Plant Molecular Biology (2005) 59:85–97
DOI 10.1007/s11103-004-5112-0


# Chemical- and irradiation-induced mutants of indica rice IR64 for forward and reverse genetics

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Received 19 May 2004; accepted in revised form 19 October 2004

*Key words:* functional genomics, mutagenesis, *Oryza sativa,* phenotype database

**Abstract**

IR64, the most widely grown *indica* rice in South and Southeast Asia, possesses many positive agronomic characteristics (e.g., wide adaptability, high yield potential, tolerance to multiple diseases and pests, and good eating quality,) that make it an ideal genotype for identifying mutational changes in traits of agronomic importance. We have produced a large collection of chemical and irradiation-induced IR64 mutants with different genetic lesions that are amenable to both forward and reverse genetics. About 60,000 IR64 mutants have been generated by mutagenesis using chemicals (diepoxybutane and ethylmethanesulfonate) and irradiation (fast neutron and gamma ray). More than 38,000 independent lines have been advanced to $M_4$ generation enabling evaluation of quantitative traits by replicated trials. Morphological variations at vegetative and reproductive stages, including plant architecture, growth habit, pigmentation and various physiological characters, are commonly observed in the four mutagenized populations. Conditional mutants such as gain or loss of resistance to blast, bacterial blight, and tungro disease have been identified at frequencies ranging from 0.01% to 0.1%. Results from pilot experiments indicate that the mutant collections are suitable for reverse genetics through PCR-detection of deletions and TILLING. Furthermore, deletions can be detected using oligomer chips suggesting a general technique to pinpoint deletions when genome-wide oligomer chips are broadly available. $M_4$ mutant seeds are available for users for screening of altered response to multiple stresses. So far, more than 15,000 mutant lines have been distributed. To facilitate broad usage of the mutants, a mutant database has been constructed in the International Rice Information System (IRIS; http://www.iris.irri.org) to document the phenotypes and gene function discovered by users.

## Introduction

Rice is not only the most important food crop but a model plant that has attracted broad interests in basic and applied research. The publication of the draft rice genome (Feng *et al.*, 2002; Goff *et al.*, 2002; Sasaki *et al.*, 2002; Yu *et al.*, 2002) presents exciting opportunities to assign function to each of the estimated 50,000 genes, many of which are potentially useful for improvement of rice as well

as other cereals. To achieve this goal, diverse genetic resources including germplasm, near-isogenic lines, mapping and mutant populations held and developed by rice-growing countries and plant breeding institutions are important to the identification of genetic variation useful for trait improvement.

Among these genetic resources, mutant stocks with discrete genetic lesions are essential to determining gene function and dissecting biochemical and metabolic pathways. Many national and international projects have been active in the production of rice mutants (Hirochika *et al*., 2004; Leung and An, 2004). The most popular approach is insertional mutagenesis using T-DNA and *Ac*/*Ds* insertions (Krysan *et al*., 1999; Jeon *et al*., 2000; Jeong *et al*., 2002; Upadhyaya *et al*., 2002; Greco *et al*., 2003; Wu *et al*., 2003) and transposons (Altmann *et al*., 1995; Hirochika, 2001; Miyao *et al*., 2003). So far, insertion mutations have been produced primarily in *japonica* rice because of the amenability to tissue culture and transformation by *Agrobacterium*-mediated method.

Chemical and ionizing radiation mutagenesis have been routinely used to generate genetic variability for breeding research and genetic studies. More than 2200 crop varieties were released by the end of the last century using irradiation mutagenesis; among them 434 are rice varieties (Maluszynski *et al*., 2000). The morphological mutations also provided genetic markers for the development of linkage maps. However, chemical and irradiation-induced mutants have not been the mainstay of gene identification tools because the mutations are not physically tagged, requiring considerable effort to isolate the gene after a phenotype has been identified. Yet, with high throughput genotyping, the efficiency in detecting genetic polymorphism (point mutations or deletions) has been significantly improved (Borevitz *et al*., 2003; Henikoff and Comai, 2003; Winzeler *et al*., 2003). 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 rice, there are several advantages of using chemical and irradiation mutagenesis to produce mutant populations suitable for both forward and reverse genetics. First, mutant populations can be produced using any genotypes. Second, 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). Third, it provides a large allelic series as a complement to the knockout mutants produced by insertional mutagenesis or transformation methods (over- and under-expression).

We have chosen IR64, the most widely grown indica rice cultivar in Southeast Asia, to produce an *indica* rice mutant collection. The wide adaptability of and large number of valuable agronomic characteristics present in IR64 make it an ideal genotype to develop a comprehensive mutant population. We used four mutagenic treatments to create mutants with a range of sizes in genetic lesions. We describe here the characteristics and attributes of the mutant population and its utility in forward and reverse genetics. We discuss our experience in producing and maintaining this mutant collection in the hope that such information can be useful to others who are interested in launching a large-scale production of mutants in various rice genotypes or other plant species.

