a treatment can cause sterility and non-viability, whereas too mild a treatment results in a low density of mutations and will require more screening to obtain an adequate allelic series. Seed mutagenesis, even repeated treatments under identical conditions, can be variable and different species may require different dosages (for examples see Till et al. 2003a, 2003b). As a result, optimizing mutagenesis may involve multiple attempts using a range of mutagen concentrations, to produce the best trade-off between fertility and mutation rate. Determining the mutation rate is best done by TILLING, and it requires carrying about 800 plants to the M₂ stage before deciding which conditions worked best. This pilot process entails TILLING of 3-6 genes in 768 plants from a test population and it is the only reliable way to estimate a mutation rate. Pilot-scale screening also can identify other factors that might limit the efficiency of high-throughput TILLING, such as insufficient DNA purity.

Rice has proved technically challenging to mutagenize to achieve a sufficient mutation density, although recent efforts to achieve the critical threshold of mutations have been successful (B. Till et al., unpublished data). To date, the Seattle TILLING Project has screened several rice pilot populations that have mutation frequencies lower than what we judge sufficient for a successful TILLING service. Some seed-mutagenized populations of *indica* had a mutation frequency of approximately one per Mb (Wu et al. 2005). A population mutagenized by the floral dip method had a better mutation frequency of approximately 1.7 per Mb (Nori Kurata, Brad Till, Jennifer Cooper et al., unpublished data). Testing of mutagenic treatments is ongoing with *indica* rice at IRRI. A recurrent EMS-mutagenesis scheme has increased the mutation density up to 1.3 per Mb based on screening about 1,600 M₂ plants with 11 genes (F. Qui and H. Leung, unpublished data). Most recently, populations of *japonica* rice mutagenized by Dr Tom Tai at the USDA-ARS of Davis have displayed the best density of mutations measured so far, allowing the isolation of multiple mutants in several tested genes (B. Till, T. Tai et al., unpublished data). It was concluded that the latter populations would be suitable for use in a large-scale TILLING project. Consequently, a scale-up of TILLING libraries derived from these populations is in progress at the UC Davis Genome Center. A public service is anticipated as early as Spring 2007 which will be run from the Genome Center of UC Davis, with seed distribution from the Dale Bumpers Rice Stock Center in Arkansas, and will be modeled on services previously established for *Arabidopsis*, maize and *Drosophila*.

8.6.2 Other Technical Improvements in Rice TILLING

Suzuki et al. (2005) have simplified the TILLING procedure for use in rice by replacing the fluorescence primers with non-labeled primers in PCR amplification, and using the capillary gel electrophoresis with the HAD-GT12 Genetic Analyzer (eGene Inc., CA, USA) that can separate DNA fragments below 2 kb within eight minutes. This modified system could detect SNPs at any DNA regions examined between *indica* and *japonica* varieties and also test-pooled DNA samples with a capability of detecting one heterozygous mutant in a pool of six plants. In such a test screening of 700 M₂ MNU-induced mutant lines for mutations in a 600 bp known intragenic region, they could detect 10 candidate mutant lines, six of which were confirmed by sequencing.

Recently, Raghavan et al. (2006) have optimized an agarose gel method to simplify detection without the specialized equipment (see flow chart in Figure 8.1). The group showed that the SNP detection by agarose gel corresponded perfectly with those based on LiCor genotyper. It was possible to detect mutations in an 8-fold DNA pool. Screening efficiency was also increased by scanning amplicons as large as 3 kb. The real advantage of agarose TILLING is the elimination of the need for labeled primers, which represents a significant cost reduction, making the technique much more affordable for laboratories with modest budgets.

8.6.3 TILLING Case Studies for Specific Traits

TILLING has been advocated as an important tool for agricultural improvement through the identification of new variants and as a means to validate gene function. For wide adoption, however, it is important to know the efficiency of identifying allelic variants that yield detectable phenotypes. The strategy adopted at IRRI has been to focus on a few genes to illustrate the potential of identifying useful variants with agronomically-important phenotypes. The first case deals with screening induced mutations in members of a gene family conditioning disease resistance. The second case concerns the detection of natural variation in a candidate gene with putative function in the drought response pathway. While these experiments are far from complete, they illustrate different challenges and may offer some useful hints to guide future applications.

