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Transgenic Plants as Gene-Discovery Tools

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1. Introduction

Mutation study is an important strategy to dissect gene functions. Classical chemical or irradiation mutagenesis is one of the most powerful screen approaches to uncover genes involved in certain genetic pathways. However, the tedious works to hunt down the gene corresponding to a mutant allele by map-base cloning make an intrinsic limitation of this approach. Loss-of-function mutations resulting from Transferred DNA (T-DNA) insertion or Mobile Genetic Elements (MGE) insertion have overcome this shortcoming. Transgenic approaches are also useful to circumvent the difficulties in the study of gene function because of genetic redundancy or lethality. Gain-of-function mutations achieved by activated expression of endogenous genes by transcription enhancer or by specific gene over-expression through transformation have also revealed function of many plant genes. This review will describe the major loss or gain-of-function mutagenesis approaches by T-DNA vector transformation or endogenous MGE and those available insertion mutant resources which have greatly facilitated the researchers to extensively identify gene functions in the past few years.

2. Loss-of-function mutations

The most conventional tool for the functional analysis of all genes in a certain organism is to generate indexed loss-of-function mutagenesis on a whole genome scale. For example, the creation of gene-indexed loss-of-function mutations for all genes has been achieved decades ago in the unicellular budding yeast *Saccharomyces cerevisiae* by gene replacement via homologous recombination (Ross-Macdonald et al. 1999; Winzeler et al. 1999; Giaever et al. 2002). However, genome wide gene disruptions with confirmed index information are not so easy for the multi-cellular eukaryotes. Loss-of-function can be achieved by chemicals

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such as ethyl methannesulfonate (EMS) or by high energy irradiation such as fast neutrons to introduce random mutations (Ostergaard and Yanofsky 2004). These methods are easy to generate large scaled mutagenesis, but the mutation site identifications by traditional forward genetic method need tedious work. Although popular to individual research groups who focus on certain aspect of their interested field, such kinds of methods are not convenient for systematic research of gene functions on a genome wide scale.

Loss-of-function mutations achieved by T-DNA insertion or MGE insertion provide practicable methods to identify the genes disrupted by these transfer elements because their insertion sites can be explored by PCR base methods such as tail-PCR, plasmid rescue, inverse PCR or adapter PCR (Liu et al. 1995; Liu and Whittier 1995; Spertini et al. 1999; Yamamoto et al. 2003). *Arabidopsis* is currently the only multi-cellular organism reported possible to achieve saturation of mutation with accurate index for each genes because *Arabidopsis* owns several crucial advantages than other species: genome with small size, easily to be transformed, self-pollination, short life cycle and bulk storage of seeds. Rice is the second better learned species that has been widely used for establishing systematic insertion mutant libraries because rice is one of the most important crops and easy to be transformed by *Agrobacterium* mediated transformation. So this review will introduce the basic strategies of the major transgenic approaches using *Arabidopsis* and rice as model plants and summary the emerged transgenic plant resources which have become the most powerful gene discovery tools in the plant kingdom.

2.1 Saturated T-DNA insertion

The ultimate goal of genome research is to characterize the function of all the genes. As the major step towards this goal, the genome sequencing projects of several plant species were already completed. However, functional genomics studies are still in progress to deduce the functions of all the sequenced genes. Saturation mutagenesis by T-DNA insertion is one of the most successful approaches for analysis of systematic gene functions in the past decade years benefitting from the continuous improvement of transgenic techniques (Parinov and Sundaresan 2000). The *Agrobacterium* vacuum infiltration method for *Arabidopsis* transformation was developed to avoid the complex transgenic process of tissue culture based method including introduction of DNA by particle bombardment or *Agrobacterium* and plant regeneration (Bechtold 2003). This method was further modified and the transgenic *Arabidopsis* can be easy achieved by simply dip the floral tissue into a solution containing sucrose, surfactant Silwet L-77 and *Agrobacterium tumefaciens* carrying target genes (Clough and Bent 1998). The basic scheme for the generation of T-DNA insertion mutants by flora dip method for *Arabidopsis* is shown in Figure 1. *Agrobacterium* harbouring the binary vector was used to transform *Arabidopsis* by floral dip method. The T-DNA region between left border (LB) and right border (RB) of the binary vector was randomly transformed into the genome of host plant. The transgenic plants can be screened by selection marker depends on the T-DNA vector used (For example: *NPTII* is the selection marker of pROK2 T-DNA vector which is used to generate Salk insertion mutant lines). The flanking sequence at both sides of insertion can be further sequenced by PCR based method.Taken the advantage of flora dip method, a vast number of T-DNA insertion lines have been generated which represent almost saturated insertions into *Arabidopsis* genes in past ten years.

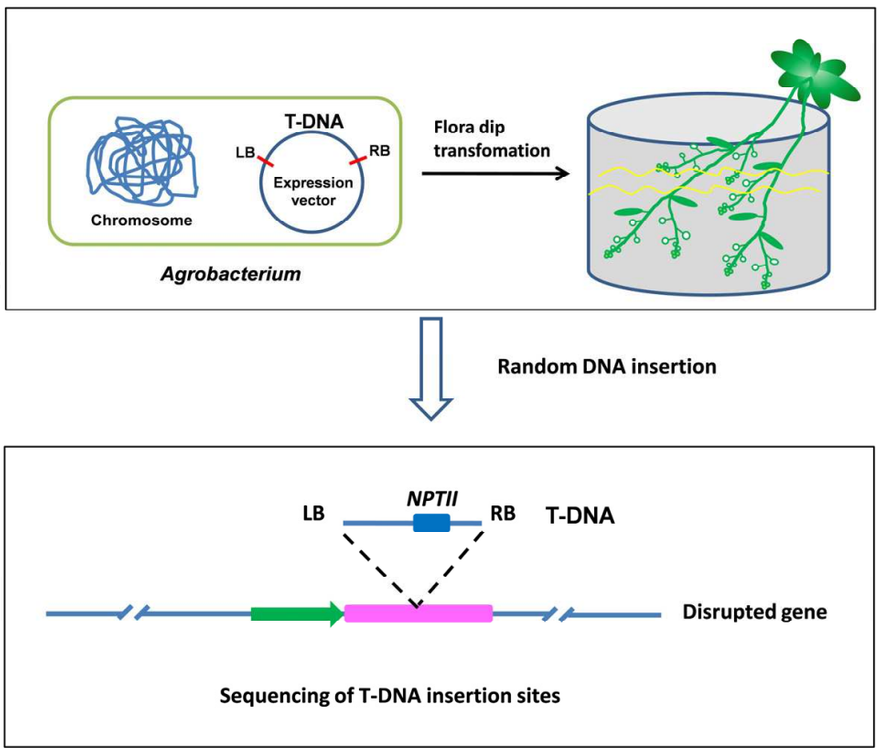

Fig. 1. A basic scheme for the T-DNA insertion method by flora dip in *Arabidopsis*.