## Generation of mutants stocks

### *Seed sources*

In 1998, we began the production of mutants using IR64 breeder seed. The breeder seeds were maintained and propagated under field conditions and selected for phenotypic uniformity. This stock was used for the initial mutagenesis; however, because of concern of heterogeneity in the breeder seeds, we produced a seed stock using progeny derived from a single IR64 plant (designated as IR64-21). This single plant was fingerprinted with 50 SSR markers to establish a reference genotype. From 780 plants from IR64-21, we produced a permanent seed stock that served as the source for all subsequent mutagenesis.

### *Mutagenesis*

Dry seeds were mutagenized by gamma ray (GR) at 250 GY, and by fast neutron (FN) at 33 GY at the International Atomic Energy Agency (IAEA), Austria with the assistance of Drs. F. Zapata, P. Donini, and Rownak Afza. The irradiation dosage was chosen based on previous experience in

mutation breeding programs. The irradiation doses of 250 GY GR and 33 GY FN resulted in approximately 97% and 70% survival of $M_1$ plants, respectively. Treated seeds were directly sown in soil in the greenhouse and advanced by single seed descent. The $M_1$ plants, at maximum tillering, were trimmed to a single main tiller per plant to produce seeds. $M_2$ plants (one $M_2$ per family) were grown in a greenhouse to advance to the next generation. Ten $M_3$ plants per line were grown in the field to produce approximately 0.5 kg seeds per $M_4$ line for storage and distribution.

Diexpoxybutane (DEB) mutagenesis was performed as described previously (Leung *et al*., 2001). Seeds were soaked in aqueous solution of 0.004% or 0.006% DEB in a shaker at 30 °C for 13 h with gentle shaking. The seeds were washed thoroughly with running water for at least 30 min and sown directly and advanced by single seed descent as described above.

Since we were interested in achieving high-density mutation in the ethyl methanesuphonate (EMS)-mutagenized population, we first conducted a kill curve analysis using a wide range of EMS concentrations (0.2–2.0%). Five EMS concentrations (0.4, 0.6, 0.8, 1.0 and 1.6%) were then chosen to generate IR64 mutant sub-populations to observe mutation frequency empirically. Seeds were presoaked in distilled water for 24 h and then treated with EMS solution in a shaker at 28 °C for 12 h with gentle shaking at 60 rpm. Treated seeds were washed thoroughly under running water before sowing into plastic trays.

Since each mutagen may produce different sizes of genetic lesions, we designated our mutants with a letter prefix (D for DEB, G for gamma ray, F for fast neutron, and E for EMS) to indicate how the mutant population was produced. For example, a D1855-1-2 line contains seed from plant number 2 of a $M_3$ family obtained from plant number 1 of a $M_2$ family of a $M_1$ plant designated 1855 obtained by DEB mutagenesis.

## Overall characteristics of IR64 mutant collection

We chose four mutagens capable of producing a spectrum of genetic lesions. EMS is used to produce mainly GC to AT transition (Rao, 1977; Koornneef *et al*., 1982). Since the rice genome can tolerate a large number of point mutations, full genome coverage can be achieved with a relatively low number of EMS mutants. Both DEB and gamma ray are predicted to cause small deletions around one kilobase and point mutations (Recio *et al*., 2001; Lee *et al*., 2002; Wang *et al*., 2004). Fast neutron irradiation is expected to cause large deletions and chromosomal rearrangements (Shirley *et al*., 1992; Okubara *et al*., 1994; Bruggemann *et al*., 1996). Hence a collection of mutants from these treatments should yield variation from point mutations to kilobase-size deletions in the genome, making the collection useful to several genotyping approaches (Table 1).

As of September 2004, over 60,000 $M_1$ IR64 mutants have been produced using these four mutagens. About 4000 mutant lines are advanced every four months. So far, approximately 38,000 lines have been advanced to $M_4$ generation with sufficient seeds available for various screening purposes. Our target is to produce 40,000 $M_4$ lines by end of 2004. We emphasize the need to produce near-homozygous inbred lines because many conditional phenotypes can be observed only by measuring the characters quantitatively in replicated tests. By providing the same mutant lines for multiple-trait screening, we can increase the probability of identifying genes in common pathways.

## Visible mutations as indicators of variability in mutant populations

Because considerable investment is needed to propagate the lines to advanced generations, it is desirable to have an indicator of the mutation density (mutation per megabase) in a batch of mutants in the early generation. We monitored the variability in $M_1$ and $M_2$ generations. Most treated $M_1$ plants exhibited retarded growth at early seedling stage due to the toxic effects of mutagens used, but a majority of them recovered and reached maturity as the wild type. At the vegetative growth stage, the common variation observed in $M_1$ included retarded growth, white leaf stripe, pale green leaf stripe and spotted leaf. Double lemma, low-density spikelets on upper portion of panicles and sterility were also commonly observed at reproductive stage.

