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Review

Natural and artificial mutants as valuable resources for functional genomics and molecular breeding

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Abstract

With the completion of rice genome sequencing, large collection of expression data and the great efforts in annotating rice genomes, the next challenge is to systematically assign functions to all predicted genes in the genome. The generations and collections of mutants at the genome-wide level form technological platform of functional genomics. In this study, we have reviewed currently employed tools to generate such mutant populations. These tools include natural, physical, chemical, tissue culture, T-DNA, transposon or gene silencing based mutagenesis. We also reviewed how these tools were used to generate a large collection of mutants and how these mutants can be screened and detected for functional analysis of a gene. The data suggested that the current population of mutants might be large enough to tag all predicted genes. However, the collection of flanking sequencing tags (FSTs) is limited due to the relatively higher cost. Thus, we have proposed a new strategy to generate gene-silencing mutants at the genome-wide level. Due to the large collection of insertion mutants, the next step to rice functional genomics should be focusing on functional characterization of tagged genes by detailed survey of corresponding mutants. Additionally, we also evaluated the utilization of these mutants as valuable resources for molecular breeding.

Key words: Functional Genomics; Molecular Breeding; Mutagenesis; Mutants; Rice

Introduction

Functional genomics is the branch of genomics that determines the biological function of genes and their products. Both *Arabidopsis* and rice plants have been regarded as model organisms for dicots and monocots, respectively [1]. Now both *japonica* and *indica* rice genomes have been completely sequenced [2-4]. With the sequencing of rice genome, gene prediction /annotation has been carried out. Various annotation databases were set up and were freely available for public researchers. One of these databases is RiceGAAS (http://ricegaas.dna.affrc.go.jp/; [5]). The second one is the Rice Annotation Project Database RAP-DB (http://rapdb.dna.affrc.go.jp/; [6]). The third is the TIGR rice genome annotation database (now moved to Michigan State University (MSU); http://rice.plantbiology.msu.edu/; [7, 8]). The releasing of these databases has been significantly contributing to the research of rice functional genomics. As a result of ongoing annotation efforts, predicted gene numbers continue to be changed [9]. More than 50,000 genes were predicted upon publication of its draft sequence [2, 3]. Subsequently, 40,612 non-transposable element-related genes were predicted by the MSU rice genome annotation project. However, 37,544 genes were predicted to be protein-coding genes [4]. Now only 30,000 or less protein-coding genes were estimated [10]. The large differences in the annotated gene numbers suggest the necessary to further validate these annotated genes by various experimental approaches. Such necessity was

strengthened by the fact that at least 40% of predicted *Arabidopsis* genes were wrongly annotated based on subsequent validation by experiments [11]. Besides the efforts in genome-wide gene prediction, many rice gene families were also annotated by individual researchers. For example, we have identified and characterized members from 5 gene families at the genome-wide level. These included 14 rice myosin gene family members [12], 114 pollen-allergen-like genes [13], 49 rice cyclin genes [14], 111 small GTPase genes and 85 genes encoding small GTPase activating proteins [15] and 17 GRAM domain containing proteins [16]. These works together with other community annotation of rice gene families (http://rice.plantbiology.msu.edu/ca/rice_ca.shtml) significantly contributed to and complemented genome-wide gene annotations.

Additionally, large amount of rice Expressed Sequence Tags (EST) data are available in public databases including the MSU (http://rice.plantbiology.msu.edu/), NCBI (http://www.ncbi.nlm.nih.gov/dbEST/index.html), Gramene (http://www.gramene.org/Oryza_sativa_japonica/index.html) databases and so on. For example, until October 29, 2009, total of 1,249,001 EST sequences have been released into NCBI database (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). In addition to these, more than 32,000 full-length cDNA clones from *japonica* rice have been sequenced [17] (http://cdna01.dna.affrc.go.jp/cDNA/) and 10,096 *indica* full-length cDNA clones were also released [18] (http://www.ncgr.Ac.cn/cDNA/indexe.html).

With the completion of rice genome sequencing, large collection of expression data and great efforts in annotating rice genomes, the next challenge is to systematically assign functions to all predicted genes in the genome. To broadly assign functions to unknown genes, various old approaches are improved and new methods are developed. The different methodologies have been developed to form their own fields within the functional genomics technological platform and are termed transcriptomics, proteomics, metabolomics and phenomics [19]. However, all tools to identify functions of genes are based on the analyses in phenotypic variations between wild type and its mutant. Therefore, the generations and collections of mutants at the genome-wide level form the technological platform of functional genomics.

On the other hand, during long breeding history, farmers and breeders have been selecting new rice varieties with better agronomic traits such as higher yield, improved resistance to various diseases and better quality of grains and so on. These varieties were developed by altering the genetic makeup of the crop. Therefore, genetic variation is the basis of breeding selection. The variation may be produced by natural and artificial mutations as well as sexual crossing. Among the hundreds and thousands of variations, elite germplasms may be developed, which form the important resource for rice breeding. The evidence has shown that a breakthrough might be achieved when such germplasms have been found and used for rice breeding practice. For example, the rice yield has been greatly improved by the utilization of dwarf germplasm [20]. Similarly, other important germplasms such as cytoplasmic male sterile lines and photoperiod/temperature-sensitive male sterile lines have led to the development of various hybrid rice combinations, which have further improved crop yields by 20-30% when compared with the conventional varieties [21]. Therefore, it is very important for us to collect, to generate, and to evaluate rice germplasms for better serving rice breeding.

In this review, we will focus on the collections and characterizations of large rice mutants generated from various methods of mutagenesis such as maize two-element *Ac*/*Ds* system and T-DNA insertion mutagenesis and so on. We also review the applications of these tools and mutants in identifying gene functions and in rice breeding.

Natural Mutagenesis and Map-based Cloning

Natural mutants were generated during species evolution. Generally, the ratio of natural mutation is very low at only 10^{-5}-10^{-8} in higher plants. However, a large collection is still available during long evolutionary history. Some of such mutants were harmful or neutral and might be lost during evolution. Others might exhibit higher resistance to various abiotic / biotic stresses or have some specific agricultural traits, which were valuable germplasm resources for rice breeding. One example is the utilization of dwarf germplasm Dee-geo-woo-gen from China and release of rice variety IR8, which was developed from the dwarf line [22]. Another example is the application of cytoplasmic male sterile (CMS) and photoperiod-sensitive genic male sterile rice lines, which are widely utilized to develop hybrid rice seeds for commercial release [23].

Table 1. Some of rice genes functionally characterized through mapped-based cloning.

Gene Name	Protein description	Functions/Descriptions	References
SPL28	clathrin-associated adaptor protein complex 1	involved in the regulation of vesicular trafficking	New Phytol. (2009) 185: 258-274
OsCSLD4	cellulose synthase-like D4	cell-wall formation and plant growth	Plant J. (2009) 60: 1055-1069.
EP3	F-box protein	Involved in vascular bundles and parenchyma in the peduncle	Theor Appl Genet. (2009) 119: 1497-1506
TID1	alpha-tubulin protein	dwarfism and right helical growth in rice	Genes Genet Syst. (2009) 84: 209-218.
OsCESA4	Cellulose synthase	cell wall biosynthesis	Plant Mol Biol. (2009) 71: 509-524.
D88	esterase	affecting architecture of rice plant	Plant Mol Biol. (2009) 71: 265-276.
HTD2	hydrolase	negatively regulating tiller bud outgrowth	Planta. (2009) 230: 649-658.
qPE9-1	the keratin-associated protein 5-4 family member	an integral role in regulation of rice plant architecture including panicle erectness	Genetics. (2009) 183: 315-324.
MER3	a ZMM protein	required for normal meiotic crossover formation, but not for presynaptic alignment in rice	J Cell Sci. (2009) 122: 2055-2063.
NYC3	an alpha/beta hydrolase-fold family protein	regulation of chlorophyll degradation	Plant J. (2009) 59: 940-952.
EP	keratin-associated protein 5-4	conferring high grain yield in rice	Theor Appl Genet. (2009) 119: 85-91.
DLT	a new member of the GRAS family	plays positive roles in brassinosteroid signaling in rice.	Plant J. (2009) 58: 803-816.
OPB	knox transcription factor	a positive regulator of class B floral homeotic gene	Plant J. (2009) 58: 724-736.
OsVPE1	cysteine protease	plays a crucial role in the maturation of rice glutelins	Plant J. (2009) 58: 606-617.
Pi5-1 and Pi5-2	coiled-coil-nucleotide-binding-leucine-rich proteins	required for rice Pi5-mediated resistance to M. oryzae	Genetics. (2009) 181: 1627-1638.
SLR1	DELLA protein	dwarf phenotype	Mol Genet Genomics. (2009) 281: 223-231.
Pikm	NBS-LRR containing protein	required to confer Pikm-specific rice blast resistance.	Genetics. (2009) 180: 2267-2276.
BC10	a DUF266-containing and Golgi-located type II membrane protein	required for cell-wall biosynthesis in rice	Plant J. (2009) 57: 446-462.
Ehd2	zinc finger transcription factor	promotes flowering by up-regulating Ehd1	Plant Physiol. (2009) 148: 1425-1435.
ALK	soluble starch synthase II (SSSII)	controls the gelatinization temperature of rice	Sci China C Life Sci. (2003) 46: 661-668.
qLTG3-1	unknown protein	controlling low-temperature germinability in rice	Proc Natl Acad Sci U S A. (2008) 105: 12623-12628.
S5	aspartic protease	a major regulator of the reproductive barrier and compatibility of indica-japonica hybrids	Proc Natl Acad Sci U S A. (2008) 105: 11436-11441
RL9	GARP protein	regulates the leaf abaxial cell fate in rice	Plant Mol Biol. (2008) 68: 239-250.
Nal1	unknown protein	affects vein patterning and polar auxin transport	Plant Physiol. (2008) 147: 1947-1959.