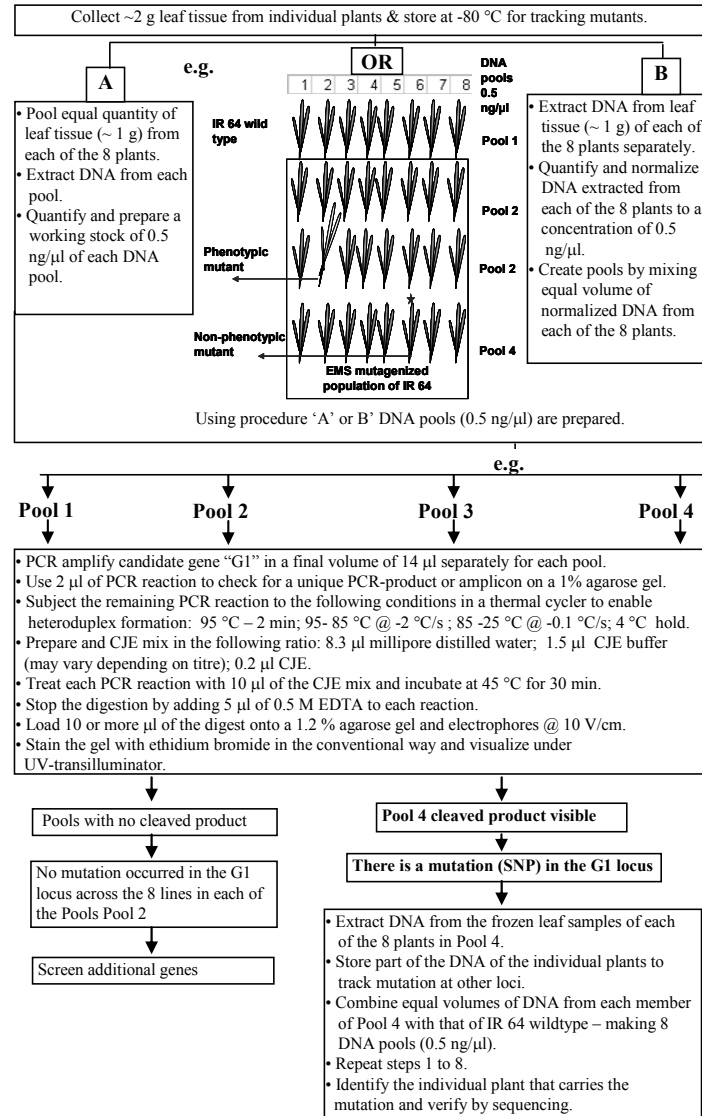


Fig. 8.1. Rice TILLING on agarose gel. The procedure is similar to standard TILLING in terms of DNA pooling, PCR, and CEL1 digestion. The main difference is the use of agarose method for detecting cleaved products. This obviates the need for label primers in PCR and special genotyping platforms. CJE=celery juice extract as a source of CEL1 restriction enzyme

Gene Family Members Associated with Disease Resistance QTL

Several chromosomal regions harboring oxalate oxidase (OXO) and oxalate oxidase-like protein (OXL) have been shown to be associated with quantitative disease resistance in mapping populations (Ramalingam et al. 2003; Liu et al. 2004). The *OsOXO* cluster has four members on Chromosome 3. The *OsOXL* genes are clustered on Chromosome 8 (12 members) and Chromosome 12 (4 members). The 12 members on Chromosome 8 have different expression patterns that do not show an obvious relationship with resistance phenotype expressed in different genotypes (R. Davidson and J. Leach, Colorado State University, USA, unpublished data). Because of the quantitative effect, it is difficult to determine if individual members of the family or combinations of these members confer disease resistance. One approach is to identify mutant alleles in each of the gene members and determine their phenotypes: an ideal problem for TILLING to address.