To determine whether the variation expressed in $M_1$ plants followed Mendelian inheritance, we

*Table 1.* Current status of IR64 mutants induced by chemical and irradiation mutagenesis.

| Mutagen | Types of mutation | Number of $M_1$ plant mutagenized | Number of lines advanced to $M_4$ | Target population size | Number of lines distributed[a] | Reverse genetics tool |
|---|---|---|---|---|---|---|
| Fast neutron | Large deletions, translocations 1–2 kb | 10,000 | 8073 | 9000 | 4004 | PCR deletion screen |
| Gammy ray | Deletions, point mutation Kilobase-range | 28,000 | 6188 | 20,000 | 485 | PCR deletion screen |
| Diepoxybutane | Deletions, point mutation | 18,000 | 15,226 | 15,000 | 4605 | PCR deletion screen, TILLING |
| EMS | Point mutation | 9000 | 8650 | 20,000 | 7174 | TILLING |
| Total | | 65,000 | 38,067 | 64,000 | 16,268 | |

[a] Includes duplicated lines sent to different users.

observed the segregation pattern of $M_2$ progeny from 63 putative $M_1$ mutants induced by 1.6% EMS. The mutant phenotypes included white leaf stripe, pale green leaf stripe, spotted leaf, retarded growth, low-density spikelet and low fertility. Phenotypes observed in 61 $M_1$ plants did not appear in the $M_2$ generation, indicating that the mutations were not transmitted through the seed. One $M_1$ mutant (E15934) with double lemma and low fertility showed dominant segregation in the $M_2$ progeny. A second mutant showed spotted leaf (E18110) appeared to be inherited in a dominant manner. In general, dominant mutations observed in $M_1$ generation are rare but our assessment could be biased because many dominant mutations could be lethal and escaped detection as many $M_1$ plants died in early seedling stage.

In the $M_2$ generation, we used 30–40 visible phenotypes to survey the variability in the four populations produced by different mutagenesis (Table 2). Albino was the most frequently observed phenotype in all mutagenized populations. Among the four mutagens, 1.6%EMS treatment gave the highest frequency of albino (8.7%). Xantha, lethality, dwarfism, white leaf stripe and chlorina were other frequently observed mutations. The amount of phenotypic variation observed in $M_2$, however, may be underestimated because single seed descent was used to advance the mutants and the full range of variation in $M_2$ family might not be captured. Furthermore, some traits such as heading date and tillering ability were not observable under greenhouse conditions. By planting 10 plants per $M_3$ line in the field, we were able to record the segregation of phenotypes not seen in $M_2$. For example, in the 1.6% EMS population, 5.6% of the $M_3$ lines (189 out of 3400 lines) segregated in visible phenotypes. Due to selection for healthy plants in single seed descent, the frequencies of sub-lethal phenotypes such as albino and xantha greatly decreased in $M_3$ relative to those in $M_2$ (Table 2). In contrast, dwarfism, narrow-leaf, reduced culm number and lesion mimic (spotted leaf) mutants were observed more frequently.

Almost all mutant categories can be found among the four populations produced by different mutagens (Table 2) but the frequencies of some specific mutants varied among the populations. For example, we observed a high frequency of *spl1* (*sp*otted *l*eaf) mutations in DEB, GR, and FN populations but only one *spl1* mutant was identified in the high-dose (1.6%) EMS population. The *Spl1* locus is known to reside on the short arm of chromosome 12 near the centromere (Chazou He, Chinese Academy of Sciences, personal communication). It is unknown whether the location or sequence of the *Spl1* locus affects the sensitivity of the gene to different mutagenic agents.

*Table 2.* Frequency of visible mutations of IR64 produced by different mutagens.