Gene Name	Protein description	Functions/Descriptions	References
OsCYT-INV1	alkaline/neutral invertase	root cell development and reproductivity in rice	Planta. (2008) 228: 51-59.
NAAT1	nicotianamine aminotransferase	iron accumulation	Plant Physiol. (2007) 145: 1647-1657.
Pi37	nucleotide binding site leucine-rich repeat protein	related to blast resistance	Genetics. (2007) 177: 1871-1880.
Du1	pre-mRNA processing (Prp1) family member	regulates starch biosynthesis through affecting the splicing of Wxb pre-mRNAs in rice	Plant Mol Biol. (2007) 65: 501-509.
OsGluRS	glutamyl-tRNA synthetase	related to thermo-sensitive chlorophyll deficient	Planta (2007) 226: 785-795.
YGL1	the Chl synthase	chlorophyllide esterification in chlorophyll biosynthesis	Plant Physiol. (2007) 145: 29-40.
Pi36	a rice coiled-coil nucleotide-binding site leucine-rich protein	confers race-specific resistance to the blast fungus	Genetics (2007) 176: 2541-2549.
NYC1	chloroplast-localized short-chain dehydrogenase/reductase	involved in light-harvesting complex II and grana degradation during leaf senescence	Plant Cell (2007) 19: 1362-1375.
LAZY1	a novel and unique protein	gravity signaling	Plant Cell Physiol. (2007) 48: 678-688.
Pi2	protein with a nucleotide-binding site and leucine-rich repeat (LRR) domain	resistance to Magnaporthe grisea	Mol Plant Microbe Interact. (2006) 19: 1216-1228.
FON4	a small putatively secreted protein	regulates apical meristem size in rice	Plant Physiol. (2006) 142: 1039-1052.
CYP81A6	cytochrome P450	confers resistance to two different classes of herbicides	Plant Mol Biol. (2006) 61: 933-943.
Chl1 and Chl9	ChlD and ChlI subunits of Mg-chelatase	chlorophyll synthesis and chloroplast development	Plant Mol Biol. (2006) 62: 325-337.
Pi-d2	a receptor-like kinase	conferring rice blast resistance	Plant J. (2006) 46: 794-804.
GH2	a primarily multifunctional cinnamyl-alcohol dehydrogenase	synthesize coniferyl and sinapyl alcohol precursors in rice lignin biosynthesis	Plant Physiol. (2006) 140: 972-983.
EUI	cytochrome P450 monooxygenase	gibberellins metabolism	Plant Cell (2006) 18: 442-456.
Pi9	a nucleotide-binding site-leucine-rich repeat protein	blast resistance	Genetics (2006) 172: 1901-1914.
EUI1	cytochrome P450 monooxygenase	gibberellins metabolism	Plant Cell Physiol. (2006) 47: 181-191.
SKC1	a member of HKT-type transporters	involved in regulating K(+)/Na(+) homeostasis under salt stress	Nat Genet. (2005) 37: 1141-1146.
NiR	ferredoxin-nitrite reductase	rice tissue cullture regeneration	Proc Natl Acad Sci U S A. (2005) 102: 11940-11944.
HTD1 /OsCCD7	CCD protein	negative regulation of the outgrowth of axillary buds	Planta (2005) 222: 604-612.
qUVR-10	cyclobutane pyrimidine dimer (CPD) photolyase	ultraviolet-B resistance	Genetics (2005) 171: 1941-1950.
D11	a novel cytochrome P450 (CYP724B1)	brassinosteroid biosynthesis	Plant Cell (2005) 17: 776-790.

Gene Name	Protein description	Functions/Descriptions	References
D3	an F-box leucine-trich repeat (LRR) protein	controlling axillary bud activity	Plant Cell Physiol. (2005) 46: 79-86.
CPT1	NPH3 family member	required for phototropism of coleoptiles and lateral translocation of auxin	Plant Cell (2005) 17: 103-115.
Spl11	a U-box/armadillo repeat protein	a negative regulator of plant cell death and defense with E3 ubiquitin ligase activity	Plant Cell (2004) 16: 2795-2808.
ACE1	polyketide synthase/peptide synthetase	signals pathogen attack to resistant rice	Plant Cell (2004) 16: 2499-2513.
Xa26	an LRR receptor kinase-like protein	conferring resistance to Xanthomonas oryzae pv. oryzae in rice	Plant J. (2004) 37: 517-527.
Rf-1	PPR motif containing protein	fertility restorer	Plant J. (2004) 37: 315-325.
PLA1	cytochrome P450, CYP78A11	a timekeeper of leaf initiation in rice	Proc Natl Acad Sci U S A. (2004) 101: 875-880.
D2	cytochrome P450, CYP90D	catalyzes the steps from 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone and from teasterone to 3-dehydroteasterone in the late BR biosynthesis pathway	Plant Cell (2003) 15: 2900-2910.
BC1	COBRA-like protein	the biosynthesis of the cell walls of mechanical tissues	Plant Cell (2003) 15: 2020-2031.
Rf-1	pentatricopeptide repeat-containing protein	promotes the processing of aberrant atp6 RNA of cytoplasmic male-sterile rice	FEBS Lett. (2003) 544: 99-102.
Spl7	a heat stress transcription factor	A rice spotted leaf (lesion-mimic)	Proc Natl Acad Sci U S A. (2002) 99: 7530-7535.
Hd6	alpha subunit of protein kinase CK2	involved in photoperiod sensitivity	Proc Natl Acad Sci U S A. (2001) 98: 7922-7927.
IAA28	the Aux/IAA family member	lateral root development	Plant Cell (2001) 13: 465-480.
Hd1	a homolog of CONSTANS in Arabidopsis	the promotion of heading under short-day conditions and in inhibition under long-day conditions	Plant Cell (2000) 12: 2473-2484.
Pi-ta	cytoplasmic receptor proteins of the nucleotide binding site (NBS) class	rice blast resistance	Plant Cell (2000) 12: 2033-2046.
Dwarf 1	the alpha-subunit of GTP-binding protein	gibberellin signal transduction	Proc Natl Acad Sci U S A. (1999) 96: 10284-10289.
Pib	nucleotide binding site (NBS) and leucine-rich repeats (LRRs)-containing protein	rice blast resistance	Plant J. (1999) 19: 55-64.
Xa1	nucleotide binding site (NBS) and leucine-rich repeats (LRRs)-containing protein	bacterial blight-resistance	Proc Natl Acad Sci U S A. (1998) 95: 1663-1668.
Xa27	R-gene protein	disease resistance	Nature (2005) 435: 1122-1125.
xa13	MtN3/saliva family member	conferring disease resistance against bacterial blight	Genes Dev. (2006) 20: 1250-1255.

Map-based cloning is a widely-used method to isolate genes using such mutants. Genetic analysis is the first step to use these mutants for identifying gene functions, by which we know that how many genetic loci control the mutated phenotype. The next step is to finely map these loci to rice genome and then to clone the mutated gene confirmed by genetic complementation experiments. In fact, many genes have been

isolated and functionally characterized by using natural mutant lines. For example, the rice *Xa21* and *Xa27* gene, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6, was isolated by map-based cloning from a natural mutated rice variety [24, 25]. Another such example is the isolation of rice semidwarf gene *sd-1*, which encodes a gibberellin 20-oxidase [26]. Up to now, at least 67 rice genes have been isolated and functionally characterized by the map-based cloning (Table 1).

Physical Mutagenesis and Deleteagene Detecting System

In 1930, Muller observed that mutation could be induced by X-rays [27]. Subsequent researches found that the most efficient mutagenesis was mediated by fast neutron bombardment [28]. A short deletion of DNA fragment was usually observed following the bombardment. Thus, a truncated gene might be detected by genomic subtraction and its functions could be identified by corresponding mutant phenotype. One example is the isolation and characterization of *Arabidopsis ga1-3* gene [29]. Currently, a new reverse genetics method has been developed to identify and isolate such mutants [30, 31]. This method was named as Deleteagene. In this system, DNA samples were extracted from the fast neutron-treated plants and were used to screen for deletion mutants by polymerase chain reaction (PCR) using specific primers flanking the targeted genes. Li et al. (2001) has generated an *Arabidopsis* population of 51,840 lines by fast neutron mutagenesis [30]. This library was then used for screening deletion mutants of 25 gene loci, among which deletion mutants were obtained for 21 (84%) gene loci. Similarly, they also generated a rice fast neutron mutant pool with 24,660 lines and similar method was successfully used for identification and isolation of targeted genes. Evidence showed that this method can be efficiently used for the identification of small genes or tandemly arrayed genes [30]. Wu et al. (2005) reported the generation of around 10,000 rice mutant lines by the fast neutron bombardment and around 20,000 lines by γ-ray [32]. Since the establishment of the method, many genes have been isolated and functionally characterized including the phytochrome family gene PHYC [33] and phytochrome-interacting transcription factor *PIF3* [34], 3 genes encoding TGA transcription factors TGA2, TGA5, TGA6 [35] and so on.

Chemical Mutagenesis and Tilling Detecting System

Chemical mutagenesis is mediated by certain chemical reagents. One of the most frequently used reagents is ethyl methane sulfonic acid (EMS). This alkylating agent can efficiently induce chemical modification of nucleotides, which results in various point mutations including nonsense, missense and silent mutations, among which silent mutations can not generate any modification in phenotype and thus can not be used for mutagenesis. In *Arabidopsis*, EMS mainly induces C to T changes resulting in C/G to T/A substitutions and at a low frequency, EMS generates G/C to C/G or G/C to T/A transversions by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors [36]. Based on codon usage in *Arabidopsis*, the frequency of EMS-induced stop codon and missense mutations has been calculated to be ~5% and ~65%, respectively [37].

In *Arabidopsis*, at least 125,000 M1 lines should be generated in order to achieve saturation of EMS mutagenesis [38]. However, it is not difficult to produce such a population since viable seeds can be used for EMS treatment. The difficulty is how to detect single-nucleotide polymorphisms or substitutions in these mutation lines in a large scale. Based on the technology in detecting single-nucleotide polymorphisms [39, 40], McCallum et al (2000) established a new detecting method named as TILLING (Targeting Induced Local Lessions In Genomes) [37, 41] complemented with denaturing high-performance liquid chromatography (DHPLC). These technologies allow chemically induced mutant pools to be used for reverse genetics. With help of automation, robust and rapid detection makes it possible to screen a wide range of mutant pools in a short time and to avoid the laborious process of forward genetic screening [42, 43]. Now the technology has been used in various species including animals and plants and some improved methods were also provided [44-51].

In rice, around 18,000 and 9,000 mutants were generated from diepoxybutane and EMS mutagenesis, respectively [32]. Total of 10 genes were screened using TILLING and independent mutations were detected in two genes: *pp2A4* encoding serine/threonine protein phosphatase catalytic subunit and *cal7* encoding callose synthase, suggesting the feasibility of this screen method in chemical mutagenesis. In another report, they screened 10 genes including *Os1433* (LOC_Os02g36974), *OsBZIP* (LOC_Os01g64000), *OsCALS8R* (LOC_Os01g55040), *OsDREB* (LOC_Os01g07120), *OsEXTE* (LOC_Os10g33970), *OsMAPK* (LOC_Os07g38530) *OsPITA* (LOC_Os12g18360), *OsR1A* (LOC_Os05g41290), *OsRPLD1* (LOC_Os01g07760) and *OsTPS1* (LOC_Os02g44230). Independent mutants were detected for all 10 genes [52]. They also

found that multiple nucleotide changes can be detected in each gene [52], suggesting that they have developed a useful method for more reliable and exact functional identification of a gene.