2.2 The enhancer trap system

Besides to disrupt the gene functions by direct insertion for large-scale discovery of gene function, T-DNA can also be utilized to identify novel regulatory elements. As a further development of T-DNA random insertion strategy, the enhancer trap system is established by random integration of a report gene cassette as the T-DNA into the genome. This reporter cassette includes a minimum or truncated promoter which is not able to drive the expression of the report gene unless activated by the endogenous regulatory sequence close to the integration site (Figure 2). Therefore, the expression of reporter gene implies the existence of enhancer element close to the insertion site. Because the T-DNA cassette of enhancer trap system can both generate gene mutations and report the presence of enhance element around the insertion site, the enhancer trap system was widely applied in the bacterium, Drosophila, *Arabidopsis*, moss and rice to unveil gene functions and identify regulator elements (Casadaban and Cohen 1979; Sundaresan et al. 1995; Bellen 1999; Campisi et al. 1999; Hiwatashi et al. 2001; Zhang et al. 2006).

As a successful example, the Rice Mutant Database (RMD) is established with the enhancer trap system and maintained by National Center of Plant Gene Research (Wuhan) at Huazhong Agricultural University. The enhancer trap T-DNA fragment used by RMD carries three critical components as indicated in Figure 2:

a. -48 CaMV minimum promoter. This truncated promoter is unable to drive gene expression unless there is a transcription enhancer element close to it.

b. A recombinant DNA sequence encoding an artificial transcriptional activator by the combining of GAL4 and VP16. GAL4 is a DNA bind domain which specifically binds to the Upstream Activator Sequence (UAS) and V16 is a transcriptional activator domain derived from a herpesvirus protein (Utley et al. 1998) which is able to activate the expression of gene adjacent to the UAS.
c. The *β-Glucuronidase* (*GUS*) report gene downstream of the 6 tandems UAS (6×UAS).

The enhancer trap T-DNA including these three components is randomly integrated into host genome by *Agrobacterium*-mediated transformation. If the integration site is by chance neighbour to a host transcriptional enhancer element, the -48 CaMV minimum promoter will drive the expression of the GAL4/VP16 recombinant transcriptional activator which will then bind to 6×UAS and activate the expression of the GUS report gene. Some of the enhancer elements regulate spatial- or temporal gene expression and thus the transgenic lines with the T-DNA inserted near these enhancer element show expression of the report gene in a tissue specific pattern. Besides causing gene mutations and providing an efficient approach for identification of the transcriptional enhancer, enhancer trap T-DNA can create certain pattern lines which are useful to ectopic express target genes in certain tissues simply by cross the pattern lines with the target lines which are transformed by the target genes driven by 6×UAS. Figure 2 shows the basic scheme for the enhance trap system: The T-DNA of enhance trap vector includes -48 CaMV promoter, *GAL4/VP64* transcription activator and *GUS* reporter under control of 6×UAS. The transgenic lines were obtained by *Agrobacterium* mediated transformation of rice callus. The enhance trap T-DNA was randomly integrated into the rice genome and the enhancer nearby the insertion site activates the expression of *GAL4/VP64* which further promote the expression of *GUS* report gene in the Pattern line. The Target line was created through the transformation of host plant with T-DNA containing 6×UAS :: *Target gene*. By crossing Target line with Pattern line, the target gene will express in the same pattern as that of the *GUS* report gene due to the trans-activation of 6×UAS regulator by GAL4/VP64 transcription activator.

2.3 Mobile Genetic Elements (MGE) insertion

In addition to T-DNA insertion lines, MGE insertion is also a popular approach to generate large number of mutations. MGEs which can move around within the genome include several kinds of mobile DNA elements such as transposon or retrotransposon. Transposons describe the DNA which can be cut away from one site and paste to other place within the genome. Retrotransposon however, make themselves a copy and then paste to other position within the genome.

Several transposable elements identified in maize have been used to obtain large population of insertions in genes for functional genomics studies. For example, the maize transposable element *Activator* (*Ac*) first identified by McClintock (Mc 1950) is a kind of transposon widely used for creating MGE insertions. *Ac* element can insert themselves into genes and cause insertion mutations to create a recessive allele. The mutations caused this way are unstable because the *Ac* element can be excised from the inserted gene by the transposase which is coded by Ac element itself. *Dissociation* (*Ds*) element is usually stable because they are incapable of excising itself from the inserted gene unless with the help of *Ac* element.

Researchers combine these two mobile elements and named it as the *Ac/Ds* system to generate mutant populations. Generally, the individual *Ds* parental lines and *Ac* parental lines are created by transformation of *Ac* element and *Ds* element independently into the host organism. Then the two parental lines are crossed to induce the translocations of the *Ds* element in the next generation. For example as shown in Figure 3, *Ac* parent line and *Ds* parent line are created by transformation of host plants with the *Ac* element and *Ds* element respectively. By crossing *Ac* parent line and *Ds* parent line, *Ds* element is activated by *Ds* element to transfer from one position to another position within the genome which will create random disruption of gene functions in the following generations. Stable *Ds* insertion mutant lines can be created by genetic method to make *Ac* element segregated away with *Ds* element by combination of a positive selection marker on *Ds* element and a negative selection marker on *Ac* element (Sundaresan et al. 1995). Besides in maize, the *Ac* element has shown translocation activity in *Arabidopsis* (Fedoroff and Smith 1993). With the *AC/Ds* or other similar MGE systems, several research groups have generated mutant resources with a high proportion of single-copy transposon insertions (Sundaresan et al. 1995; Martienssen 1998; Tissier et al. 1999; Ito et al. 2002; Kuromori et al. 2004; Ito et al. 2005; Nishal et al. 2005)