| Phenotypic category[a] | EMS (0.8%) | | EMS (1.6%) | | DEB (0.006%) | | Fast neutron (33 GY) | | Gamma ray (250 GY) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $M_2$ | $M_3$ | $M_2$ | $M_3$ | $M_2$ | $M_3$ | $M_2$ | $M_3$ | $M_2$ | $M_3$ |
| Albino | 6.37 | 3.15 | 8.74 | na | 4.47 | 1.13 | 8.57 | 6.25 | 5.03 | 3.96 |
| Xantha | 0.41 | na | 1.56 | na | 0.60 | na | 0.28 | 0.10 | 0.54 | 0.40 |
| Lethal | 1.44 | 1.40 | 4.51 | 1.18 | 0.80 | 0.07 | 1.11 | 1.51 | 0.60 | 0.53 |
| Dwarfism[b] | 1.44 | 4.81 | 0.55 | 0.74 | 1.53 | 2.79 | 0.98 | 2.50 | 0.40 | 2.50 |
| White leaf stripe | 0.82 | 1.16 | 2.02 | 0.71 | 1.27 | 0.14 | 0.98 | 1.11 | 0.40 | 1.59 |
| Pale green leaf | 0.31 | 0.33 | 0.86 | 0.12 | 0.40 | 0.34 | 0.07 | 0.91 | 0.13 | 0.40 |
| Pale green leaf stripe | 0.31 | 0.39 | 0.37 | 0.12 | 0.07 | 0.14 | na | 0.07 | na | 0.07 |
| White leaf spot | 0.47 | na | 0.15 | 0.06 | 0.20 | na | 0.14 | na | na | na |
| Broad leaf | 0.10 | 0.49 | 0.06 | 0.12 | 0.07 | 0.07 | 0.07 | na | 0.27 | 0.67 |
| Narrow leaf | 0.21 | 2.16 | 0.83 | 0.97 | 0.27 | 0.48 | 0.56 | 2.87 | 0.20 | 2.53 |
| Erect leaf | na | 0.17 | na | 0.06 | na | 0.07 | na | 0.07 | na | 0 |
| Chlorotic upper half | na | na | na | 0.06 | 0.13 | 0.07 | 0.14 | 0 | na | 0.53 |
| Dark green leaf | 0.10 | 0.19 | 0.06 | 0.24 | 0.07 | 0.07 | 0.07 | 0.20 | 0.27 | na |
| Semi-rolled leaf | 0.10 | 0.17 | 0.61 | 0.41 | 0.07 | 0.27 | 0.07 | 0.40 | 0.07 | 0.33 |
| Zebra | na | na | 0.03 | 0.03 | na | 0.07 | na | 0.07 | na | 0.33 |
| Chlorina | 0.41 | 1.40 | 0.86 | 0.21 | 1.47 | 0.07 | 1.95 | 0.07 | na | 0.20 |
| Spotted leaf (*spl*) | 0.10 | 0.66 | 0.12 | 0.74 | 0.20 | 0.48 | 0.28 | 0.93 | 0.07 | 1.33 |
| Twisted leaf | 0 | 0 | 0.18 | 0 | 0.07 | na | 0.14 | na | 0.20 | na |
| Uniculm | na | 0.17 | 0.15 | 0.18 | na | 0.07 | na | 0.33 | na | 0.13 |
| Reduced culm number | na | 1.49 | 0.06 | 0.27 | na | 0.55 | na | 2.02 | na | 0.53 |
| Increased culm number | na | 0.17 | 0.03 | 0.15 | na | 0.14 | na | 0.07 | na | 0.13 |
| Early heading | na | 0.20 | 0.12 | 0.03 | na | na | na | 0.10 | na | na |
| Late heading | na | 0.49 | 0.12 | 0.77 | na | na | na | 0.67 | na | 0.26 |
| Open hull | na | 0.19 | 0.03 | 0.03 | na | na | na | 0.30 | na | 0.07 |
| Golden hull | 0 | 0.17 | 0 | 0 | na | na | na | 0.20 | na | na |
| Purple hull | 0 | 0.17 | 0 | 0.06 | na | na | na | na | na | 0.10 |
| Broad grain | 0 | 0.33 | 0 | 0 | na | na | na | 0.07 | na | 0.13 |
| Awned | na | 0.33 | 0.03 | 0.12 | na | 0.20 | na | 0.50 | na | 0.13 |
| Low density spikelet | 0.41 | 0.17 | 0.03 | 0.03 | na | na | na | na | na | na |

na = Data not available.
[a] Each trait is based on observation of 750–3400 independent mutant lines.
[b] Dwarfism includes dwarf and semi-dwarf.

## Mutant database

We have developed a database 'Mutant View' in the International Rice Information System (IRIS; http://www.iris.irri.org) that presents the description of mutants identified from visual inspection of the mutant lines planted in the field and from multiple stress screens. This database also serves as a portal for users to request materials. The mutant database consists of two main components: an in-house Laboratory Information Management System (LIMS) and the public website accessible via http://www.iris.irri.org. The in-house system is implemented as a MySQL relational database consisting of five tables (Figure 1). It serves as the laboratory and field notebook to store data regarding mutagen used, familial relationship, phenotypes, seed availability and other information from field evaluation. After each cropping season, data from field observations are curated and then exported to the public WWW website. The current database view allows query using a hierarchical tree of controlled vocabulary terms. Currently, we have over 3000 mutants in the database described by over 90 distinct phenotype trait values, many with digital images. The range of information in the database will be expanded as more functional and molecular data are returned from investigators evaluating the mutants.