Agrobacterium Transferred DNA (T-DNA) Mutagenesis and Functional Characterization of Rice Genes

With the development of high efficient *Agrobacterium*-mediated transformation of rice [53], T-DNA mutagenesis has become a major method to generate a large collection of insertion mutants. Generally, T-DNA can be randomly and stably inserted into plant genome, which made it possible to generate a population saturated with insertions, i.e. having at least one insertion in each gene [54]. In *Arabidopsis*, at least 225,000 independent T-DNA insertion lines have been created that represent near saturation of the gene space; the precise locations were determined for more than 88,000 T-DNA insertions, which resulted in the identification of mutations in more than 21,700 of the approximately 29,454 predicted *Arabidopsis* genes [55].

In rice, several research groups have contributed to the generation of T-DNA insertion lines. For example, An's group has generated approximately 100,000 insertion lines [56, 57]. Around 42,000 T-DNA insertion lines have been generated by Zhang and Wu's group [58, 59]. Recently, Hsing et al. (2007) have reported the generation of 55,000 T-DNA insertion lines [60]. Several other groups also independently produced T-DNA insertional mutant lines in rice [61-63]. According to the previous reports, the average copy number of T-DNA inserts per line is 1.4-2.0 [64]. Thus, more than 450,000 T-DNA tags have now been generated in rice (Fig. 1A). Recent progresses on the generation of T-DNA insertion lines have been reviewed by several researchers [65-67]. If there are only 30,000 or less protein-coding genes in rice genome [10], these populations are large enough to find a knockout in a given gene, assuming that T-DNA is randomly inserted into a chromosome. This suggestion was strengthened by the fact that T-DNA have been observed to insert preferentially in gene-rich regions [58-59, 68-70].

After T-DNA insertion, various phenotypes have been observed including changed growth rates, different plant statues, pollen and seed fertility and so on [67, 71-74]. Those visible differences in phenotypes could significantly contribute to the identification of gene functions. Since the establishment of T-DNA insertion populations, at least 43 genes have been functionally characterized by T-DNA insertion mutants (Table 2). For example, a knockout line of *OSMADS3* by T-DNA insertion shows homeotic transformation of stamens into lodicules and ectopic development of lodicules in the second whorl near the palea where lodicules do not form in the wild type but carpels develop almost normally [75]. Their data show that this gene plays a crucial role in regulating stamen identity.

Transposon Mutagenesis and Its Application on Functional Identification of Rice Genes

Maize transposon *Ac* and *Ds* elements have been successfully used as insertion mutagens for rice insertion mutagenesis. For obtaining stable insertion lines, a two-element *Ac*/*Ds* tagging system has been established. In this system, two different transgenic parental lines were used for sexual crossing. One parental line contains *Ac* element, in which *Ac* is immobilized and provides only *Ac* transposase under the control of 35S promoter. Another parental line is transgenic *Ds* plant, in which *Ds* is also non-autonomous element and provides only two wings of *Ds* element (5′ *Ds* and 3′ *Ds*). Thus, in both *Ac* and *Ds* parental lines transposon *Ac* or *Ds* can not mobilize by themselves. However, after crossing between *Ac* and *Ds* plants, *Ds* element will be mobilized and inserted into different genome positions under the presence of *Ac* transposase. In the next generation, these lines containing only *Ds* element and without *Ac* transposase were selected. Therefore these *Ds* insertional lines will be stable since the plants contained no *Ac* transposase. Besides *Ac* and *Ds*, other transposons such as *En* and *Spm* were also used to generate transposon insertion mutants [76].

Currently, multiple research groups have been greatly contributed to the large collection of transposon insertion mutants and various databases have been set up for better utilization of these resources (Fig. 1B; [65-67, 76-84]). Totally, more than 153,000 transposon insertion lines have been generated, providing valuable resources for the survey of functional genomics.

Table 2. Some of rice genes functionally characterized through T-DNA insertion mutants

Gene Name	Protein description	Functions/Descriptions	References
COW	A new member of the YUCCA protein family	Required for maintaining water homeostasis and an appropriate root to shoot ratio	Plant Mol Biol. (2007) 65: 125-136.
FON1	LRR receptor kinase	Controling vegetative and reproductive development by regulating shoot apical meristem size	Mol Cells (2006) 21: 147-152.
GLR3.1	Glu receptor-like protein	the maintenance of cell division and individual cell survival in the root apical meristem	Plant Cell (2006) 18: 340-349.
OsAGO7	Argonaute (AGO) family protein	Leaf development	Planta (2007) 226: 99-108.
OsAT1	Similar to acyltransferase	Related to disease resistance	Plant Mol Biol. (2007) 63: 847-860.
OsCAO1 and OsCAO2	Chlorophyll a oxygenase	Chlorophyll b biosynthesis	Plant Mol Biol. (2005) 57: 805-818.
OsCHLH	the largest subunit of the rice Mg-chelatase	Chlorophyll biosysthesis	Plant Cell Physiol. (2003) 44: 463-472; J Biol Chem. (2004) 279: 6874-6882.
OsCP1	Cysteine protease	Pollen development	Plant Mol Biol. (2004) 54: 755-765.
OsGA20ox1	Gibberellin (GA) 20-oxidase	Regulation of plant stature	Plant Mol Biol. (2005) 55: 687-700.
OsGLU1	Membrane-bound endo-1,4-beta-D-glucanase	Plant internode elongation	Plant Mol Biol. (2006) 60: 137-151.
OsGNA1	Glucosamine-6-P acetyltransferase	maintaining normal root cell shape	Plant Physiol. (2005) 138: 232-242.
OsGSK1	Glycogen synthase kinase3-like protein	Stress signal transduction and floral development	Plant Mol Biol. (2007) 65: 453-466.
OsHMA9	P1B-type ATPase family protein	A metal efflux transporter	Plant Physiol. (2007) 145: 831-842.
OsLFL1	B3 DNA-binding domain-containing transcription factor	Regulating Flowering time	J Plant Physiol. (2007) 165: 876-885.
OsLG1	SBP domain containing transcription factor	Controlling ligule and auricle development	Plant Mol Biol. (2007) 65: 487-499.
OSMADS3 and OSMADS58	C-class MADS box protein	Determinacy of the floral meristem	Plant Cell (2006) 18: 15-28.
OsMADS50	MADS-box protein	Flowering activator	Plant J. (2004) 38: 754-764.
OsPHYB	Phytochrome B	Negative regulator of brassinolide-regulated growth and development	Plant Cell Environ. (2007) 30: 590-599.
OsPPDKB	Pyruvate orthophosphate dikinase	Modulating carbon metabolism during grain filling	Plant J. (2005) 42: 901-911.
OsRRM	Spen-like protein	Regulating cell development in rice endosperm	Cell Res. (2007) 17: 713-721.
OsSSIIIa	Starch synthase III	Starch synthesis in endosperm	Plant Cell Rep. (2007) 26: 1083-
RIP1	WD40 repeat protein	A regulator of late pollen development	Plant Cell Physiol. (2006) 47: 1457-1472.
Udt1	bHLH transcription factor	Maintaining tapetum development	Plant Cell (2005) 17: 2705-2722.
Wda1	Integral membrane protein	Involving in Cuticle and Wax Production in Rice Anther Walls and Is Required for Pollen Development	Plant Cell (2006) 18: 3015-3032.
OsCPL1	CTD phosphatase-like	development of the abscission layer and seed shattering	Plant J. 2009 doi: 10.1111/j.1365-313X.2009.04039.x
OsPE	Hypothetical protein	multiple embryos	Funct Integr Genomics (2009) DOI 10.1007/s10142-009-0139-6

Gene Name	Protein description	Functions/Descriptions	References
OsEF3	nematode responsive protein-like protein	affect root development and kilo-grain weight by delaying cell division or cell elongation	Plant Biol (Stuttg) (2009) 11: 751-757.
OsBC1L	COBRA-like protein	as a regulator controlling the culm mechanical strength	Plant Mol Biol. (2009) 71: 469-481.
HTD2	hydrolase	negatively regulating tiller bud outgrowth	Planta. (2009) 230: 649-658.
OsCBT	calmodulin-binding transcription factor	act as a negative regulator on plant defense	Mol Cells (2009) 27: 563-570.
OGR1	pentatricopeptide repeat-DYW protein	essential for RNA editing in rice mitochondria	Plant J. (2009) 59: 738-749.
ETR2	ethylene receptor	floral transition and starch accumulation	Plant Cell (2009) 21: 1473-1494.
PAIR3	coiled-coil motifs containing protein	homologous chromosome pairing and synapsis in meiosis	Plant J. (2009) 59: 303-315.
OsMRP5	ABC transporter gene 5	Phytic acid metabolism in rice seeds	Theor Appl Genet. (2009)119: 75-83.
OsIAA1	a member of rice Aux/IAA family	auxin and brassinosteroid hormone responses and plant morphogenesis	Plant Mol Biol. (2009) 70: 297-309.
OsATG10b	an autophagosome component	plays an important role in the survival of rice cells against oxidative stresses.	Mol Cells (2009) 27: 67-74.
GF14c	14-3-3 protein	acts as a negative regulator of flowering in rice by interacting with the florigen Hd3a	Plant Cell Physiol. (2009) 50: 429-438.
FC1	cinnamyl-alcohol dehydrogenase	controls culm mechanical strength in rice	Plant Mol Biol. (2009) 69: 685-697.
OsALDH7	aldehyde dehydrogenase7	maintaining seed viability by detoxifying the aldehydes generated by lipid peroxidation	Plant Physiol. (2009) 149: 905-915.
Os-CASTOR and Os-POLLUX	ion channel proteins	indispensable for mycorrhizal symbiosis in rice	Plant Physiol. (2009) 149: 306-317.
RID1	Cys2/His2-type zinc finger transcription factor	a master switch from vegetative to floral development in rice	Proc Natl Acad Sci U S A. (2009) 105: 12915-12920.
PDF1B	peptide deformylase	OsPDF1B is essential for the development of chloroplast	Plant Cell Physiol. (2009) 49: 1536-1546.
WSL1	beta-ketoacyl CoA synthase	biosynthesis of cuticular waxes on rice leaf.	Planta. (2008) 228: 675-685.