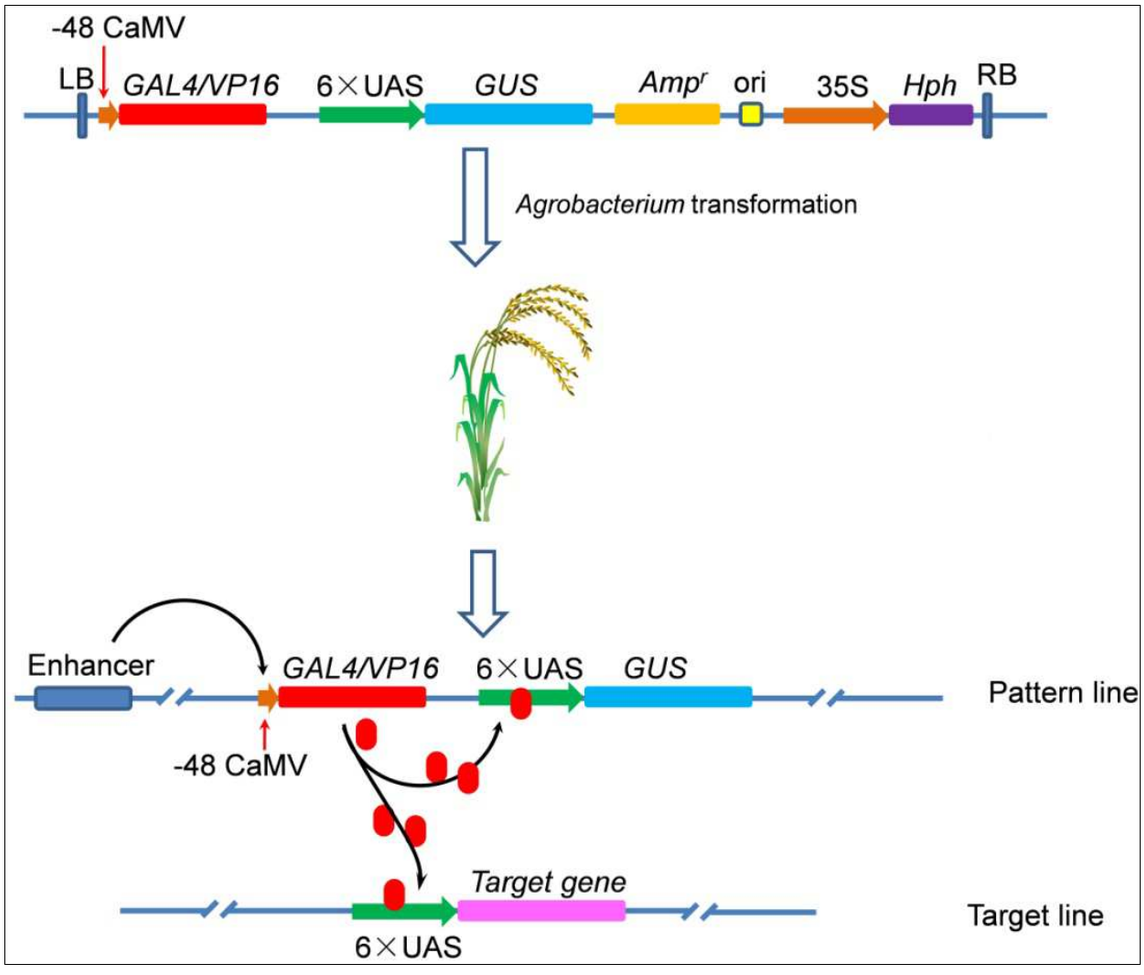

Fig. 2. Overview for the enhance trap system used by RMD.

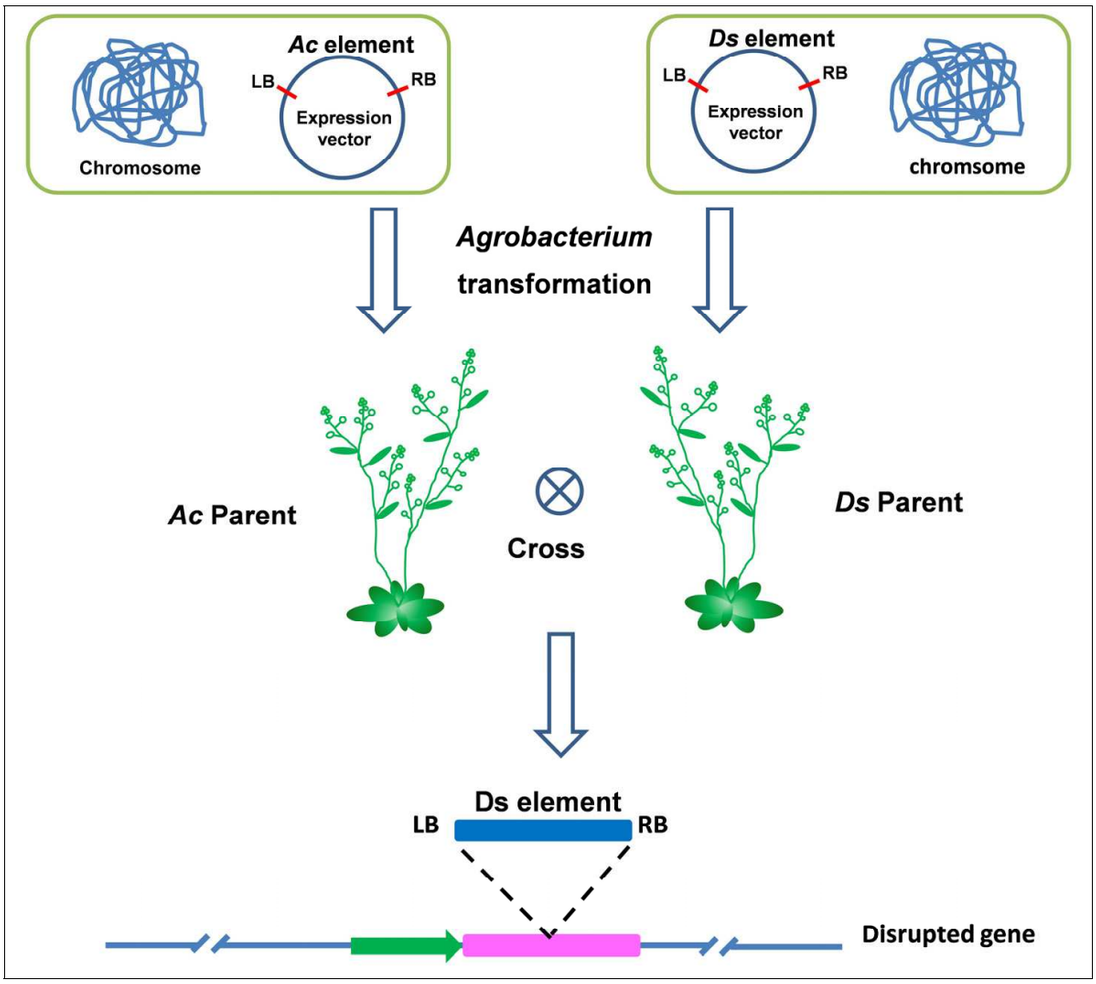

Fig. 3. Scheme for the generation of insertion mutant lines by *Ds* transposon.

