Mutant Resources in Rice for Functional Genomics of the Grasses[W]

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Rice (*Oryza sativa*) is the reference genome for the grasses, including cereals. The complete genome sequence lays the foundation for comparative genomics to the other grasses based on genome structure and individual gene function (Devos, 2005; International Rice Genome Sequencing Project, 2005). The basic complement of monocot genes in rice can be examined by functional genomics studies because of the many advantages of rice as a system for genetic analysis, as well as the worldwide development of resources.

The analysis of mutants by forward and reverse genetics approaches is an effective way to study gene function. Knockout (KO) mutations, which abolish gene expression and display a phenotype, provide a direct causal relationship between the gene sequence and its biological function. However, not all gene mutations display a KO mutant phenotype, primarily due to gene redundancy, because plant genomes have been shown to have large segmental genomic duplications, as well as tandem duplications of gene families (Yu et al., 2005; Sterck et al., 2007). In many cases, the redundancy is partial or unequal, due to overlap in expression of duplicated genes (Briggs et al., 2006), or the gene activity is required only under some specific conditions, such as biotic/abiotic stresses where the mutant phenotype can be observed.

The use of molecular tags or DNA insertions, such as transposons or T-DNA, is favored for mutations because their genome positions can be easily monitored to determine the correlations between tagged genes and phenotypes. The limitations in identifying gene functions by KO mutations alone are resolved by employing heterologous DNA insertions with engineered properties to monitor the expression of tagged genes using entrapment vectors or to alter the expression of tagged genes using activation tagging (Pereira, 2000).

The International Rice Functional Genomics Consortium, combined with many national programs, set a goal to generate mutant resources toward discovering the function of all rice genes, primarily through reverse genetics approaches (Hirochika et al., 2004). This resource update describes the generation of over 200,000 insertion flanking sequence tags (FSTs), which tag two-thirds of the predicted protein-coding genes, with one-half of the protein-coding genes estimated to have knockout mutations. The insertion sequences comprise the endogenous *Tos17* retrotransposon showing the highest insertion rate in exons, as well as engineered maize (*Zea mays*) *Ds/dSpm* transposons and *Agrobacterium* T-DNA, which function as enhancer trap (ET), gene trap (GT), or activation tags (ATs). In addition, chemical and physical mutagen-derived mutant populations have been developed that are available for TILLING and other high-throughput screens. The extensive number and variety of mutant resources described here for rice are very amenable for dissecting the functions of genes of interest in other grasses.

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DEVELOPMENT OF RICE MUTANT RESOURCES

Insertion Mutants

With the sequencing of plant genomes, it was recognized that insertion mutants indexed by their position in the genome would be very suitable for systematic analysis of annotated genes by reverse genetics (Parinov and Sundaresan, 2000). Arabidopsis (*Arabidopsis thaliana*) genome sequence-indexed mutants can now be found in databases (http://signal.salk.edu/Source/AtTOME_Data_Source.html), which comprise a total of 379,674 inserts tagging 30,280 of the predicted 33,003 genes.

In rice, the two-component maize transposon *Ac-Ds* (Chin et al., 1999; Upadhyaya et al., 2002; Greco et al., 2003; Kolesnik et al., 2004) and *En/Spm-dSpm* (Greco et al., 2004; Kumar et al., 2008) systems have been well characterized, and good genetic selection schemes have been developed to select for transpositional activity useful for large-scale mutagenesis (Hirochika et al., 2004; Zhu et al., 2007). In addition, the endogenous rice *Tos17* retrotransposon is active in specific genotypes and conditions and is an effective insertion mutagen in the rice genome (Miyao et al., 2003). The development of efficient protocols for rice transformation has helped in the generation of a large number of transgenic rice plants bearing low-copy T-DNA insertions (Jeon et al., 2000; Sallaud et al., 2003).

In addition to KO or loss-of-function mutagenesis, the engineering of transposon and T-DNA constructs offers immense flexibility in fashioning the insertion sequences to detect adjacent gene expression or activate the expression of adjacent genes by activation tagging, resulting in gain-of-function mutations. These modified insertions can contribute to gene function discovery of redundant genes and those having lethal mutant effects.

Gene Entrapment

To facilitate the analysis of genes based on their expression patterns, GT and ET constructs have been designed that carry a reporter gene and can display the expression pattern of an adjacent trapped gene (Sundaresan et al., 1995). The reporter gene pattern in ET inserts reflects the adjacent plant gene enhancer activity, and in GT inserts the adjacent gene promoter activity. ET and GT constructs have been used in both T-DNA and *Ac-Ds* transposons in rice, yielding interesting gene expression patterns and entrapped genes, which support their widespread generation and use for complementing KO mutagenesis (Hirochika et al., 2004; An et al., 2005).