Since a large collection of transposon insertion lines were generated during 2000's, not so many genes have been functionally identified by such insertion mutants. Currently, at least 9 genes have been functionally characterized by *Ds* insertion mutants including *ANTHER INDEHISCENCE1, BRANCHED FLORETLESS 1, CSL1, OSH6, OsKS1, OSMYOXIB, OsNAC2, OSNOP* and *OsPE* (Table 3). These data suggested the feasibility and potential of transposon insertion lines as a tool to decipher gene functions.

In our lab, we have used a two-element *Ac/Ds* gene trap system to tag rice genes. In this system, an immobilized version of *Ac,* in which the transposase gene is under the control of cauliflower mosaic virus (CaMV) 35S promoter was used. The non-autonomous *Ds* element carries the *bar* gene encoding phosphinothricin acetyltransferase conferring resistance to phosphinothricin (herbicide Basta), which serves as a positive selection marker and a modified promoterless *gusA* gene encoding β-glucuronidase as a reporter gene. The *gusA* gene used in the *Ds* construct has the intron and triple splice acceptor sequences upstream of the ATG codon to trap the expression of tagged genes at 3' *Ds*. The synthetic green fluorescence protein (sGFP) was used under maize ubiquitin promoter as negative selection markers within both the *Ac* and the *Ds* T-DNA constructs as a negative selection marker.

These two constructs were then introduced into rice genome by Agrobacterium-mediated transformation. Transgenic *Ac* and *Ds* rice plants were used as parent lines for sexual crossing. In next generation, stable *Ds* insertion lines were obtained by selecting Basta positive and GFP negative plants. Homozygous *Ds* insertion lines were obtained after the fifth/sixth generations by self-crossing. These homozygous lines were used for phenotype investigation.

Table 3. Some of rice genes functionally characterized through transposon insertion mutants

Gene Name	Protein description	Functions/Descriptions	References
ANTHER INDEHISCENCE1	MYB transcription factor	Anther development	Plant Physiol. (2004) 135: 1514-1525.
BRANCHED FLORETLESS 1	EREBP/AP2 domain containing protein	Mediating the transition from spikelet to floret meristem	BMC Plant Biol. (2003) 3: 6.
CSL1	-	Involving in the regulation of leaf initiation and developmental transition	Plant Cell Rep. (2007) 26: 421-427.
OSH6	Homeobox protein	Bract differentiation, especially at the basal nodes of panicles	Planta. (2007) 227: 1-12.
OsKS1	Kaurene synthase	Catalyzing the second step of the gibberellin (GA) biosynthesis pathway	Plant Cell Rep. (2005) 23: 819-833.
OSMYOXIB	Myosin protein	Pollen development	Dev Biol. (2007) 304: 579-592.
OsNAC2	NAC domain containing protein	Regulating shoot branching	New Phytol. (2007) 176: 288-298.
OSNOP	C2-GRAM domain containing protein	Pollen development	Plant Mol Biol. (2005) 57: 835-853.
OsPE	Hypothetical protein	multiple embryos	Funct Integr Genomics. 2009. DOI 10.1007/s10142-009-0139-6.

In this system, the germinal transposition frequency of *Ds* was estimated as an average of 51% by analyzing 4413 families. Study of *Ds* transposition pattern in siblings revealed that 79% had at least two different insertions, suggesting late transposition during rice development. Analysis of 2057 *Ds* flanking sequences showed that 88% of them were unique, whereas the rest within T-DNA. The insertions were distributed randomly throughout the genome; however, there was a bias toward chromosomes 4 and 7, which had two times as many insertions as that expected. A hot spot for *Ds* insertions was identified on chromosome 7 within a 40-kbp region. One-third of *Ds* flanking sequences was homologous to either proteins or rice ESTs, confirming a preference for *Ds* transposition into coding regions. Analysis of 200 *Ds* lines on chromosome 1 revealed that 72% insertions were found in genic region. Anchoring of more than 800 insertions to yeast artificial chromosome (YAC)-based EST map showed that *Ds* transposes preferentially into regions rich in expressed sequences. High germinal transposition frequency and independent transpositions among siblings show that the efficiency of this system is suitable for large-scale transposon mutagenesis in rice [78].

Additionally, we have performed a systematic analysis to survey the transposition activities of *Ac/Ds* parent lines in the following generations. We found that high somatic and germinal transposition frequencies were maintained as late as T4 and T5 generations; thus the propagation of parental lines did not induce transposon silencing. Moreover, the stably transposed *Ds* element was active even at the F5 generation, since *Ac* could remobilize the *Ds* element as indicated by the footprint analysis of several revertants. Strikingly, substantial transgenic silencing was not observed in any of the generations tested. We analyzed the timing of transposition during rice development and provide evidence that *Ds* was transposed late after tiller formation. Our study validates the *Ac/Ds* system as a tool for large-scale mutagenesis in rice, since the *Ds* elements were active in the starter and insertion lines even in the later generations [85].

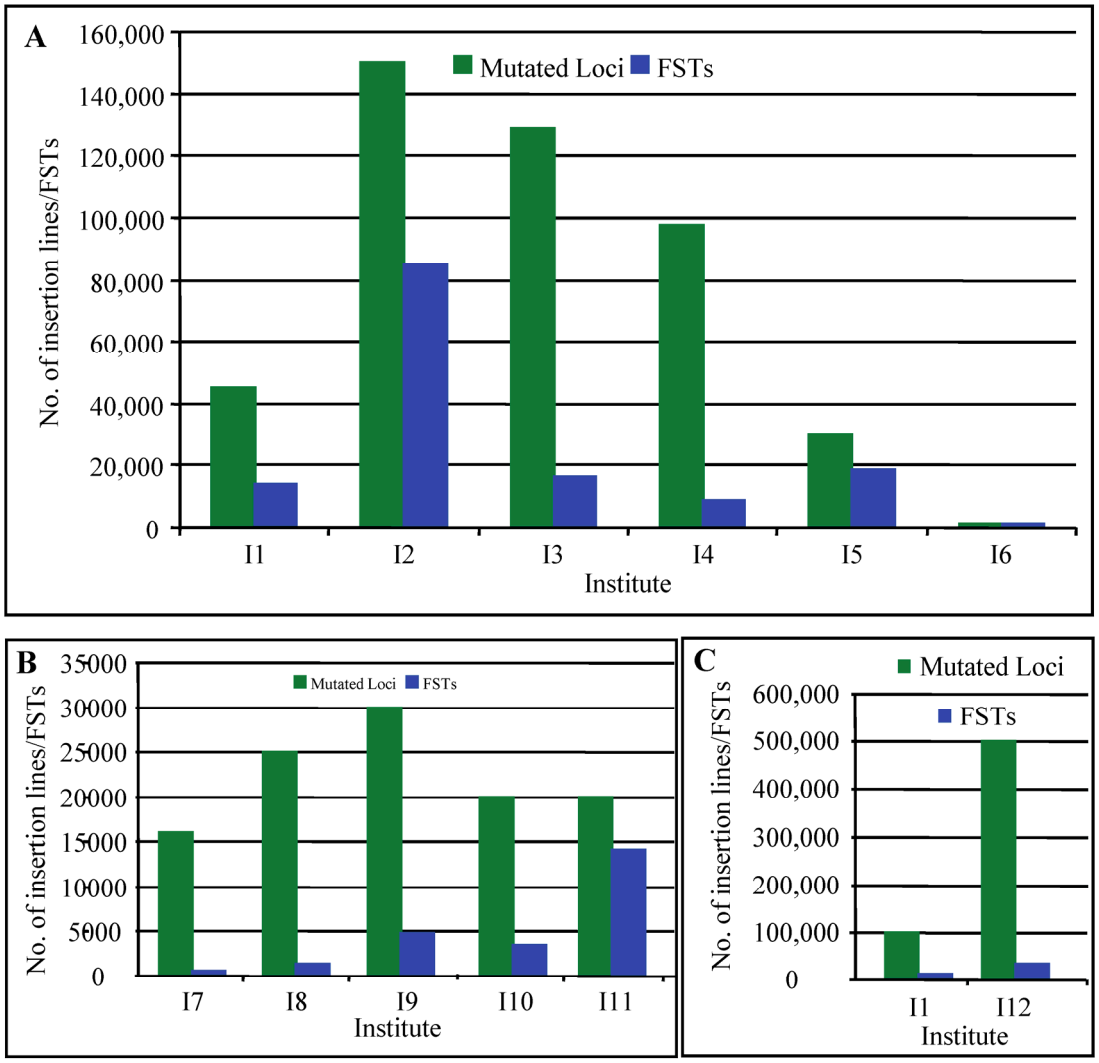

Figure 1. Collection of insertion mutants in rice. This figure summarizes the collection of T-DNA (A), Ac/*Ds*/Spm/dSPM transposon (B) and retrotransposon Tos17 (C) insertion lines in rice. Green columns indicate the numbers of mutated loci carried out in each institute and blue columns indicate the numbers of insertion lines with FSTs. I1, including Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Institut National de la Recherche Agronomique and Centre National de la Recherche Scientifique; I2, Pohang University of Science and Technology; I3, Huazhong Agricultural University, China; I4, Shanghai Institute of Plant Physiology and Ecology, China; I5, Institute of Plant and Microbial Biology, Academia Sinica, Taiwan; I6, Zhejiang University, China; I7, CSIRO Plant Industry, Australia; I8, Centre de Coopération Internationale en Recherche Agronomique pour le Développement; I9, Gyeongsang National University, Korea; I10, Temasek Life Sciences Laboratory, Singapore; I11, University of California, Davis; I12, National Institute of Agrobiological Sciences, Japan. The data are based on the following references: [56-60, 62, 63, 65-67, 76-83, 105, 107, 111, 121-124].