A controlled vocabulary (CV) for mutant phenotype and data collection method is used for data entry. Phenotype CV is a collection of

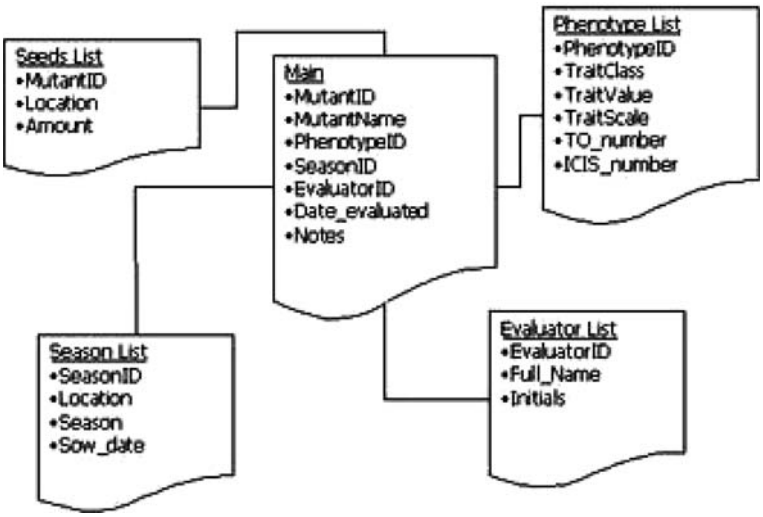


*Figure 1.* Database schema and Laboratory Information Management System for IR64 mutants.

trait-value-scale terms obtained from previous rice mutant publications, the Standard Evaluation System (SES) descriptions, and from *Tos17* phenotype CV used by the laboratory of Hirohiko Hirochika at National Institute of Agrobiological Sciences, Japan (http://www.nias.affrc.go.jp). We also include novel descriptions of mutations not yet published. We are in the process of mapping the IR64 mutant phenotype CV to the phenotype ontology standards of public initiatives such as the Plant Ontology Consortium (http://www.plantontology.org) and the NIAS *Tos17* phenotype vocabulary, so that terms originating from each CV can be used to search for comparable mutants across other public mutant databases using internet protocols such as the BioMOBY web services technology (http://www.biomoby.org). As results of genotypic characterization of mutants become available in the future, such information will also be cross-linked to the mutant stocks.

## Forward Genetics

### *Phenotyping*

From the screening of morphological mutants based on 35 phenotypic categories, we found an average of 8.3% visible mutants across the four populations as observed in $M_2$ and $M_3$ generations (Figure 2). It is more difficult to estimate the variation for conditional traits because of the differences in experimental conditions. Table 3 shows the identification of altered response to three diseases (blast, bacterial blight, tungro virus) and brown plant hopper. Both gain and loss of resistance mutants were recovered. The data suggest a mutant detection rate of 0.1% for a broad category of traits such as altered disease resistance but a low frequency of 0.02% for a highly specific trait, such as a change in response to tungro viruses (P. Cabauatan and I. Choi, unpublished data). These frequencies are similar to those observed in *Arabidopsis*. Thus, the data suggest that the mutation population harbors a large amount of genetic variability that can be revealed when the mutants are subjected to appropriate phenotypic screens. So far, around 15,000 mutant lines have been distributed to users within and outside of IRRI for screening a variety of phenotypes, including response to salinity and drought, phytic acid content, sensitivity to plant hormones, and non-host resistance.

### *Detection of genomic changes using genome-wide chip*

Although abundant mutations and phenotypic variability exist in the IR64 mutant collection, it is essential to increase the efficiency with which mutated genes can be isolated. Borevitz *et al.* (2003) described the use of *Arabidopsis* oligonucleotide (oligo) chip to detect single feature polymorphisms (SFP) in ecotypes of *Arabidopsis*. Based on the same concept, Chang *et al.* (2003) reported preliminary results on using the Syngenta GeneChip®,

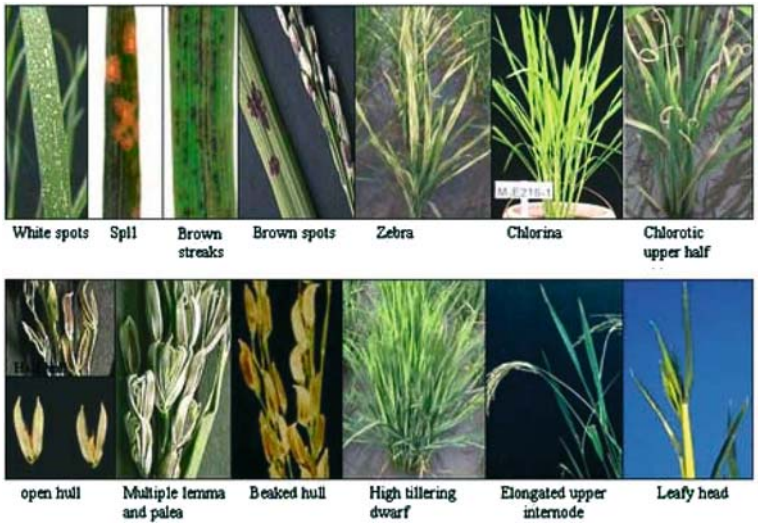


*Figure 2.* A sampling of IR64 morphological mutants observed in $M_2$ and $M_3$ generations. Additional mutant images and descriptions are available at http://www.iris.irri.org.