At IRRI, the simplified agarose method has been adopted for all TILLING operations (Fig. 8.1; Raghavan et al. 2006). From screening approximately 800 M₂ DNA samples, 11 SNPs in the oxalate oxidase genes in a high-dose (2%) EMS population were identified. Of them, five SNPs cause synonymous changes leading to changes in amino acids (Table 8.2). All five SNPs are G/C to A/T transitions consistent with that expected from EMS mutagenesis. Interestingly, while the estimated mutation density for this mutant population is low (about one per Mb), a good number of mutations can be identified in a specific gene family in a relatively-small population of 800 lines. Of the five mutants evaluated for disease response, only one appeared to show reduced resistance in preliminary analysis. This illustrates the need to assemble a large collection of allelic mutations or to combine multiple mutations in a single genotype in order to reveal phenotypic changes.

Table 8.2. Identification of mutations in members of gene families of oxalate oxidase (*OsOXO*) and oxalate oxidase-like protein (*OsOXL*) in an EMS-induced IR64 mutant collection

Gene name	TIGR Locus ID	Chromosome	Mutant line identified	Mutation	Amino acid change
<i>OsOXO-4</i>	LOC_Os03g48780	3	M3E93	C to T	Pro to Leu
<i>OsOXL-7</i>	LOC_Os08g09010	8	M3E715	G to A	Arg to Lys
<i>OsOXL-6</i>	LOC_Os08g09000	8	M3E97	C to T	Ser to Phe
<i>OsOXL-9</i>	LOC_Os08g09040	8	M3E183	C to T	Arg to Val
<i>OsOXL-9</i>	LOC_Os08g09040	8	M3E543	C to T	Pro to Leu

Drought Response Candidate Gene

A modified procedure of TILLING, called EcoTILLING (Comai et al. 2004), was applied to identify natural allelic variants in a gene coding a putative ethylene-responsive element binding protein 3 (ERF3) harboring an AP2 domain at 136.6 cM on Chromosome 1 (Wang 2005). This locus falls within a drought QTL region centered on 146 cM which is correlated with yield components under stress. The genetic variation at this locus was examined in a collection of 905 rice lines of the mini-core germplasm collection at IRRI (Table 8 3). The germplasm collection in essence is considered a large natural mutant bank.

Table 8.3. Summary of nucleotide diversity in ERF3 region based on analysis of 905 rice lines

Nucleotide Polymorphism	5' upstream 847 bp	5' UTR 90 bp	CDS 708 bp	3' UTR 97 bp
SNPs	21 (2.48)	1 (1.11)	5 (0.71)	1 (1.03)
Insertions/deletions	4 (0.47)	0 (0.00)	2 (0.28)	1 (1.03)
Informative sites ^a	15 (1.77)	0 (0.00)	3 (0.42)	2 (2.06)
Total	25 (2.95)	1 (1.11)	7 (0.99)	2 (2.06)

^aInformative sites: nucleotide substitutions resulting in changes in either *cis*-acting factors or amino acids; Figures in parenthesis are % sites polymorphic (i.e., number of polymorphisms/total no. of bp in each region)

The percentage of polymorphic sites shows that 5' upstream region is the most polymorphic among the four screened regions. In this germplasm collection, the average SNP frequency of the ERF3 noncoding region was one SNP per 65 bp and of the coding region (CDS) was one SNP per 141 bp. This frequency is lower than that of maize, where the SNP frequency in US elite inbred germplasm was one SNP per 48 bp in noncoding regions and one SNP per 131 bp in coding regions (Bhatramakki et al. 2002).