Gene Trap, Promoter Trap and Enhancer Trap in T-DNA or Transposon Mutagenesis

Not all genes can be functionally identified by mutagenesis. One of the reasons is that many genes are functionally redundant and mutation of such genes may not lead to an easily recognizable phenotype. Another reason is that many genes function at multiple stages of development and mutations of these genes may lead to early lethality. Therefore, it is necessary to develop a system to monitor gene expression patterns to better understand functions of these genes. Gene trap, promoter trap or enhancer trap is a system that allows gene activity to be monitored by creating gene fusions with a reporter gene. In an enhancer trap, a reporter gene has a minimal promoter that is only expressed when inserted near cis-acting chromosomal enhancers. Reporter genes in

gene trap and promoter trap have no promoter, so that reporter genes expression can occur only when the reporter gene inserts within a transcribed chromosomal gene, creating a transcriptional fusion. Expression of a promoter trap reporter gene requires that it be inserted into an exon, leading to a transcriptional fusion. In contrast, gene trap constructs contain one or more splice acceptor sequences preceding the reporter gene. Thus reporter genes can be detected even if insertion occurs in an intron since splicing from the splice donor sites to the splice acceptor sites in the reporter gene results in fusion of upstream exon sequences to the reporter gene [86]. Currently, this system has been widely used for T-DNA or transposon mutagenesis. The GUS reporter gene is the mostly used gene for various trap systems in rice. In an enhancer trap *Ds* insertion population, around 8% of the lines were detected with GUS expression in panicles [81]. For T-DNA promoter trap lines, histochemical GUS assays were carried out in the leaves and roots from 5353 lines, mature flowers from 7026 lines, and developing seeds from 1948 lines. The data revealed that 1.6-2.1% of tested organs were GUS-positive and that their GUS expression patterns were organ- or tissue-specific or ubiquitous in all parts of the plant [56]. In our lab, 2852 *Ds* lines were subjected to GUS assay and the result showed that around 8.1% of the lines were with GUS activities [87]. Some of the examples are shown in Figure 2.

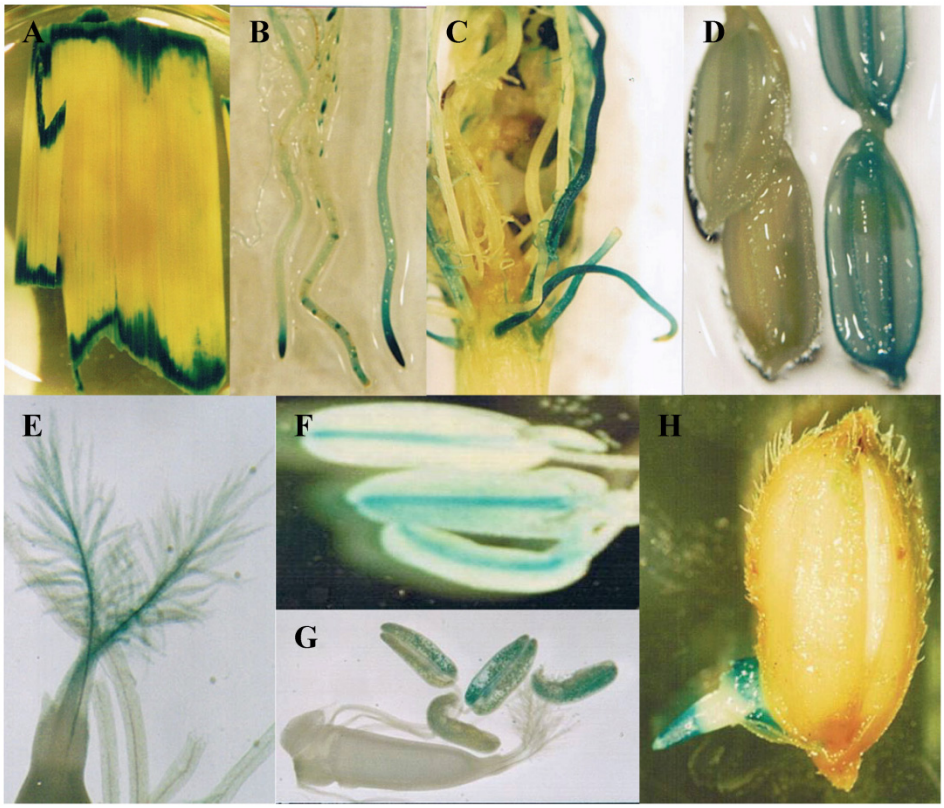

Figure 2. Expression of GUS in gene trapped *Ds* insertion lines. These images show various GUS expression patterns. (A) Expressed in wounded leaves. (B) Expressed in root tips. (C) Expressed in lateral roots. (D) Expressed in grain hulls (left image is WT control). (E) Expressed in stigma. (F) Expressed in connective tissues of anthers. (G) Expressed in pollens. (H) Expressed in geminated seeds.

These analyses suggested that *Ds*-tagged genes exhibited different expression patterns due to the *Ds* insertion into different genomic positions. Furthermore, the multimerized transcriptional enhancers from the cauliflower mosaic virus 35S promoter were positioned next to the left border of the T-DNA to make activation tagged lines [57]. Histochemical GUS assays have revealed that the GUS-staining frequency from those lines is about twice as high as that from lines without the enhancer element. This result sug-

gests that the enhancer sequence presented in the T-DNA improves the GUS-tagging efficiency [57]. In another report, a CaMV35S enhancer was cloned in eight tandem repeats. This octamer configuration may serve as more potent activator than the traditional tetramer, as gene distances as far as 12.5 kb from the ATG start codon led to gene activation [60]. Thus, most insertions of the CaMV35S enhancers into the rice genome (excluding insertions in exons and introns that lead to gene knockout) have the potential to activate at least one native gene based on the average gene density of one gene per 9.9 kb in the rice genome [4]. Recently, a versatile transposon-based activation tag vector system was used for functional genomics in cereals and other monocot plants to further enhance rice gene expression [88]. All these data showed that gene trap, promoter trap and enhancer trap in T-DNA or transposon mutagenesis can be used as efficient tools to trap gene expression and to analyze their functions.

Besides the activation tagging system, gain-of-function type mutants may also be obtained from over-expression of individual rice genes. Both over-expression and gene silencing (see below) have been widely used for the annotation of gene functions. Currently, many rice genes have been functionally characterized by the over-expression of their genes. For example, the biological function of *ETHYLENE RESPONSE2 (ETR2)* was annotated by comparing the difference between gain-of-function and knockout mutants [89]. To investigate the over-expression mutants at the genome-wide level, special binary vectors have been designed to globally over-express all genes in an organism [90-92]. Up to now, at least 45,000 FOX hunting rice lines have been generated [93, 94].

Tissue Culture Mutagenesis and Retrotransposon *Tos17*

Tissue culture is also an efficient tool to induce various mutations, which is called somaclonal variations [95]. Tissue culture mutagenesis formed the important resources for rice breeding [96, 97]. However, little is known about the application of this technique in functional genomics until that some transposon elements in maize can be activated during tissue culture, indicating that some tissue culture-derived genetic variability may be the result of insertion or excision of transposable elements, or both [98]. Subsequent studies showed that active DNA transposon elements were also observed during rice tissue culture [99-101]. In addition to transposons, active retrotransposons were also detected during rice tissue culture [102]. Differentiated from transposons, retrotransposons are mobile genetic elements that transpose through reverse transcription of an RNA intermediate. One of these retrotransposons was named as *Tos17*, a widely utilized retrotransposon in rice [102]. One to five copies of *Tos17* elements can be detected in normal growth conditions, varying with different rice varieties. For example, two copies of *Tos17* were detected in *japonica* variety Nipponbare. These *Tos17* elements have usually no activity in normal growth conditions. However, *Tos17* will be activated during tissue culture and its copy number will increase to 5-30 [102]. For example, at least 5 new insertions of *Tos17* were induced during 3- to 9-month tissue culture. Although *Tos17* is actively transcribed during tissue culture, no transcript of *Tos17* was detected in plants regenerated from tissue culture [102-104], suggesting that transposition is active only under tissue culture conditions. This result indicated that *Tos17* could be used for mutagenesis to generate stabilized insertion lines. Subsequent studies showed that insertion sites were mostly found in genic regions and preferably in coding sequences [105, 106]. In 2001, 32,000 rice lines were generated from *Tos17*, containing 256,000 insertions [103]. Now they have produced around 50,000 insertion lines (Fig. 1C; [107]). Phenotypic investigation of these insertion lines indicated that nearly half of the lines showed more than one mutant phenotype; the most frequently observed phenotype was low fertility, followed by dwarfism [107]. These phenotype data have been submitted into *Tos17* mutant database with a dataset of sequences flanking *Tos17* insertion points in rice genome (http://tos.nias.affrc.go.jp/).

Since the identification and characterization of *Tos17*, many genes have been isolated and functionally characterized through *Tos17* insertion lines. Currently, at least 24 genes have been characterized by *Tos17* insertion (Table 4). For example, *oshkt2;1* is the first mutant that greatly diminishes sodium influx into plant roots. Further investigator showed that OsHKT2;1 is the central transporter for nutritional Na^+ uptake into K^+ starved rice roots [108].

Table 4. Some of rice genes functionally characterized through retrotransposon *Tos17*

Gene Name	Protein description	Functions/Descriptions	References
COW	A new member of the YUCCA protein family	Required for maintaining water homeostasis and an appropriate root to shoot ratio	Plant Mol Biol. (2007) 65: 125-136.
FON1	LRR receptor kinase	Controling vegetative and reproductive development by regulating shoot apical meristem size	Mol Cells (2006) 21: 147-152.
GS1	Glutamine synthetase	Play important in development and grain filling	Plant J. (2005) 42: 641-651.
MYBGA	GARE-interacting transcription factor	tissue-specificly regulating a-amylase expression by sugar and GA signaling interference	Plant Cell (2006) 18: 2326-2340.
OsABA1	Zeaxanthin epoxidase	The epoxidation of zeaxanthin	Plant Physiol. (2001) 125: 1248-1257.
OsCAO1 and OsCAO2	Chlorophyll a oxygenase	Chlorophyll b biosynthesis	Plant Mol Biol. (2005) 57: 805-818.
OsCHLH	The largest subunit of the rice Mg-chelatase	Chlorophyll biosysthesis	Plant Cell Physiol. (2003) 44: 463-472.
OsCLC-1 or OsCLC-2	Voltage-gated chloride channel protein	May play a role in the transport of chloride ions across the vacuolar membrane	Plant Cell Physiol. (2006) 47: 32-42.
OsGAMYB	Transcriptional regulator of gibberellin (GA)-dependent alpha-amylase expression	Induction of alpha-amylase in aleurone and floral organ development	Plant Cell (2004) 16: 33-44.
OsHKT2;1	Sodium ion transport protein	Mediating large Na+ influx component into K+-starved roots for growth	EMBO J. (2007) 26: 3003-3014.
OsMKP1	A calmodulin-binding mitogen-activated protein kinase phosphatase	Involving in the negative regulation of rice wound responses	Plant Cell Physiol. (2007) 48: 332-344.
OsMT2b	Metallothionein	A reactive oxygen species (ROS) scavenger	Plant Physiol. (2004) 135: 1447-1456.
OsSIG1	Sigma factor	Maintaining photosystem I activity via regulated expression of the psaA operon in rice chloroplasts	Plant J. (2007) 52: 124-132.
OsTPC1	Voltage-gated Ca(2+)-permeable channel protein	a key regulator of elicitor-induced hypersensitive cell death and mitogen-activated protein kinase activation	Plant J. (2005) 42: 798-809.
PAIR1	Coiled-coil motif-containing protein	Homologous chromosome pairing and cytokinesis in male and female meiocytes	Plant Cell (2004) 16: 1008-1020.
PAIR2	HORMA domain containing protein	Essential for homologous chromosome synapsis in rice meiosis I	J Cell Sci. (2006) 119: 217-225.
PHYA	Phytochrome A	Controling photomorphogenesis	Plant Cell (2001) 13: 521-534.
SSI	Starch synthase I	Involving in the synthesis of amylopectin chains	Plant Physiol. (2006) 140: 1070-1084.
Udt1	bHLH transcription factor	Maintaining tapetum development	Plant Cell (2005) 17: 2705-2722.