Activation Tagging

T-DNA AT populations have been developed using vectors with cauliflower mosaic virus 35S enhancer multimers, the inserts characterized by FSTs and phenomic data for forward and reverse genetics screens (Jeong et al., 2002; Chern et al., 2007; Hsing et al., 2007; Wan et al., 2009). Recently, an *Ac-Ds* AT system has also been developed (Qu et al., 2008) using convenient markers for selection of multiple transposants from a few starter transformed lines. In both these AT systems, activation of adjacent genes is observed, albeit 52.7% of the T-DNA lines and 20.8% of the *Ds* tags activate adjacent genes, which can be as far away as 10 kb from the AT enhancer.

Chemical and Physical Mutagenesis

Chemical agents, such as ethyl methanesulfonate (EMS), methyl nitrosourea, and diepoxybutane, or physical methods like fast-neutron, γ-rays, and ion beam irradiation can cause a high density of mutations that can saturate the genome (Hirochika et al., 2004). Mutant populations have been generated in rice in which the point mutations can be screened by TILLING and larger deletions by PCR-based screens (Wu et al., 2005; Till et al., 2007). The IR64 (Wu et al., 2005) and the Nipponbare (Till et al., 2007) populations, as well as other unpublished populations also shown in Table I, offer different backgrounds and mutation spectrum. The IR64 mutant collection comprises a total of 66,891 mutant lines in the M4 generation. Of these, about 15,000 are γ-ray-induced mutants, each carrying 30 to 40 deletions per genome, thus contributing to a conservative estimate of over 500,000 mutations in the collection (H. Leung, unpublished data). Screening for mutations in such populations can be done using genome-wide chips or other high-throughput genotyping technologies. These populations and/or the DNA pools are presently available to screen for specific genes on a small scale.

UTILITY OF MUTANT RESOURCES FOR FUNCTIONAL GENOMICS IN RICE

Forward and Reverse Genetics in Rice

The first rice genes identified by insertional mutagenesis were with *Tos17* in a forward genetics screen for viviparous mutants (Agrawal et al., 2001), and simultaneously in a reverse genetics screen for inserts in phytochrome A genes (Takano et al., 2001). With T-DNA, genes were identified by forward screens (Jung et al., 2003), by reverse genetics PCR-based screens for mutations in specific genes (Lee et al., 2003), as well as expression-based GT screens (Kang et al., 2005). Likewise, the maize *Ac-Ds* transposon system also yielded tagged genes (Zhu et al., 2003, 2004).

Because the complete genome sequence became available, the generation of FST information of mutant populations has made the mutants more accessible to address biological questions. Table I shows the different mutant populations available and the FSTs that can be screened for inserts in genes of interest. Such queries can be made in silico, thus providing a conve-

Table I. *Mutant resources, contributors, and databases*

DEB, Diepoxybutane; EMS, ethyl methanesulfonate; CIRAD, Centre de Coopération Internationale en Recherche Agronomique pour le Développement; CNRS, Centre National de la Recherche Scientifique; INRA, Institut National de la Recherche Agronomique; IPMB, Institute of Plant and Microbial Biology; IRD, Institut de Recherche pour le Développement; IRRI, International Rice Research Institute; NIAS, National Institute of Agrobiological Sciences; POSTECH, Pohang University of Science and Technology; SIPP, Shanghai Institute of Plant Physiology and Ecology.