Tos17 is one kind of copia-like retrotransposons in rice (Hirochika et al. 1996) ,which can duplicate and paste to elsewhere in the genome. *Tos17* owns several special features that make it suitable for engineering large scale insertion mutagenesis:

a. The copy number of *tos17* is quiet low, ranging from one to five among rice cultivars. For example, the genome of cv. Nipponbare, the selected cultivar for the IRGSP (International Rice Genome Sequencing Project)(Sasaki and Burr 2000), contains only two native copies of *tos17*.
b. Transposition of tos17 is inactive under normal conditions but only activated in the callus by tissue culture and then becoming stable again in the regenerated plants (Hirochika et al. 1996; Miyao et al. 2003).
c. The transposition site of *tos17* prefers gene-dense regions over centromeric heterochromatin regions with a three times higher insertion frequency in genic regions than in intergenic regions (Hirochika et al. 1996; Hirochika 2001; Miyao et al. 2003; Piffanelli et al. 2007).
d. Its size is just a little bit over 4kb and its insertion sequence is clearly known for flanking sequencing.

Tos17 is stably present in genome during the normal life cycle of rice. By the tissue culture of the rice callus, the transcription of *tos17* is activated and the reverse transcript DNA fragments are integrated into new places in the genome which creates disruption of genes. The original *tos17* and its duplications become silence again in the regenerated insertion mutant plants (Figure 4). Taking these advantages of *tos17*, Large-scale *tos17* T-DNA mutant library of Nipponbare has been established by tissue culture and stably preserved by normal generation (Miyao et al. 2003; Sallaud et al. 2004; Miyao et al. 2007; Piffanelli et al. 2007).

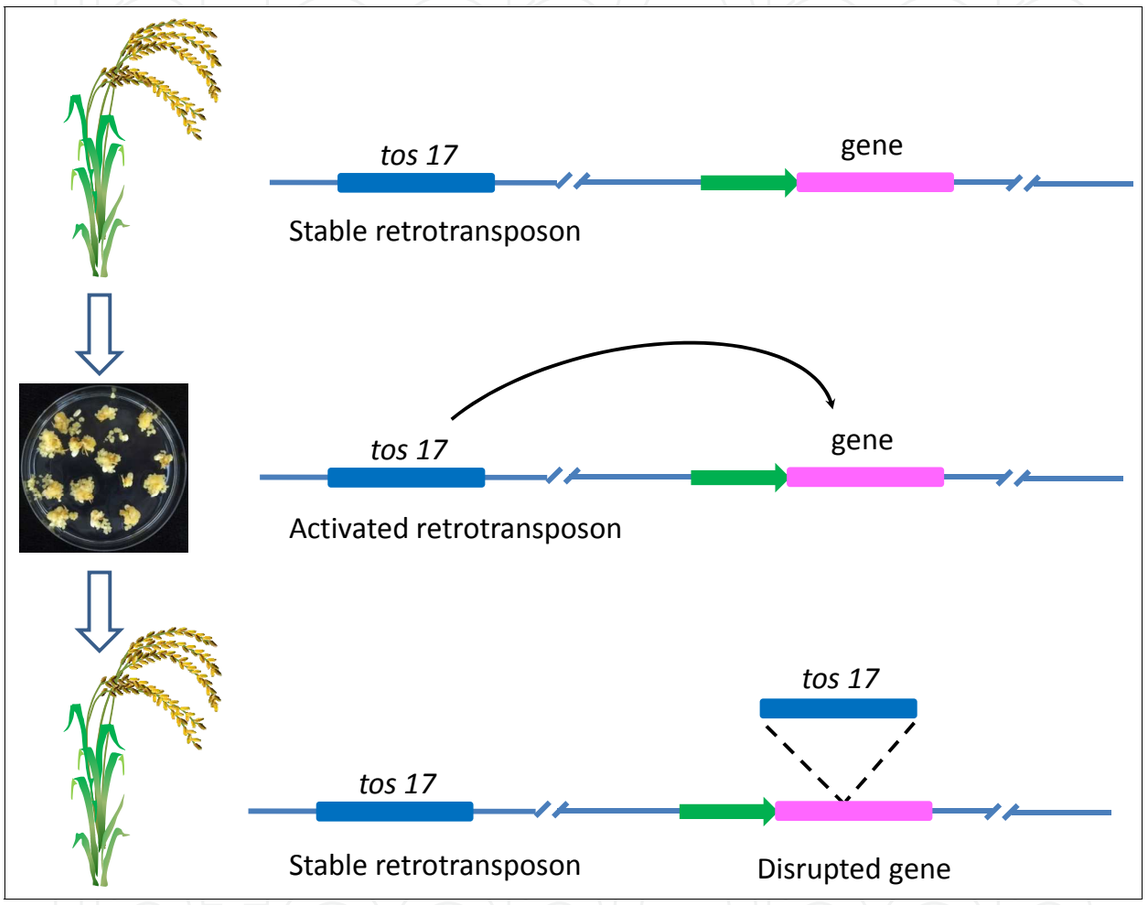

Fig. 4. Scheme for the generation of *tos17* insertion mutant lines by tissue culture.

3. Gain-of-function

Screen for loss-off-function mutations is a primary tool for dissecting a genetic pathway. However, because many genes belong to gene families, loss-of-function screens are not always possible to identify genes that act redundantly. In addition, some genes are critically required for the survival of plants. The homozygote mutants of these genes will not be available for entire function research because of embryonic or gametophytic lethality. As another option, gain-of-function technologies were developed to compensate the limitations of loss-of-function approaches or confer new function in transgenic plants, which is achieved through activation expression of endogenous genes by transcription enhancer which is randomly introduced into the genome or through ectopic gene over-expression driven by constitutive promoter.