Gene Name	Protein description	Functions/Descriptions	References
OsSSI2	Fatty-acid desaturase	Negative regulation of defense responses in rice	Mol Plant Microbe Interact. (2009) 22: 820-829.
RIM1	NAC-domain protein	As a host factor that is required for multiplication of Rice dwarf virus (RDV)	Plant J. (2009) 57: 615-625.
OsIPD3	a Ca(2+)/calmodulin-dependent protein kinase	required for root symbiosis with AM fungi in plants	New Phytol. (2008) 180: 311-315.
OsRecQ1	a QDE-3 homologue in rice	participate in the process that allows inverted repeat DNA to be transcribed into dsRNA, which can trigger RNA silencing	Plant J. (2008) 56: 274-286.
OsJar1	GH3 auxin-responsive promoter	involved in phytochrome and jasmonate signalling	Plant Cell Environ. (2008) 31: 783-792.

Gene Silencing Mutagenesis and its Application on Functional Identification of Rice Genes

Silencing a gene is also an efficient tool to determine its functions. Several methods can be used to silence a gene. For example, anti-sense or co-suppression was frequently observed in transgenic plants [109, 110]. However, in some cases, only partial functions can be suppressed by anti-sense or co-suppression. Among RNA silencing methods, RNA interference (RNAi) is now widely used for gene silencing. During silencing, double-stranded RNA (dsRNA) is processed into 20- to 25-nt short interfering RNA (siRNA) and microRNA (miRNA) by RNaseIII-like enzymes called Dicers1. siRNAs and miRNAs guide RNA-induced silencing complexes (RISCs) to suppress gene expression at the level of transcription, RNA stability or translation. siRNAs are 21-23 nucleotide double-stranded RNA molecules. Once incorporated into RISC they facilitate the cleavage and degradation of their recognized mRNA. MicroRNAs are single-stranded RNAs of 22-nucleotides that are processed from ~70-nucleotide hairpin RNA precursors by Dicers. Similar to siRNAs, miRNAs can silence gene activity through destruction of homologous mRNA or blocking its translation in plants.

The key to utilize the RNAi silencing in plants is how to transfer double strand RNAs into cells. In animals, RNAi can be initiated by injecting or feeding dsRNA into cells [111]. However, these methods can not be used in plants. Currently, at least two types of constructs have been used for RNAi-mediated silencing in plants. One is by virus-based vectors to transfer dsRNAs into plant cells. However, the virus-based silencing method can not be genetically inherited. Another one is by hairpin RNA (hpRNA)-mediated gene silencing. In this method, the target gene is cloned as an inverted repeat spaced with an unrelated sequence and is driven by a strong promoter, such as the 35S CaMV promoter for dicots or the maize ubiquitin 1 promoter for monocots. When an intron is used as the spacer, the efficiency becomes very high: almost 100% of transgenic plants show gene silencing [112]. However, this technique cannot be applied to genes whose silencing may block plant regeneration or result in embryo lethality. To obviate these potential problems, a chemical-inducible Cre/loxP (CLX) recombination system was used to trigger the expression of an intron-containing inverted-repeat RNA (RNAi) in plants [113]. In addition, a vector for high-throughput cloning of target genes as inverted repeats has been constructed for genome-wide analysis of gene functions [114]. Another such RNAi vector is based on the spreading of RNA targeting (transitive RNAi) from an inverted repeat of a heterologous 3'UTR [115]. Thus, the RNAi-mediated gene silencing can be used as a tool not only to analyze gene functions of a single gene or a gene family but also for genome-wide analysis of gene functions.

Now, this RNAi-mediated gene silencing has been successfully used for identification of gene functions in rice plants. In less than 7 years from 2003, at least 71 genes were functionally characterized by the RNAi silencing (Table 5). These genes included genes encoding various transcription factors, flowering-related proteins, pathogen/membrane-related proteins, various protein kinases and cell division-related proteins and so on. These results suggested that various genes can be silenced by RNAi and this method may be universally employed for characterizing various rice genes.

Table 5. Some of rice genes functionally characterized through gene silencing mutants

Gene Name	Protein description	Functions/Descriptions	References
CEBiP	Lysin domain containing protein	Perception and transduction of chitin oligosaccharide elicitor	Proc Natl Acad Sci USA. (2006) 103: 11086-11091.
DSH1	Dihydrosphingosine C4 hydroxylase	Stigma development	Plant Cell Physiol. (2007) 48: 1108-1120.
Ehd1	B-type flowering response regulator	Short-day promotion of flowering	Plant Physiol. (2007) 145: 1484-1494.
Os8N3	A member of the MtN3 gene family	A host susceptibility gene for bacterial blight	Proc Natl Acad Sci USA. (2006) 103: 10503-10508.
OsARD	Aci-reductone dioxygenase	Metabolism of methionine and ethylene	Gene (2005) 360: 27-34.
OsBP-5	DNA-binding protein with a SAP-like domain	Transcriptional regulation of the rice Wx gene.	J Biol Chem. (2003) 278: 47803-47811.
OsBP-73	SAP-like domain protein	Cell proliferation	Plant Mol Biol. (2003) 52: 579-590.
OsBZR1	Brassinazole-resistant 1 protein	Brassinosteroid signal transduction	Proc Natl Acad Sci USA. (2007) 104: 13839-13844.
OsDCL1	Dicer or Dicer-like protein	Affecting microRNA accumulation and causing developmental defects	Plant Physiol. (2005) 139: 296-305.
OsDMC1	Meiosis-specific protein	Meiosis during homologous pairing	Plant Mol Biol. (2007) 65: 31-42.
OsDOS	CCCH-type zinc finger protein	Delaying leaf senescence	Plant Physiol. (2006) 141: 1376-1388.
OsDR8	Pathogen-induced defense-responsive protein 8	Disease resistance and thiamine accumulation	Plant Mol Biol. (2006) 60: 437-449.
OsGEN-L	The RAD2/XPG nuclease family protein	DNA metabolism required for early microspore development	Plant Cell Physiol. (2005) 46: 699-715.
OsGLU1	Endo-1,4-beta-D-glucanase	Plant internode elongation	Plant Mol Biol. (2006) 60: 137-151.
OsHAP3	HAP3/nuclear factor-YB (NF-YB)/CCAAT binding factor-A	Chloroplast biogenesis	Plant J. (2003) 36: 532-540.
OsLFL1	B3 DNA-binding domain-containing transcription factor	Regulating flowering time	J Plant Physiol. (2007) 165: 876-885.
OsMADS1	MADS-box protein	A repressor of overdevelopment of lemma and palea	Planta. (2006) 223: 882-890.
OsMADS16	MADS-box protein	Identities of lodicules and stamens	Plant Mol Biol. (2003) 52: 957-966.
OsMADS2	MADS-box protein	Floral organ patterning	Genetics (2003) 165: 2301-2305.
OsMADS50	MADS-box protein	Flowering activator	Plant J. (2004) 38: 754-764.
OSMADS58	C-class MADS box protein	Determinacy of the floral meristem	Plant Cell (2006) 18: 15-28.
OsSSI2	Fatty-acid desaturase	Negative regulation of defense responses in rice	Mol Plant Microbe Interact. (2009) 22: 820-829.
OsMET1	Cytosine-5 DNA methyltransferase	Gene silencing	Planta (2004) 218: 337-349.
OsMT2b	Metallothionein	A reactive oxygen species (ROS) scavenger	Plant Physiol. (2004) 135: 1447-1456.
OsNAC2	Transcription factor	Tiller development	New Phytol. (2007) 176: 288-298.
OsPDK1	Pyruvate dehydrogenase kinase	Regulating mitochondrial pyruvate dehydrogenase activity	Plant Cell Physiol. (2006) 47: 244-253.
OsPIN1	A PIN1 family protein	Auxin-dependent adventitious root emergence and tillering	Plant Cell Physiol. (2005) 46: 1674-1681.
OsRAD21-3	N terminus of Rad21 / Rec8 like protein	Pollen development	Plant J. (2007) 51: 919-930.