*Table 3.* Frequency of conditional mutants with altered response to biotic stresses identified in disease and insect screens.

| Trait | Altered response relative to IR64 | | Number of mutant lines screened | Mutant recovery (%) |
|---|---|---|---|---|
| | Loss of function | Gain of function | | |
| Resistance to blast only | 1 | 3 | 2271 | 0.18 |
| Resistance to bacterial blight only | 2 | 5 | 6341 | 0.11 |
| Resistance to both blast and bacterial blight[a] | 0 | 2 | 5337 | 0.04 |
| Resistance to tungro viruses (RTSV and RTBV)[b] | 1 | 1 | 18,449 | 0.01 |
| Resistance to brown planthopper[c] | 2 | 1 | 2251 | 0.13 |

[a] Not including lesion mimics mutants.
[b] I. R. Choi, unpublished data. RTSV, Rice Tungro Spherical Virus; RTBV, Rice Tungro Bacilliform Virus.
[c] P. Kadirvel, M. Cohen, M. Maheswaran, and H. Leung, unpublished data.

which contains short 24- mer oligos representing 24,000 rice genes, to detect genetic lesions in rice deletion mutants. Genes/probes that generate hybridization signals fivefold below those of the wild type (based on significant *t*-test) will be considered candidate genes falling within the deleted regions. Full sequences of the candidate genes are then used to design primers to amply the fragments in wild type and mutant DNA to obtain additional supportive evidence for the deleted genes.

Mutants containing mutations in two known genes were selected to test the gene chip approach. The first was a dwarf mutant, which contains a deletion in *d1* (AB028602) encoding a heterotrimeric G protein (Ashikari *et al*., 1999). This mutant was selected from GR-induced mutants. The second set of mutants contained deletions in the bacterial blight resistance gene *Xa21*. These mutations were induced by diepoxybutane and fast neutron mutagenesis of 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 (C. Wu *et al*., unpublished data). The GeneChip® arrays successfully detected the single copy *d1* gene. However, the result for detecting a known deletion at the *Xa21* locus was ambiguous due to cross hybridization between members of the gene family.

The ability to detect deletions using oligo arrays depends on both the completeness of the genome coverage and the size and position of the deletions relative to the oligos designed from a gene. The Syngenta GeneChip® experiments and Southern hybridization analyses indicate that many of the deletions in our gamma ray and fast neutron-induced lines are greater than 1 kb. Since large deletions (>100 kb) may include multiple genes, the precision of gene identification would be reduced. Another potential problem associated with the chip-based technique is the high number of mutations per genome that may complicate the interpretation of the hybridization signals. It may be necessary to backcross interesting mutant lines to IR64 to reduce background mutations that give positive hits on the chip but are not relevant to the target gene. Alternatively, an allelic series can help pinpoint the gene. If an allelic series is not available, it may be possible to use pooled DNA from segregants with common phenotypes (analogous to bulked segregant analysis) as a means to 'mask' the irrelevant genetic lesions. Thus, we expect broad utility of the mutant collection for gene discovery when complete genome rice oligonucleotide chips are widely accessible.

## Reverse genetics

### *PCR screening*

The concept of detecting deletions by high-throughput DNA screen was first developed in nematode (Liu *et al*., 1999), in which a primer pair defining a target gene was used to screen DNA pools of deletion strains. Li *et al*. (2001) applied a similar approach to detect deletions in *Arabidopsis* mutants induced by fast neutron, and an example of detecting a deletion in a rice gene was included in the study to illustrate the generality of the PCR screening approach. We have developed a modified pooling and detection method to detect mutations in the IR64 mutants. The optimal pool size for detection of mutant rice line DNA was determined by performing reconstruction experiments using a deletion mutation in the *Xa21* gene of rice line IRBB24. One variant in pools of DNA from 200 wild type plants was detected. Based on this information, we developed pools of 1:100 using DNA from 8500 DEB and FN mutants (Manosalva *et al*., 2003).

To identify rice lines with deletions in disease defense response genes, information on more than 100 different putative defense genes (cDNA and genomic sequence, where available) was gathered by database searches. Based on this information, defense response gene primers were designed to be either gene-specific (e.g., usually to the untranslated regions of the genes) as well as gene-family-specific (e.g., to conserved regions of gene family members). Using PAL (phenylalanine ammonia lyase) gene family-specific primers we found a mutant line that exhibited variant bands after PCR amplification. Nested PCR was performed to confirm the polymorphisms occurred in the mutant line but not in IR64 or other mutant lines. The two PCR fragments that co-segregated with the PAL deletion were cloned and sequenced; the data suggest that the deletions occurred in two different PAL family members. Since some family members of PAL are predicted to function in disease defense responses, we are currently determining if the PAL mutations result in altered defense responses. The PCR-based screening technique is being used to screen for more defense response gene mutants and for other types of mutations as requested by collaborators.