3.1 Activation tagging

Activation tagging is a gain-of-function method that generates transgenic plants by T-DNA vectors with tetrameric cauliflower mosaic virus (CaMV) 35S enhancers which can lead to an enhancement expression of adjacent genes in the distance ranging between 0.4 to 3.6kb from the insertion site (Weigel et al. 2000). Differently from the action of the complete CaMV 35S promoter, CaMV 35S enhancers can activate both the upstream and downstream gene transcription. In addition, it has been reported that in at least one case, rather than led to constitutive ectopic expression, CaMV 35S enhancers elevate transcriptional activity based on the native gene expression pattern (Weigel et al. 2000).

Activation tagging technique was firstly developed by Walden and colleagues in decades years ago (Hayashi et al., 1992). Since then, several large scale activation tagging mutant resources have been generated and activation tagging method was widely used to isolate new genes. As an early example, the activation-tagging technique was used in tissue culture to identify cytokinin-independent mutants in *Arabidopsis* and *CKI1* gene whose overexpression can bypass the requirement for cytokinin in the regeneration of shoots was identified (Kakimoto, 1996). Based on the original activation tagging vectors, Weigel and colleagues (2000) developed new generation of vectors possessing resistance to the antibiotic kanamycin or herbicide glufosinate which is low toxic to humans or easy to select transgenic plants in soil in large scale. By screening a set of the transgenic lines, they identified 11 dominant mutants with obviously morphological phenotypes and 9 of them were confirmed due to the activation of adjunct genes by reproducing the phenotype in a new set of transgenic lines through overexpression of the adjunct candidate genes on both sides of insertion.

To accelerate the recapitulation process of phenotype resulted from the enhancer of T-DNA insertion, a new activation-tagging method has been developed using a pair of plasmids including pEnLOX and pCre. pEnLOX contains multimerized CaMV 35S transcriptional enhancers flanked by two *lox P* sites on both sides while pCre includes the *cre* gene which can remove the DNA sequence between two *lox P* sites (Pogorelko et al. 2008). the activation-tagging lines containing the pEnLOX were named the E-lines, and the helper lines containing pCre was named the C-lines. By crossing the E-lines with the C-lines, the CaMV 35S enhancers can be removed from the chromosome coming from E-lines and thus the reversion from mutant phenotypes to the wild-type phenotype may be detected in the next generation.

Activation tagging has also been applied to generate rice activation-tagging lines (Jeong et al. 2002; An et al. 2005; Jeong et al. 2006; Hsing et al. 2007; Wan et al. 2009). Based on the basic activation tagging technology, a dual function T-DNA vectors have been developed for both promoter trapping and CaMV 35S enhancers activation tagging(Jeong et al. 2002). By analysis of the gene expression in these rice activation tagging lines, the authors reported the activated the expression of genes located up to 10.7 kb from insertion site of the enhancers (Jeong et al. 2006). The activation tagging vector pSK1015 is used as an example in Figure 5. The T-DNA contains *BAR* gene as transgenic plant selection marker and 4×35 enhancers as activation element. The activation tagging T-DNA is integrated into the genome of host plants by *Agrobacterium* mediated transformation. The expressions of both side genes around the inserted T-DNA enhancer are elevated.

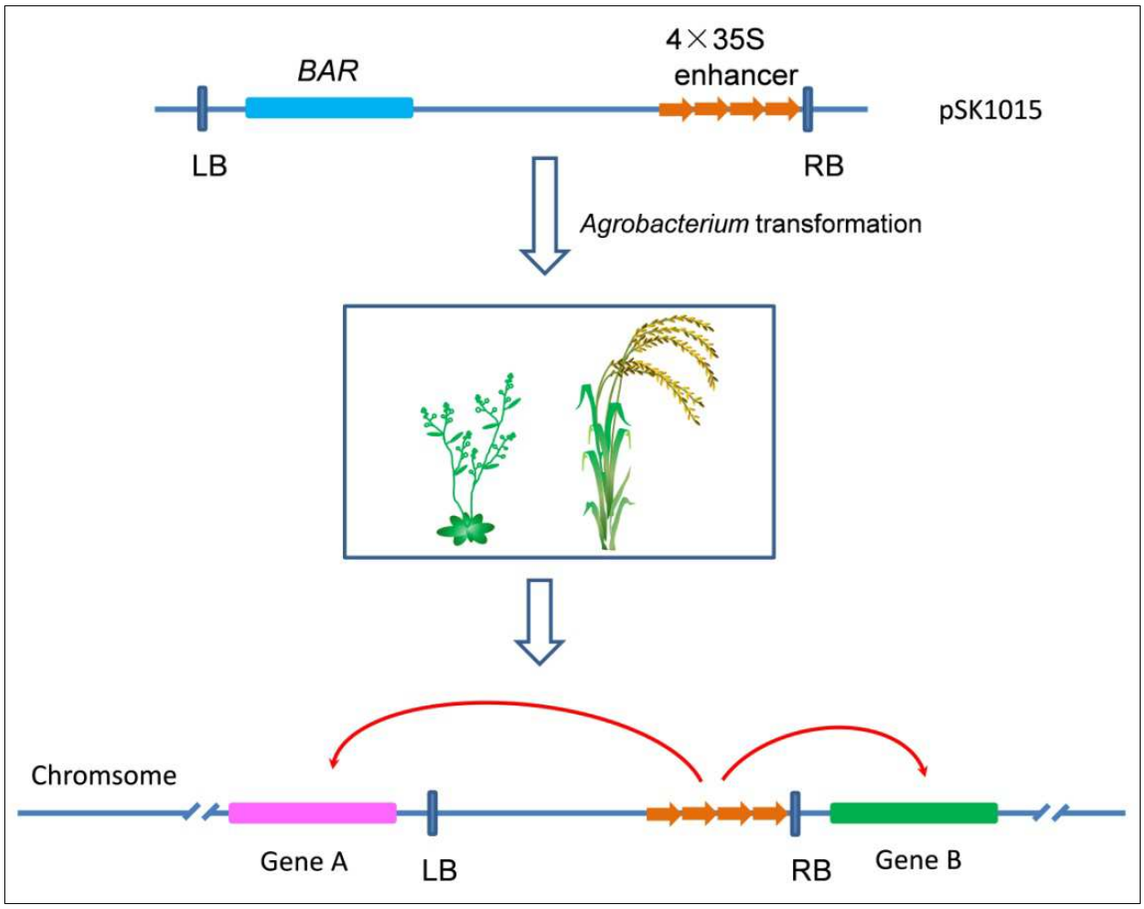

Fig. 5. Scheme for the generation of activation tagging transgenic plants.