Gene Name	Protein description	Functions/Descriptions	References
OsRAD21-4	N terminus of Rad21 / Rec8 like protein	Required for efficient meiosis	Plant Mol Biol. (2006) 60: 533-554.
OsRMC	Receptor-like protein kinase	Root development and coiling mediated by jasmonic acid signalling	Plant Cell Environ. (2007) 30: 690-699.
OsSPY	O-linked N-acetylglucosamine transferase	Negative regulator of gibberellin signaling	Plant J. (2006) 48: 390-402.
OsSRT1	NAD(+)-dependent histone deacetylases	Inducing DNA fragmentation and cell death	Plant Physiol. (2007) 144: 1508-1519.
OsWRKY89	WRKY transcription factor	In responsive to rice blast and planthopper as well as UV-B irradiation	Plant Mol Biol. (2007) 65: 799-815.
OsXTH8	Xyloglucan endotransglucosylase/hydrolase	Cell elongation	Plant Physiol. (2004) 136: 3670-3681.
SDG714	Histone H3K9 methyltransferase	*Tos17* DNA methylation and transposition	Plant Cell (2007) 19: 9-22.
SnRK1A	Ser/Thr protein kinase	Sugar Signaling during Germination and Seedling Growth	Plant Cell (2007) 19: 2484-2499.
Ugp1	UDP-glucose pyrophosphorylase	Pollen callose deposition	Plant Cell (2007) 19: 847-861.
YAB3	Transcription factor	Rice leaf development	Plant Physiol. (2007) 144: 380-390.
ETR2	ethylene receptor	floral transition and starch accumulation	Plant Cell (2009) 21: 1473-1494.
PAIR3	coiled-coil motifs containing protein	homologous chromosome pairing and synapsis in meiosis	Plant J. (2009) 59: 303-315.
Os-CASTOR and Os-POLLUX	ion channel proteins	indispensable for mycorrhizal symbiosis in rice	Plant Physiol. (2009) 149: 306-317.
OsPRP3	proline-rich protein	determining extracellular matrix structure of floral organs	Plant Mol Biol. (2009) 72: 125-135.
OsNAC4	plant-specific transcription factor	plant hypersensitive cell death	Plant Signal Behav. (2009) 4: 740-742.
OsCPL1	conserved carboxy-terminal domain (CTD) phosphatase	represses differentiation of the abscission layer during panicle development	Plant J. (2009) doi: 10.1111/j.1365-313X.2009.04039.x.
RFT1	mobile flowering signal protein	a major floral activator under long day length conditions.	Development (2009) 136: 3443-3450.
OsMADS22 and OsMADS55	SVP-group MADS-box proteins	negative regulators of brassinosteroid responses	Plant J. (2008) 54: 93-105.
OsERO1	ER membrane-localized oxidoreductase	required for disulfide bond formation in the rice endosperm	Proc Natl Acad Sci U S A. (2009) 106: 14156-14161.
HDA704 and HDA710	Histone deacetylases	Multiple functions	Biochem Biophys Res Commun. (2009) 388: 266-
BU1	Helix-Loop-Helix Protein	Involved in Brassinosteroid Signaling and Controls Bending of the Lamina Joint in Rice	Plant Physiol. (2009) 151: 669-680.
OsBRR1	leucine-rich repeat receptor kinase	involved in rice blast resistance	Planta. (2009) 230: 377-385.
TDC	tryptophan decarboxylase	leaf senescence	Plant Physiol. (2009) 150: 1380-1393.

Gene Name	Protein description	Functions/Descriptions	References
OsDEG10	small RNA-binding protein	involved in abiotic stress signaling	Biochem Biophys Res Commun. (2009) 380: 597-
OsCP	cysteine protease	involved in the process of suspension-cultured rice cells proliferation	Biochim Biophys Acta. (2009) 1794: 459-467.
OsSPX1	SPX domain containing protein	involved in phosphate homeostasis in rice	Plant J. (2009) 57: 895-904.
OsGSR1	a member of the GAST (GA-stimulated transcript) gene family	involved in crosstalk between gibberellins and brassinosteroids in rice	Plant J. (2009) 57: 498-510.
OsBADH2	homologous to betaine aldehyde dehydrogenase	aroma accumulation	BMC Plant Biol. (2009) 8:100.
OsSGT1	salicylic acid glucosyltransferase	contribute to the SA signaling mechanism by inducing up-regulation of SAG in rice plants.	Plant J. (2009) 57: 463-472.
OsId1	zinc finger protein	the activation of Ehd1 by OsId1 is required for the promotion of flowering	Plant J. (2009) 56: 1018-1029.
Ostrxm	thioredoxin m isoform	the redox regulation of chloroplast target proteins	Plant Physiol. (2009) 148: 808-817.
OsXIP	xylanase inhibitor protein	involved in plant defense mechanisms against phytopathogens	Plant Cell Physiol. (2009) 49: 1122-1127.
OsTudor-SN	cytoplasmic-localized, cytoskeletal-associated RNA binding protein	storage protein RNA transport and localization	Plant J. (2009) 55: 443-454.
IDEF2	A novel NAC transcription factor	regulating the genes involved in iron homeostasis in plants.	J Biol Chem. (2009) 283: 13407-13417.
OsMT2b	Metallothioneins	involved in root development and seed embryo germination	Plant Physiol. (2008) 146: 1637-1650.
OsTDL1A	TAPETUM DETERMINANT1-like protein	binding to the LRR domain of rice receptor kinase MSP1, and is required to limit sporocyte numbers	Plant J. (2009) 54: 375-387.
Hd3a and RFT1	flowering signal related proteins	floral activators under Short day length conditions	Development. (2008) 135: 767-774.
OsLCY	lycopene beta-cyclase	related to photo-oxidation	Plant J. (2008) 54: 177-189.
UGPase1	UDP-glucose pyrophosphorylase 1	pollen development and seed carbohydrate metabolism	Plant J. (2008) 54: 190-204.
DH1	LOB (Lateral Organ Boundaries) domain-like protein	required for glume formation in rice	Plant Mol Biol. (2008) 66: 491-502.
RAR1	zinc-binding protein	function in innate-immune responses	Plant Cell. (2007) 19: 4035-4045.
OsWRKY31	WRKY transcription factor	the auxin response and the defense response in rice	Cell Res. (2008) 18: 508-521.
Oshox4	homeodomain-leucine zipper (HD-Zip) transcription factor	negative function in gibberellin responses	Plant Mol Biol. (2008) 66: 289-301.

In order to use this RNAi-mediated gene silencing at the genome-wide level, we have designed a binary vector to globally silence rice genes (Fig. 3). Currently, in plants, genome-wide RNAi technology has been developed only in Arabidopsis [116]. However, this method was based on whole genome sequence information and was costly. The new system is based on the construction of a cDNA library and subsequent RNAi constructs followed by transformation; thus, it is relatively cheap and can be used for other plants since no sequence information is required to generate such system. On the other hand, gene silence-based phenotypes can be investigated during the first or second generation; thus, it is a time-saved system. In this system, 300-500 bp 5′-UTR fragments are generated from a normalized cDNA library and are then cloned into the Gateway pENTR vector, which carry two recombination sites (attL1 and attL2) for LR clonase reactions (Fig. 3). These cloned UTR fragments are then transferred into a pANDA destination vector, which was developed by Miki and Shimamoto (2004) [117], through recombinase reactions. Thus, a RNAi silencing vector library is constructed, where UTR fragments are inserted into two regions flanked by two inverted repeats. This library can be used for transformation to develop a collection of transgenic rice plants integrated with different RNAi silencing T-DNAs.

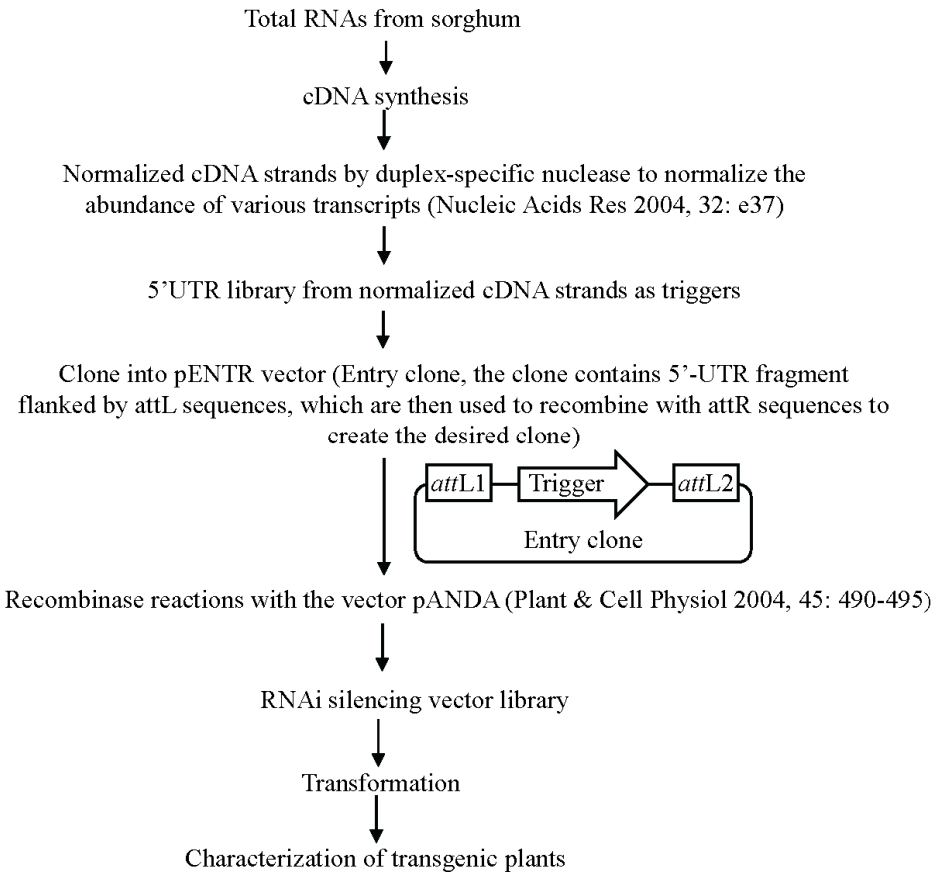

Figure 3. Binary vector construction for genome-wide gene silencing. This figure shows the detail of the construction of gene silencing vector.

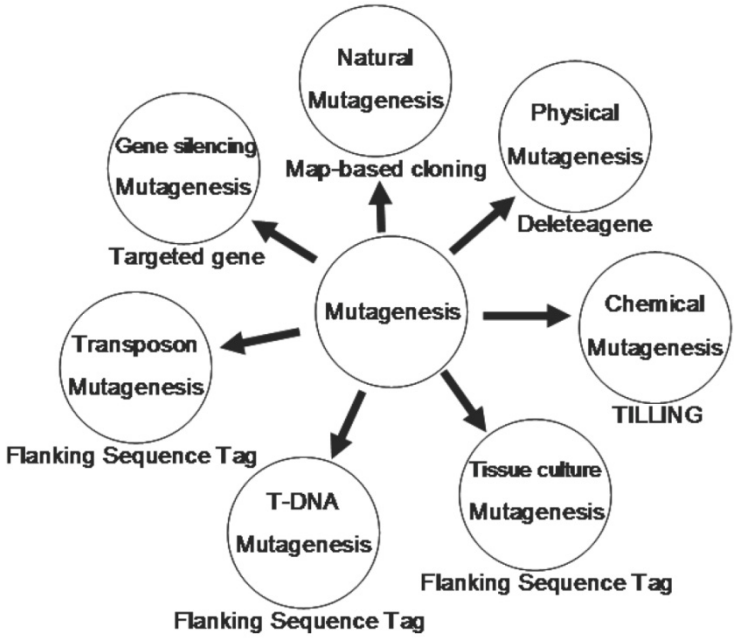

Figure 4. The technological platform for rice functional genomics. This figure shows various tools used to generate mutants and the strategies to screen these mutants.