The experiment to associate molecular variation to phenotype was done with the drought physiology group at IRRI who assayed drought-response phenotypes of over 400 lines under field and greenhouse conditions. The phenotyping data were then tested for association with SNP haplotype data at the ERF3 locus. Preliminary analysis suggested a positive association between a SNP haplotype and biomass under stress within a collection of

indica germplasm (N =217). Recognizing that multiple loci are likely to be involved, a larger panel of >20 drought response candidate genes is being tested in this collection of germplasm to understand the molecular mechanisms of drought tolerance response (K.L. McNally and E. Naredo, IRRI, unpublished data).

TILLING with Phenotype-enriched Mutant Subsets

Another approach adopted by IRRI researchers is a combination of forward- and reverse genetics wherein a particular phenotype-enriched mutant subset is first selected phenotypically for subsequent TILLING using a small set of genes presumed to be involved in imparting the phenotype in question. As a test case, genes in the starch biosynthesis pathways have been targeted. A large collection of mutants is being screened first for abnormal grains (in an inexpensive phenotypic screen) and then TILLING is being applied using a small set of known genes involved in starch biosynthesis. Adopting this strategy, several mutants have been obtained (Douglas Willoughby and Melissa Fitzgerald, IRRI, unpublished data). Currently, this approach is also adopted in screening for mutations in genes related to small RNA metabolism by using a set of >200 mutant lines with visible morphological variation based on the assumption that most of the genes involved in small RNA metabolism also affect development in either vegetative or reproductive stages (Taeko Sasaki and Jehan Sasongko, IRRI, unpublished data).

8.7 Future Prospects

Now that we have the complete rice genome sequence information, it is instructive to re-read the short essay by Hieter and Boguski (1997) on “Functional Genomics: it’s all how you read it”, published just a year before the rice genome sequencing project was conceived. In their commentary, they pointed out the essence of “functional genomics” which is the “development and application of global (genome-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics”. The new science entails high-throughput and computational analyses and it promises to narrow the gap between sequence information and function and eventually phenotypes. But they also cautioned that “functional genomics, however, will not replace the time-honored use of genetics, biochemistry, cell biology, and structural studies in gaining a detailed understanding of the biological

mechanisms". These comments are as relevant now as they were a decade ago when only the yeast genome was completely sequenced.

With whole genome sequence available, it is theoretically possible to test the relationship between molecular variation and phenotypes at every gene by forward and reverse genetics. A prerequisite to understanding the functions of each gene, and its interactions with other genes, is the identification of biological variants that carry the loci and alleles of interest. Chemical- and irradiation-induced mutants are particularly valuable for understanding gene-phenotype relationship because SNPs and indels represent the majority of genomic variations in natural germplasm. Furthermore, techniques that enable a high-throughput sampling of allelic variation in multiple genes in large collections of mutants or natural germplasm are critical in the post-sequencing era. In this chapter, we have surveyed the present state-of-the-art in chemical and irradiation mutagenesis, resources available to accelerate gene discovery and single nucleotide detection technologies for forward as well as reverse genetics. In this context, TILLING represents a promising tool because it is relatively simple to implement and can serve to identify induced and natural variants in germplasm and mutant collections for almost any crop species.

The main benefit of TILLING lies in its potential to identify a large series of mutations ranging from knockouts to subtle missense mutations. There is already a large collection of insertion and activation lines with flanking sequence tags (FST). The current OryGenes database (<http://orygenesdb.cirad.fr>) has about 80,000 available tagged sequences on the rice genome. On average, there is a 50% success rate of finding tagged mutations of a gene of interest (A. Pereira, personal communication). While the success rate will continue to rise with enlarging mutant populations, mutations discovered by TILLING can fill in the missing gaps by producing a rich allelic series of point/indels mutations. With modest investment, almost any laboratory can conduct TILLING using local mutant populations and germplasm collection. At present, the limitation of TILLING mutant populations is largely biological - the ability to produce a sufficiently-large allelic series such that the desired knockouts or knock-down mutations can be uncovered. Also, there is still a paucity of empirical data to indicate the number of mutations needed for phenotypic evaluation of agronomic traits. Thus, more convincing examples are needed to demonstrate the benefits of TILLING in generating useful diversity in induced mutations.

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