3.2 Fox hunting system

FOX hunting system (full-length cDNA overexpressor gene hunting system) is used to ectopic expression of full-length cDNAs (fl-cDNA) in plants to generate systematic gain-of-function mutant populations (Figure 6). The most significant difference between FOX hunting system and other transgenic gene discovery methods is that FOX need to construct a large number of expression vectors containing as many as possible the independent fl-cDNAs, while other methods generally depend on one or a small number of expression vectors. Although the construction of numerous vectors seems labour-intensive, FOX hunting system shows unique benefits in discovery of new gene functions and potential utility of heterologous genes in improvement of agronomic traits.

Firstly, FOX hunting system can be flexibly used to systematically research of gene functions by generation of transgenic plants (also named FOX lines) expressed the fl-cDNA derived from the same species or heterologous host.

The first systematic gain-of-function transgenic population by FOX hunting system was produced by overexpression of *Arabidopsis* fl-cDNAs in *Arabidopsis* and these FOX lines showed various physiological and morphological phenotypes. Thus far, FOX approach has

been applied to generate several other FOX population using *Arabidopsis* or rice as model plants by the transformation of rice with rice fl-cDNA or the transformation of *Arabidopsis* with rice fl-cDNA. In addition, FOX hunting technology is an idea approach to systematically investigate the gene functions for those plants such as maize and wheat, the genome of which are too large or not easy to be sequenced.

Secondly, FOX hunting system is powerful to identify lead genes from relative or distant species to improve the traits of plants. It's interesting that the *Arabidopsis* FOX lines with ectopic expression of some rice genes that has no homolog gene in *Arabidopsis* also display abnormal phenotypes, which demonstrates that heterologous gene expression could introduce new function among different species. FOX approach has also been used to screen salt stress tolerance genes in salt cress (*Thellungiella halophila*). As a well know example of Monsanto's huge success by the similar approach, the gene (*BAR* or *PAT*) resistance to herbicide, firstly isolated from *Streptomyces* bacterial (Thompson et al. 1987), has been widely used to generate transgenic varieties of crops including canola, cotton, maize etc., for resistance to glufosinate which interferes with the biosynthesis of the amino acid glutamine and ammonia detoxification and causes cessation of photosynthesis. Therefore, this approach has the great potential to identify desirable genes to improve the agronomic trait of crop varieties. Figure 6 shows the basic scheme of FOX hunting system: Full length cDNAs were cloned into the FOX hunting over-expression vectors. Those individual vectors were transformed into host plants by mixed *Agrobacterium*. The full length cDNA over-expression cassettes were randomly inserted into the host genome and the ectopic expression of exogenous full length cDNAs can induce abnormal phenotypes.

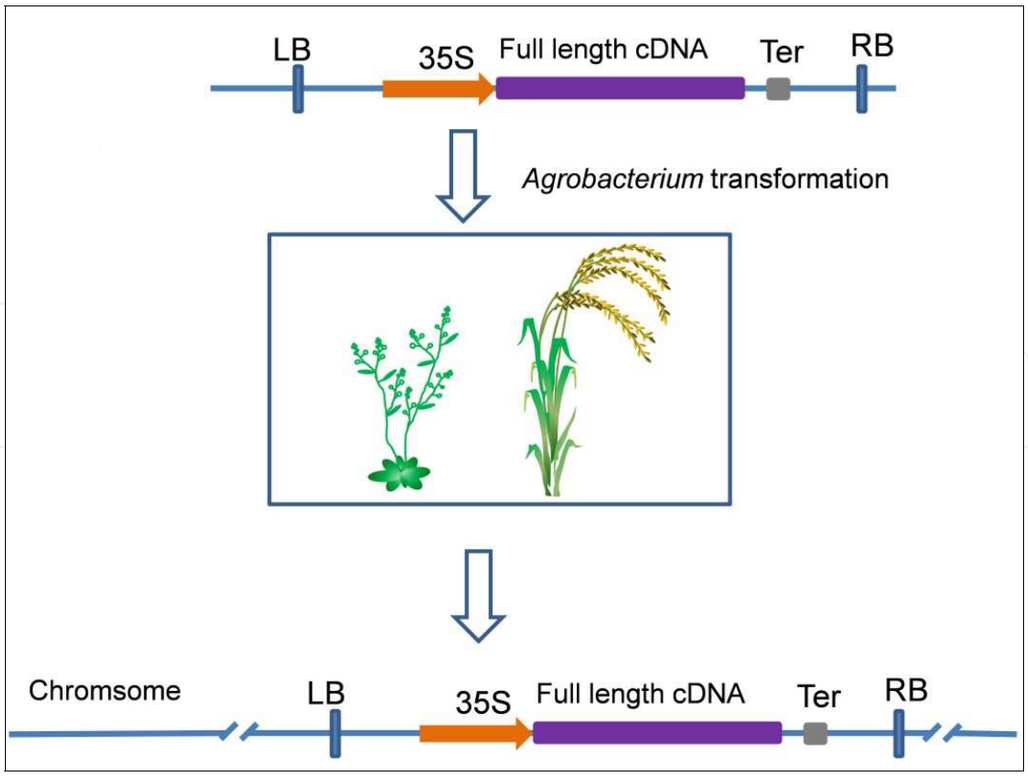

Fig. 6. Scheme for the generation of FOX hunting transgenic plants.

4. Summary of the transgenic resources

By reviewing the research progress in past few years, we are always impressed by that most of the basic scientific discoveries in plant field were using *Arabidopsis* or rice as the model plants. One of the significant reasons is that these two species are easiest to be transformed than other plants and thus the researchers can take the advantages of the public available transgenic resources to test their hypothesis in a relatively short period of time. In the following sections, we summarized the transgenic resources in Table 1 and described those representative ones for each of the methods. Some of the MGE insertion mutant lines are generated by endogenous transposon or retrotransposon rather than artificial transgene. Regarding those MGEs disrupt the gene functions in the similar way as T-DNA insertion and MGE mutant lines represent an important portion of genetics resources, we also introduce them briefly in this review for the interest of readers.