Institution	Genotype	Mutagen	Mutated Loci	FSTs/ Screen	FST Lines Availability[a]	Database Web Site	Contact
CIRAD-INRA-IRD-CNRS, Génoplante, FR	Nipponbare	T-DNA ET *Tos17*	45,000 100,000	14,137 13,745	17,414 11,488 (March 2009)	http://urgi.versailles.inra.fr/OryzaTagLine	E. Guiderdoni guiderdoni@cirad.fr
CSIRO Plant Industry, AU	Nipponbare	*Ac-Ds* GT/ET	16,000	611	Approximately 50% lines no seed	http://www.pi.csiro.au/fgrttpub	N.M. Upadhyaya narayana.upadhyaya@csiro.au
EU-OSTID, EU	Nipponbare	*Ac-Ds* ET	25,000	1,380	1,300	http://orygenesdb.cirad.fr/	E. Guiderdoni guiderdoni@cirad.fr
IRRI, PH	IR64	Fast neutron γ-ray DEB, EMS	500,000	Deletion database: 400 genes	Selected lines[b]	http://www.iris.irri.org/cgibin/MutantHome.pl	H. Leung h.leung@cgiar.org
Gyeongsang National University, KR	Dongjin Byeo	*Ac-Ds* GT	30,000	4,820	4,820	KRDD http://www.niab.go.kr/RDS	C.-D. Han cdhan@nongae.gsnu.ac.kr
NIAS, JP	Nipponbare	*Tos17*	500,000	34,844	34,844	http://tos.nias.affrc.go.jp	H. Hirochika hirohiko@nias.affrc.go.jp
NIAS, JP	Nipponbare	γ-ray ion beam	15,000 M2 7,000 M2	DNA pools	Selected lines		M. Nishimura nisimura@affrc.go.jp
POSTECH, KR	Dongjin, Hwayoung	T-DNA ET/AT *Tos17*	150,000 400,000	84,680	58,943	RISD http://an6.postech.ac.kr/pfg	G. An genean@postech.ac.kr
Huazhong Agricultural University, CN	Zhonghua 11 Zhonghua 15 Nipponbare	T-DNA ET	113,262 14,197 1,101	16,158	26,000 Dec. 2008	RMD http://rmd.ncpgr.cn	Q. Zhang qifazh@mail.hzau.edu.cn
SIPP, CN	Zhonghua 11	T-DNA ET	97,500	8,840	8,840 FST + 11,000 lines	http://ship.plantsignal.cn/home.do	F. Fu ship@sibs.ac.cn
Temasek Lifesciences, SG	Nipponbare	*Ac-Ds* GT	20,000	3,500	2,000		R. Srinivasan sri@tll.org.sg
IPMB, Academia Sinica, TW	Tainung 67	T-DNA AT	30,000	18,382	31,000	TRIM http://trim.sinica.edu.tw	Y.C. Hsing bohsing@gate.sinica.ed.tw
University of California, Davis	Nipponbare	*Ac-Ds* GT *Spm/dSpm*	20,000	*Ds* 4,735 *dSpm* 9,469	4,630 9,036	http://www-plb.ucdavis.edu/Labs/sundar	V. Sundaresan sundar@ucdavis.edu
University of California, Davis	Nipponbare	Sodium azide + MNU	6,000	TILLING screen	Selected lines	http://tilling.ucdavis.edu	L. Comai lcomai@ucdavis.edu
Zhejiang University, CN	Nipponbare Zhonghua 11	T-DNA		1,009	1,009	http://www.genomics.zju.edu.cn/ricetdna	P. Wu clspwu@zju.edu.cn
Zhejiang University, CN	Kasalath SSBM	γ-ray EMS	40,000		Selected lines	http://www.genomics.zju.edu.cn	P. Wu clspwu@zju.edu.cn

[a]Based on searching current project database, future plans, and subject to seed availability. [b]Selected lines from gene-specific PCR screens.

nient way to assess mutant populations around the world. The *Ds* and *dSpm* insertions are generated by transposition from a few starter transformed lines that can be scaled up for genome saturation and do not directly result from a regeneration process (Kolesnik et al., 2004; van Enckevort et al., 2005; Upadhyaya et al., 2006; He et al., 2007; Park et al., 2007; Qu et al., 2008). The FST resource of the endogenous *Tos17* retrotransposon are generated by regeneration process in Nipponbare (Miyao et al., 2003), which also accompanies *Agrobacterium* transformation of T-DNA yielding additional *Tos17* insertions (Piffanelli et al., 2007). The T-DNA insertions with FSTs in various genetic backgrounds comprise an extensive diverse resource (Chen et al., 2003; Sallaud et al., 2004; Jeong et al., 2006; Zhang et al., 2006; Hsing et al., 2007).

The generation of insertions accompanied by a regeneration phase, such as for T-DNA and *Tos17*, can result in a high frequency of untagged mutations in the background that can complicate genetic analysis of the mutants (H. Leung and E. Guiderdoni, unpublished data). To alleviate this problem, genetic segregation analysis and the use of multiple mutants of the gene are useful. Transposon inserts have a much lower

frequency of background mutations, leading to many genes identified by forward screens (Zhu et al., 2007), and the use of reversions that restore the wild-type phenotype is a convenient approach to prove gene-phenotype relationships.

Resources and Databases for Reverse Genetics

To facilitate the identification of insertion mutations in genes using available FST information, a number of project database Web sites are available as shown in Table I. In addition, functional genomics databases are available, such as RiceGE/SIGnAL (http://signal.salk.edu/cgi-bin/RiceGE), OryGenesDB (http://orygenesdb.cirad.fr), and Gramene (http://www.gramene.org), where the FST information has been collated and mutants can found for inserts in genes of interest. These databases link rice genes to other grass genes and thus direct functional queries to the rice mutant resources.