### *TILLING*

A high-throughput reverse genetic strategy called TILLING (Targeting Induced Local Lesions IN Genomes) (McCallum *et al.,* 2000a, b; Till *et al*., 2003) has been successfully used for study of the EMS mutant population. We first produced four small IR64 mutant populations using 0.4%, 0.6%, 0.8% and 1.0% EMS. DNA was isolated individually from the $M_2$ plants, pooled (8 genotypes per pool), and subject to screening. In collaboration with University of Washington (B. Till and L. Comai) and Fred Hutchinson Cancer Research Center (S. Henikoff), we screened DNA from approximately 2000 lines from the 0.8% and 1.0% EMS population. Of 10 genes screened so far, independent mutations were detected in two genes: *pp2A4* encoding serine/threonine protein phosphatase catalytic subunit and *cal7* encoding callose synthase, yielding a mutation density of approximately 0.5 mutation per Mb. Sequencing of the

mutated loci confirmed that they were G/C to A/T transition mutations (B. Till, personal communication). In a new population mutagenized with a higher dose of EMS (1.6%), we found an increased mutation density of 1 mutation per Mb. Although a high mutation density is desirable to increase the efficiency, the preliminary data suggest that TILLING can be a robust strategy for identifying an allelic series for any gene of interest.

## Allelic series for gene validation and relating genetic variation in germplasm

As research in rice functional genomics advances, we anticipate many candidate genes will be awaiting confirmation of function. Complementation by transformation is often considered the standard test for confirming function of a gene. However, transformation is not yet routine for some rice genotypes (particularly if they are defective mutants). Alternative means are necessary to confirm the identity of a candidate gene. The allelic series generated in the IR64 mutant collection together with other mutant collections (Hirochika *et al*., 2004) will prove very useful in future. For example, we have used several independent mutant alleles of the *Spl11* locus to confirm that the gene encodes a U-box E3 ubiquitin ligase (Zeng *et al*., 2004). Similarly, a large series of *Spl1* mutants (over 10 independent mutations) has been used to delimit the deletion within a 70 kb region on chromosome 12 (Liu *et al*., 2004).

In addition to gene identification, an allelic series caused by point mutations or indels (induced by EMS and DEB) can be useful to relate to natural variation in the rice gene pool. By comparing the allelic series in knockouts, SNP, and indels across mutants and natural germplasm, it may be possible to infer functional polymorphism as related to phenotypes observed in mutants and germplasm.

## Experience from producing and maintaining the mutant stock

### *Sensitivity of rice genotypes to mutagensis*

During the course of the study, we mutagenized other genotypes with EMS to screen for specific traits. We mutagenized four additional rice genotypes – IR231 (*indica*), DX236 (*japonica*), Jinbubyeo (*japonica*), and Jalmagna (*japonica*) and observed differences in sensitivity to EMS among the varieties. The EMS kill curves indicated that IR231 and Jinbubyeo were more sensitive to EMS treatments than DX236 and IR64 based on survival rate at 21 days (Figure 3A). At 1.2% EMS, almost all seedlings of IR231 and Jinbubyeo were killed while about 30% of IR64 and DX236 survived. When mutagen response was measured as $M_1$ sterility (average seed set per panicle relative to untreated plants), DX236 showed greater sensitivity than IR64 (Figure 3B). DX236 is a japonica variety from Korea known to exhibit cold tolerance. It was reported that fertility of *japonica* genotypes was in general more sensitive than *indica* genotypes (Rao, 1977). The lower seed set in DX236 could be due to genotypic response to the high temperature in the Philippines. The range of response among different rice genotypes should be considered in the production of additional mutant populations.

### *Mutagen doses vs. mutation density*

For the purpose of identifying mutated genes, we 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 a very large population in a single attempt. As shown in Figure 3, there is a dramatic reduction in survival of $M_1$ plants at EMS concentration higher than 1.6%. Fertility measured as seed set in $M_1$ plants was negatively correlated with dosage ($r = -0.99$) within the range of 0.2–1.0% EMS treatments.

High mutation density also may give rise to an excessive number of mutations in the genome. From the oligo-hybridization experiments, we have estimated that over 100 mutations could be present in each genome of the chemical or irradiation-induced mutants. Although the chance of inducing genes affecting a similar function is small, it is important to backcross a mutant to the wild type to remove background mutations before conducting in-depth analysis. Producing a useful mutant population therefore requires a balance between the need to produce high-density