4.1 T-DNA insertion resources

SALK T-DNA insertion database is the most successful example in the history of transgenic resource so far. In 2003, over 225,000 *Arabidopsis* T-DNA insertion mutants have been generated by the SALK Institute, resulting in more than 88,000 insertions with precise locations determined in the whole genome of *Arabidopsis* (Alonso et al. 2003). Till recently, the numbers of T-DNA insert sites mapped to the genome of *Arabidopsis* by the Salk Institute have been over 150,000. Combined with the collections of other resources (SAIL, Wisc and GABI-KAT) around the world (Sessions et al. 2002; Rosso et al. 2003; Nishal et al. 2005; Li et al. 2007), totally 25,762 genes were identified with at least one T-DNA insertion mutation, which represent nearly 83% of the entire 31,128 protein-coding and non-coding RNA genes in the *Arabidopsis* genome (http://natural.salk.edu/geno/sum.txt). In addition, large-scale genotyping of those T-DNA insertion mutant lines has been ongoing for nearly 6 years to obtain as much as possible homozygous insertion mutants. As of today, 44,122 homozygote T-DNA lines, representing 24,476 individual genes have been sent to *Arabidopsis* Biological Resource Center (ABRC) for reproduction and distribution (http://signal.salk.edu/cgi-bin/homozygotes.cgi) (http://abrc.osu.edu/). The SALK Homozygote T-DNA Collection Project is nearly completed and this resource greatly facilitates the researchers to analyze the comprehensive phenotype and further understand the systematic gene functions at a genome wide level. Some other T-DNA insertion mutant resources are available for researchers to refer and utilize, which are listed in the Table 1.

4.2 Enhancer trap resources

The earlier Enhancer trap resource is reported in 1995 by Robert Martienssen and colleagues in the Cold Spring Harbor Laboratory (CSHL). They designed an ingenious strategy which combined the *Ac/Ds* MGE insertion method, Gene trap and Enhancer trap technology to generate a large DNA insertion population and 21,661 insertion events have been mapped to the genome of *Arabidopsis* (Sundaresan et al. 1995; Martienssen 1998). NASC established by Jim Haseloff and colleagues in the University of Cambridge contains 250 GAL4-GFP enhancer-trap lines which using GFP as report gene (Haseloff et al. 1997). Based on the similar strategy, the researchers in the Department of Biology, University of Pennsylvania established Enhancertraps database providing information of *Arabidopsis* lines transformed

with Jim Haseloff's GAL4 enhancer trap vector. This database currently release 510 enhancer trap transgenic lines that show specific expression pattern in multiple kinds of organs, tissues or cell type. RMD (Rice Mutant Database) is a successful enhancer trap resource of rice generated by Qifa Zhang and colleagues as describe in section 2.2. Since first released in 2006, approximate 132,193 T-DNA insertion lines generated by this enhancer trap system have been characterized with the flanking sequence of insertion, report genes expression or phenotype of transgenic mutation. RMD currently releases the comprehensive information of the large scaled transgenic lines including the flanking sequences of T-DNA insertion sites, seed availability, reporter-gene expression patterns, as well as mutant phenotypes, etc (http://rmd.ncpgr.cn/).

4.3 MGE insertion resources

Multiple MGEs identified in maize and some of them have been exploited to generate insertion mutant resources for maize as well as other plants such as *Arabidopsis*. For example, Exon Trapping Insert Consortium (EXOTIC) utilized both *Ac/Ds* system and *Enhancer/Suppressor-mutator* (*En/Spm*) system to create large insertion mutant populations and 23,537 insertion sites have been mapped to the genome of *Arabidopsis* (Tissier et al. 1999). Also using the *Ds* element, RIKEN BioResource Center (RIKEN BRC) built a resource containing 18,566 *Arabidopsis* mutants with insertion positions mapped to the genome. As an alternative, *tos17* retrotransposon characterized in rice is employed in mutational analysis of rice genome by several institutes such as National Institute of Agrobiological Sciences (NIAS)(Miyao et al. 2003) and Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)(Sallaud et al. 2004). Because other plant species are not easy to be transformed by direct trangene method, MGE insertion may become a more and more important strategy for the functional genomics studies of those plants other than *Arabidopsis* or rice.

4.4 Activation tagging resources

As a useful supplement of loss-of-function mutant resources mentioned above, activation tagging technique was developed and widely used to generate gain-of-function resources. Currently, there are 22,600 activation-tagging lines available in SALK and NASC (Weigel et al. 2000). RIKEN also established a large scale of activation-tagging lines with the number of 32,650 (Nakazawa et al. 2003). With the same method, 47,932 T-DNA tag lines in japonica rice were generated using activation-tagging vectors in POSTECH Rice T-DNA Insertion Sequence Database (RISD) (Jeong et al. 2006). Taiwan Rice Insertional Mutant (TRIM) created about 45,000 activation tagging lines using a T-DNA vector containing an enhancer octamer(Hsing et al. 2007). As a T-DNA insertion resource that using hosts plants other than *Arabidopsis* and rice, GmGenesDB in University of Missouri created 900 activation tagging lines of soybean.

4.5 Fox hunting resources

FOX hunting system was developed by a novel gain-of-function system that used normalized full-length cDNA introduced into host plants via large scaled transformation (Ichikawa et al. 2006). The first FOX hunting resource were established by transformation of

Arabidopsis with 10,000 *Arabidopsis* full length cDNA driven by 35S promoter, which contains more than 15,000 transgenic lines with 2.6 cDNA insertions on average in the genome. By a similar approach, they created 12,000 independent FOX hunting lines by transformation of rice with rice full length cDNA (Nakamura et al. 2007). The third FOX hunting system were created by transformation of *Arabidopsis* with the rice Full length cDNA for generating a heterologous gene resource which contain more than 23,000 independent *Arabidopsis* transgenic lines that expressed rice fl-cDNAs (Kondou et al. 2009).

5. Perspective

Take advantage of the dramatic improvements in transgenic efficiency, researchers are able to generate gain-of-function and loss-of-function transgenic resources with vast numbers of transformed *Arabidopsis* and rice plants. As a general procedure to utilize the loss-of-function resources, the individual researchers firstly order their interested insertion mutant lines by web based search of related database and then perform a PCR based genotyping to confirm the T-DNA insertion in their interested gene. Following a careful analysis of phenotype of the insertion homozygote lines, the researchers will confirm the abnormal phenotype of the mutants caused by T-DNA insertion via complementary experiment which is to transform the insertion mutant with a functional intact gene to rescue the phenotype. One of the common problems faced to the researches is that how should we do if the homozygote mutants show no obviously phenotype due to homolog gene redundancy or redundant genetic pathway to other gene with similar function. The first solution is to order all the redundant mutants and cross them to create double, triple or even quadruple mutant. If the redundant genes are correlated too close on the chromosome, it would be impossible to get the homozygote multiple mutants. Fortunately, other methods such as RNA interference, artificial micro RNA technology or TILLING (Targeting Induced Local Lesions in Genome) method are developed which can combine with the genetic cross method to create loss-of-function mutant of multiple gene loci (Schwab et al. 2006; Till et al. 2007; Warthmann et al. 2008). However, genetic pathway redundancies rather than homolog gene redundancies are not rare. In such case, the researchers will have neither homolog genes nor literature information to help them to figure out which gene loci should they use to create multiple mutant. New strategy may be developed to generate multiple site mutation resources for the researchers to screen obvious phenotype and further digest the complex genetic pathway.