Properties of Insertion Mutants

We compiled 206,668 insertion FSTs from our contributing groups, which comprise 180,639 unique hits in the genome (Supplemental Table S1). The different insertion types (*Tos17*, T-DNA, *Ds*, *dSpm*) show differences in their specificity, with *Tos17* showing the highest proportion of insertions in exons (Fig. 1). A remarkably large proportion of all the inserts (62.5%) are in genic regions, including 5′ and 3′ regions, as described in Figure 1. However, many genes have multiple different insertions, with a total of 32,459 genes containing inserts out of the total 56,985 (56.9%) nuclear genes with assigned locus IDs. Among the 41,753 predicted protein-coding (see Supplemental Materials and Methods S1) rice genes, 28,545 (68.4%) have inserts in the genic region. Assuming that the most probable insertions to produce KO mutations would be those in exons, introns, and the 5′-untranslated region, the insertions were recalculated to be 21,239 (50.8%) in the protein-coding genes (Supplemental Table S1). One of the major reasons for a low frequency of insertions in genes is the actual target size, with around 13,000 genes of 1-kb size showing only around 35% bearing insertions (Supplemental Fig. S1). The insertion mutants found for the rice annotated genes, defined by the GO-slim biological process (10,232 total) and molecular function (12,765) categories (Supplemental Figs. S2 and S3), reveal an even distribution of >90% in total genic region and around 80% in the critical KO mutation target region. This reveals that a high proportion of mutations in annotated genes would most probably cause KO mutants, while the frequencies in the unannotated genes is relatively lower. However, some genes annotated to be involved in pollen-pistil interaction and pollination biological processes have a lower than expected number of mutations in the coding regions (Supplemental Fig. S3).

FUTURE DEVELOPMENT

The size of rice mutant populations generated is large and diverse to suit many functional genomics objectives in the grasses. The number of insertion mutants needed to tag every gene in rice has been estimated to be between 180,698 to 460,000 (Hirochika et al., 2004). At present, the positions of over 200,000 inserts have been determined by FSTs with KO mutations predicted for 50% of the protein-coding genes.

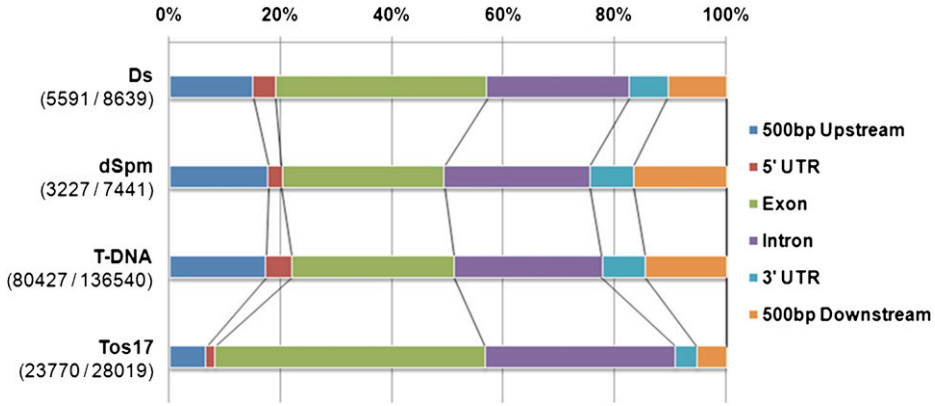

Figure 1. Distribution of insertion positions within genic regions in rice. The three classes of insertion mutagens: endogenous *Tos17* retrotransposon, *Agrobacterium* T-DNA, and maize cut-and-paste transposons (*Ds* and *dSpm*), shown with their insertion positions in different parts of genes with color code shown alongside. The numbers of individual insertion types in genes in relation to the total number of insertions are entered below the insertion name. Datasets from the following resources (Table I) were used: *Ds*, CSIRO, KRDD/GNU, OSTID, UCD; *dSpm*, UCD; *T-DNA*, CIRAD, POSTECH, RMD, SHIP, TRIM, ZJU; *Tos17*, CIRAD and NIAS. 500-bp upstream and downstream regions correspond to sequences upstream and downstream of the transcription unit (start site to site of termination). See Supplemental Table S1 for more information.

Thus, mutants of the remaining genes are required, many of them smaller genes with a lower mutation frequency. Although the total available mutants are >2,000,000 (Table I), the cataloging of the mutants by FSTs is limiting because of the manual manipulations and costs involved. However, new methods of high-throughput sequencing of multidimensional DNA pools should be able to assess the genome positions in a more cost-effective way. In addition, the chemical/physical mutagen-derived mutants would be accessible by the next generation high-throughput genotyping technologies. For those genes still inaccessible to mutation, probably due to small size, lethality, or genome position, more directed gene-specific methods using RNAi silencing would be very useful.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Gene size and distribution of insertion mutations.

Supplemental Figure S2. GO-slim molecular function categories of all tagged genes.

Supplemental Figure S3. GO-slim biological process categories of all tagged genes.

Supplemental Table S1. Genomic distribution and statistics of rice inserts.

Supplemental Materials and Methods S1. Datasets and analysis.

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