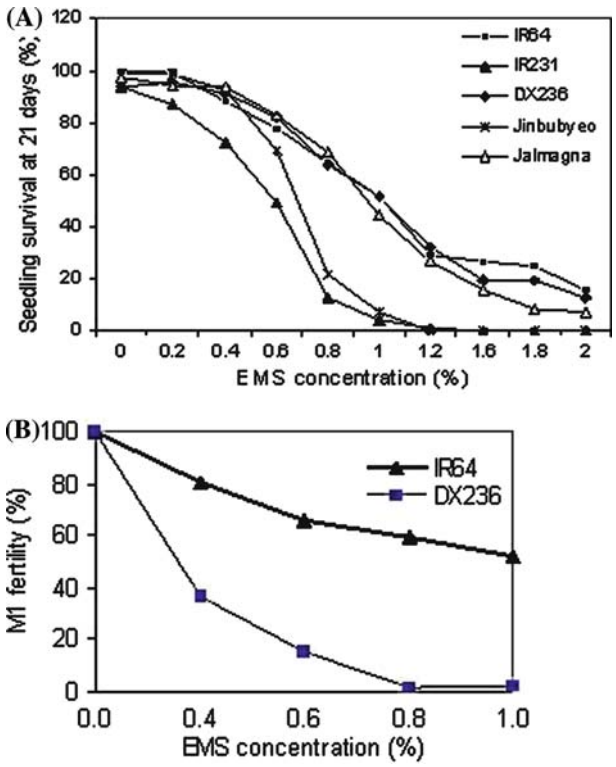


*Figure 3.* Genotypic differences of 21-day-old seedling in response to EMS concentrations. (A) Kill curves of five genotypes suggesting two levels of sensitivity. (B) Fertility of $M_1$ plants between two genotypes IR64 and DX236. Fertility measured as seed set per panicle relative to untreated populations.

mutations and the practicality of keeping a vigorous population without too many deleterious effects.

*SSR genotyping for quality control of seed stock*

Quality control is important for producing and maintaining a mutant stock at a large scale. Rice is a self-pollinating plant but occasionally outcrossings by unwanted pollen may occur especially when a mutant plant is partially or completely male sterile. However, it is not practical to have pollen control by bagging panicles in the greenhouse or the field. We have taken a retroactive approach to assure quality. We genotype the original IR64 parent with 12 SSR markers (RM151, RM266, RM251, RM335, RM334, RM204, RM320, RM264, RM278, RM333, RM224, RM101) which together identify 96 SSR alleles among common rice genotypes across the 12 linkage groups (fingerprint pattern available upon request). We use this set of markers routinely to test genotypes of mutants under study. So far, nearly all morphological mutants tested have the same SSR pattern as wild IR64; however, for conditional mutants where selection pressure is high to identify variants (such as screening for disease susceptible mutants), we observed contaminated seeds. Thus, as a standard procedure, putative mutants identified with an interesting phenotype are subject to SSR fingerprinting to ensure they are from the original IR64 seed stock. We consider this approach as a cost-effective way to maintain quality control of the mutant stock.

## Distribution

After harvesting from the field, seeds are dried to about 14% moisture, hand-cleaned, and packed in paper bags. After fumigation provided by the Seed Health Unit at IRRI, the envelopes are packed in

airtight plastic boxes kept dry with silica gel. The working collection is kept in at 22–24 °C in an air-conditioned room. Seeds kept under this condition remain viability for over three years. As long-term storage, we are in the process of keeping a small amount of $M_4$ seeds (50 seeds) of each line in aluminum foil bags at 4 °C in IRRI's International Germplasm Bank. The seeds are available upon request to IRRI (contact persons: H. Leung, h.leung@cgiar.org or Mayette Baraoidan, m.baraoidan@cgiar.org). We normally provide 50–100 seeds per line based on user's need.

## Conclusions

We have produced a large *indica* rice mutant collection as a public resource for gene discovery. The wide adaptability and unique agronomic attributes of IR64 make this mutant stock particularly useful for rice-growing countries in the tropics. Ability to evaluate the mutants in replicated tests under field conditions will be increasingly important in order to identify conditional traits that express only under agronomically relevant conditions. The value of the IR64 mutant collection will increase with increasing usage and extensive testing under a wide range of conditions.

Collectively, the rice mutants already produced (and being developed) in different countries represent perhaps the largest mutant collection in crop plants. By systematically searching for phenotypes (forward genetics) and selectively phenotyping for predicted characters in mutants identified by reverse genetics, we can close the gap between genotypes and phenotypes. This is one of the main objectives of the International Rice Functional Genomics Consortium (www.IRIS.IRRI.org/IRFGC). We anticipate growing collaboration among researchers to maximize the use of the mutant resources to accelerate gene discovery in rice.

## Acknowledgments

We thank P. Donini, F. Zapata, and R. Afza of International Atomic Energy Agency, Vienna, for conducting fast neutron and gamma ray mutagenesis, and S. Henikoff and L. Comai for their collaboration in TILLING projects. We thank the Swiss Agency for Development and Cooperation for supporting the establishment of an IR64 Mutant Bank, USDA and NSF for supporting characterization of the mutants, and Rockefeller Foundation for providing postdoctoral fellowships to C. Wu and C. Lei.

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