To compensate the shortage of loss-of-function methods, gain-of-function transgenic can cause obvious phenotype even if the gene is redundant with other gene or the loss-of-function insertion is lethal. In addition, gain-of-function method has potential to discover useful genes which can be utilized to improve the agronomic traits of economic plants. For example, salt cress (*Thellungiella halophila*) is a very salt-tolerant species which are closely related to *Arabidopsis* (90-95% DNA sequence identity). In order to isolate salt stress tolerance genes, salt cress cDNAs under CaMV 35S promoter were transformed into *Arabidopsis* in a large scale and two genes ST6-66 and ST225 were discovered to improve the salt tolerance of *Arabidopsis* (Du et al. 2008). As another example described before, *Arabidopsis* FOX lines with ectopic over expression of rice specific genes in *Arabidopsis* also show abnormal phenotypes, further indicating that genes from various germplasms could

introduce novo functions. Actually, genes from distant species have already been successfully used to improve the agronomic traits of crops such as *BAR* gene used for weed control, or *CRY* gene (encoding Bt toxin in *Bacillus thuringiensis*) used for pests control (Thompson et al. 1987; Bravo et al. 2007). We speculate that gain-of-function methods will be further engaged to identify more useful genes to improve crop traits as an important goal of plant science.

	method	Resource	Host plant	Web site for the resource	Reference
Lose of Function	T-DNA insertion	SALK Institute, ABRC	*Arabidopsis*	http://signal.salk.edu/tabout.html	(Alonso et al. 2003)
		Syngenta, SAIL	*Arabidopsis*	N/A	(Sessions et al. 2002)
		WiscDslox	*Arabidopsis*	http://www.hort.wisc.edu	(Nishal et al. 2005)
		GABI	*Arabidopsis*	http://www.gabi-kat.de	(Rosso et al. 2003; Li et al. 2007)
		INRA, FLAGdb	*Arabidopsis*	http://urgv.evry.inra.fr/FLAGdb	(Samson et al. 2002)
		POSTECH	Rice	http://www.postech.ac.kr/life/pfg/risd/index.htm	(Jeon et al. 2000)
		SHIP	Rice	http://ship.plantsignal.cn/index.do	(Fu et al. 2009)
		TRIM	Rice	http://trim.sinica.edu.tw/	(Hsing et al. 2007)
		ZJU	Rice	http://www.genomics.zju.edu.cn/ricetdna.html	(Chen et al. 2003)
	Enhancer trap	CSHL	*Arabidopsis*	http://genetrap.cshl.org/	(Sundaresan et al. 1995; Martienssen 1998)
		NASC	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=24	n/a
		University of Penn	*Arabidopsis*	http://enhancertraps.bio.upenn.edu/default.html	n/a
		RDM	Rice	http://rmd.ncpgr.cn	(Zhang et al. 2006)
	MGE insertion	EXOTIC	*Arabidopsis*	http://www.jic.bbsrc.ac.uk/science/cdb/exotic/index.htm	(Tissier et al. 1999)
		RIKEN	*Arabidopsis*	http://rarge.gsc.riken.go.jp/dsmutant/index.pl	(Ito et al. 2002; Kuromori et al. 2004; Ito et al. 2005)
		NIAS	Rice	http://www.dna.affrc.go.jp/database	(Miyao et al. 2003)
		OTL, CIRAD	Rice	http://urgi.versailles.inra.fr/OryzaTagLine/	(Sallaud et al. 2004)
		UCD	Rice	http://www-plb.ucdavis.edu/labs/sundar/Rice_Genomics.htm	(Kolesnik et al. 2004)

		CSIRO	Rice	http://www.pi.csiro.au/fgrttpub/	(Eamens et al. 2004)
		GSNU	Rice	N/A	(Kim et al. 2004)
		EU-OSTID	Rice	http://orygenesdb.cirad.fr	(van Enckevort et al. 2005)
		Maize GDB	Maize	http://www.maizegdb.org/rescuemu-phenotype.php	(Fernandes et al. 2004)
Gain of function	Activation tagging	SALK, NASC	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=59	(Weigel et al. 2000)
		RIKEN	*Arabidopsis*	http://activation.psc.database.riken.jp	(Nakazawa et al. 2003)
		Plant Research International	*Arabidopsis*	N/A	(Marsch-Martinez et al. 2002)
		TAMARA	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=71	(Schneider et al. 2005)
		NI Vaviliv Institute of General Genetics RAS	*Arabidopsis*	N/A	(Pogorelko et al. 2008)
		JIC activate line	*Arabidopsis*	http://arabidopsis.info/CollectionInfo?id=29	n/a
		RISD	Rice	http://www.postech.ac.kr/life/pfg/risd	(Jeong et al. 2006)
		TRIM	Rice	http://trim.sinica.edu.tw	(Hsing et al. 2007)
		GmGenesDB	soybean	http://digbio.missouri.edu/gmgenedb/index.php	(Mathieu et al. 2009)
	FOX hunting	RIKEN	*Arabidopsis*	http:// nazunafox.psc.database.riken.jp	(Ichikawa et al. 2006)
		RIKEN, NIAS,RIBS Okayama	*Arabidopsis*	http:// ricefox.psc.riken.jp	(Kondou et al. 2009)
		NIAS	Rice	N/A	(Nakamura et al. 2007)

Table 1. T-DNA or MGE insertion mutant resources (N/A, not available).

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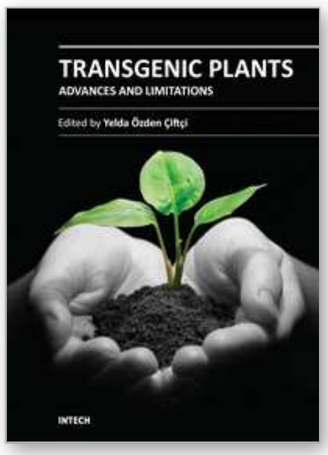

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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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