In summary, we have reviewed 7 different methods for mutagenesis including natural, physical, chemical, tissue culture, T-DNA, transposon and gene silencing mutagenesis (Fig. 4). Natural mutagenesis has been widely used to identify gene functions but this method can not be used for genome-wide analysis due to its low frequency of mutation and its difficulty in identifying mutated genes by map-based cloning. Chemical mutagenesis can be efficiently used to produce a large number of point mutants in a short period and the induced mutants can be detected by TILLING. However, multiple point mutations were sometimes observed in one mutant. Thus, it is necessary to genetically segregate these point mutants. Similarly, Physical mutagenesis can be applied for producing a large number of deletion-based mutants in a short period, and the deletion mutants can be screened by Deleteagene. However, it is also very difficult to identify a deletion mutant and its phenotype when the induced deletion occurs covering multiple genes or within an intron. Insertional mutagenesis based on T-DNAs, transposons and retrotransposons is becoming a major approach to produce a saturated mutant pool. A large number of T-DNA insertion lines have been produced in rice; but T-DNA insertional mutagenesis can be used only for those rice varieties with highly efficient transformation. The retrotransposon *Tos17* has been successfully applied for rice functional genomics. But it is also difficult to identify a mutant related to *Tos17* because there are multiple copies of *Tos17* in a mutant and only about 10 percent of mutants are tagged by *Tos17* insertion. Theoretically, *Ac/Ds* two-element system is regarded as a best approach for rice insertional mutagenesis because more than 95% of *Ds* insertion lines have only single copy of *Ds* insertion. An additional advantage is that *Ds* can be remobilized from a tagged gene in the presence of *Ac* transposase, resulting in phenotypic reversion to the wild type or giving rise to alleles with weaker phenotypes. However, it is also difficult to identify a mutant when there are *Ds* excision footprints in the mutant caused by multiple *Ds* excision-insertion events in the presence of *Ac* transposase. RNAi can efficiently silence a gene, but not all genes can be silenced. In addition, RNAi can interfere in genes with redundant and overlapping functions or gene families with high homolog in sequence, making it difficult to identify a silenced gene. So it is obvious that each method has its advantage and disadvantage and different methods should be combined to produce a saturated mutant population.

Natural and Artificial Mutants as Valuable Resources for Molecular Breeding

Large-scale phenotype investigation has been carried out in rice using several mutant resources. Chern et al (2007) reported 11 categories of the visible phenotypes including growth condition, leaf color, leaf morphology, plant morphology, mimic response, tiller, heading date, flower, panicle, seed fertility and seed morphology, which were subdivided into 65 subcategories [71]. Miyao et al (2007) also investigated around 50,000 *Tos17* insertion lines in their phenotype variation including germination, growth, leaf color, leaf shape, culm shape, spotted leaf/lesion mimic, tillering, heading date, spikelet, panicle, sterility and seed [107]. Park et al (2009) have analyzed 115,000 *Ds* insertion lines in their phenotype variation and 437 mutants from 12,162 *Ds*-tagged lines were catalogued in their agronomic traits including tillers, panicles, leaves, flowers, seed, chlorophyll content, and plant height [83]. Furthermore, several rice mutant phenotype databases are now established including *Tos17* insertion lines [107], T-DNA-tagged lines [72], and chemical- and irradiation-induced lines [32]. Kuromori et al (2009) have reviewed a detail phenome analysis [67]. However, they have not discussed their application in rice breeding. Recently, Mochida and Shinozaki (2010) have summarized the genomics and bioinformatics resources for crop improvement [118].

We have subjected around 20,000 *Ds* insertion lines to phenotypic and abiotic stress screens and evaluated these lines with respect to their seed yields and other agronomic traits as well as their tolerance to drought, salinity and cold. Based on this evaluation, we observed that random *Ds* insertions into rice genome have led to diverse variations including a range of morphological phenotypes. We have observed various variations in these *Ds* insertion lines including the differences in plant height, growth vigor, growth period of duration and stigma and so on (Fig. 5). Among the various phenotypes identified, some *Ds* lines showed significantly higher grain yield compared to wild-type plants under normal growth conditions indicating that rice could be improved in grain yield by disrupting certain endogenous genes [87]. In addition, several thousands of *Ds* lines were subjected to abiotic stresses to identify conditional mutants. Subsequent to these screens, over 800 lines responsive to drought, salinity or cold stress were obtained, suggesting that rice has the genetic potential to survive under abiotic stresses when appropriate endogenous genes were suppressed. The mutant lines that

have higher seed yielding potential or display higher tolerance to abiotic stresses may be used for rice breeding by conventional backcrossing combining with molecular marker-assisted selection. In addition, by exploiting the behavior of *Ds* to leave footprints upon remobilization, we have shown an alternative strategy to develop new rice varieties without foreign DNA sequences in their genome [87].

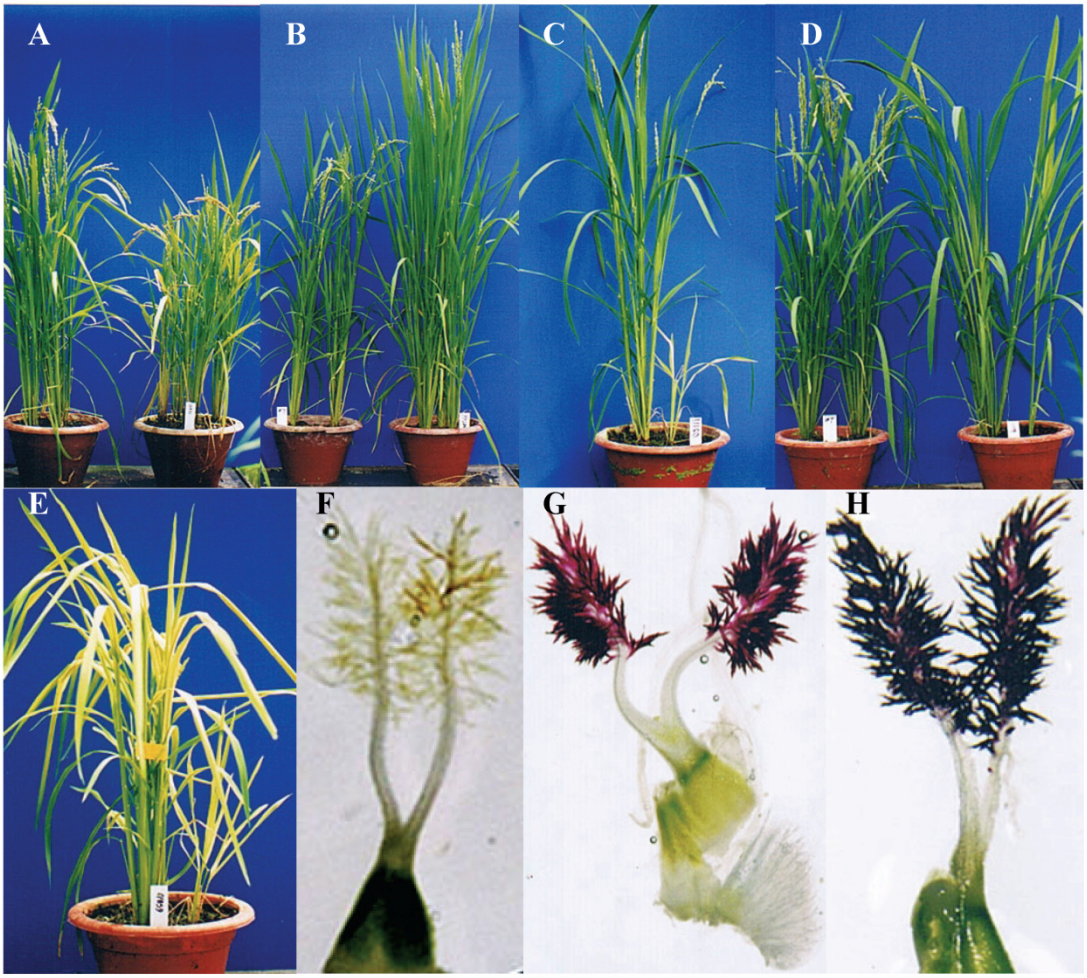

Figure 5. Phenotype investigation of *Ds* insertion lines. Left image is wild type (WT) control and right image is mutant line in A to D. (A) Dwarf phenotype. (B) Taller mutant. (C) weak-growth mutant, (D) Late-flowering mutant. E, Yellow-leaf mutant. F, Stigma in WT. G and H, Stigmas with brown color.

Phenotype screens of *Ds* insertion lines have identified two male sterile mutants. One is *Orysa sativa no pollen* (*Osnop*) mutant with a pollen-less phenotype at the flowering stage. The mutant phenotype showed linkage to *Ds* insertion into *OSNOP* gene region. This mutant contained a deletion of 65 kb chromosomal region at the site of *Ds* insertion containing 14 predicted genes. Among them, *delegen 14* was expressed only in late stage of pollen development with the highest expression at the stage of pollen release and germination by RT-PCR, Northern blotting, *in situ* hybridization, and promoter-GUS transgenic plants. Thus, this gene is the best candidate for *OSNOP*. Since this gene encoded C2 and GRAM domains, it can be assumed that this gene cross-links both calcium and phosphoinositide signaling pathways. This is the first report to suggest possible functions for this gene in plant development [119].

Another one is the myosin mutant *osmyoXIB*. This mutant showed male sterility under short day length (SD) conditions and fertility under long day length (LD) conditions. Under both SD and LD conditions, the OSMYOXIB transcript was detected in whole anthers. However, under SD conditions, the OSMYOXIB-GUS fusion protein was localized only in the epidermal layer of anthers due to the lack of 3′-untranslated region (3′-UTR) and to dilute (DIL) domain sequences following the *Ds* insertion. As a

result, mutant pollen development was affected, leading to male sterility. By contrast, under LD conditions, the fusion protein was localized normally in anthers. Despite normal localization, the protein was only partially functional due to the lack of DIL domain sequences, resulting in limited recovery of pollen fertility [120]. Since this mutant is a photoperiod sensitive male sterile line, it can be a candidate line to develop new male sterile lines for producing two-line hybrid rice.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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