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A Versatile Transposon-Based Activation Tag Vector System for Functional Genomics in Cereals and Other Monocot Plants[1]

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ABSTRACT

Transposon insertional mutagenesis is an effective alternative to T-DNA mutagenesis when transformation through tissue culture is inefficient as is the case for many crop species. When used as activation tags, transposons can be exploited to generate novel gain-of-function phenotypes without transformation, and are of particular value in the study of polyploid plants where gene knockouts will not have phenotypes. We have developed an *in cis* activation tagging *Ac*-*Ds* system in which a T-DNA vector carries a *Ds* element containing 4x CaMV enhancers along with the *Ac* transposase gene. Stable *Ds* insertions were selected using dual GFP/RFP fluorescence marker genes driven by promoters that are functional in maize (*Zea mays*) and rice (*Oryza sativa*). The system has been tested in rice, where 638 stable *Ds* insertions were selected from an initial set of 26 primary transformants. By analysis of 311 flanking sequences mapped to the rice genome, we could demonstrate the wide distribution of the elements over the rice chromosomes. Enhanced expression of rice genes adjacent to *Ds* insertions was detected in the insertion lines using semi-quantitative RT-PCR method. The *in cis* 2-element vector system requires minimal number of primary transformants and eliminates the need for crossing, while the use of fluorescent markers instead of antibiotic or herbicide resistance increases the applicability to other plants and eliminates problems with escapes. Since *Ac*-*Ds* has been shown to transpose widely in the plant kingdom, the activation vector system developed in this study should be of utility more generally to other monocots.

INTRODUCTION

Genetic mutants have always played a central role as tools for functional analysis of plant genes. Many plant genes have been isolated by the strategy of insertional mutagens. In the model plants of Arabidopsis and rice, large-scale T-DNA and transposon insertion libraries and flanking sequence tag (FST) database have been generated, which serve the plant biologists worldwide for both forward and reverse genetics studies (Parinov *et al.*, 1999; Tissier *et al.*, 1999; Jeon et al., 2000; Ito *et al.*, 2002; Kuromori et al., 2004; Ito *et al.*, 2005). Transposon insertional mutagenesis is an effective alternative to T-DNA mutagenesis when transformation through tissue culture is inefficient as is the case for many crop species. The strategy for transposon mutagenesis requires just a limited number of primary transformants, with insertions being generated through propagation.

On the other hand, although the classic insertional mutagenesis strategies play important role in plant functional genomics studies, a limitation of such methods is the difficulty of identifying genes that are redundant in plant genomes and whose knockouts do not induce phenotypes. Activation tagging is an effective approach for overcoming this limitation. Activation tagging involves introduction of a T-DNA containing regulatory sequence such as the enhancer of the CaMV 35S promoter randomly into a plant genome to enhance expression of nearby genes, which potentially resulted in a gain-of-function phenotype (Kardailsky et al., 1999; Borevitz et al., 2000; Weigel et al., 2000; Jeong et al., 2002; Mathews et al., 2003; Jeong et al., 2006; Mori et al., 2007; Hsing et al., 2007).

As for the functional genomics of cereals and monocot plants, maize transposable elements are useful tools for generating large collection of gene knockouts because high-throughput T-DNA transformation is not a vial approach for those plant species. A number of maize genes have been isolated from transposon-tagged mutants. Several studies have shown that the maize *Ac-Ds* transposable elements function in barley and mapped *Ds* launching pads were developed for study of functional genomics (Koprek et. al, 2000; Cooper et al., 2004; Singh et al., 2006; Zhao et al., 2006). Ayliffe et al. (2007) reported a novel *Ac-Ds* system in which a modified *Ds* element (*UbiDs*) carrying two maize ubiquitin 1 promoters (Christensen et al., 1996) was utilized for read-through transcription of adjacent flanking sequences.

We have been developing vectors using maize transposable elements for plant functional genomics. In rice, we previously developed an *Ac-Ds* insertional mutagenesis system in which a GFP fluorescence gene functioned as negative selection marker against the immobilized *Ac* and a BASTA resistance marker worked for selection of transposed *Ds* elements (Kolesnik et al., 2004). In the development of an *En/Spm* tagging system in rice, our transposition selection scheme was improved based on GFP and RFP double fluorescence markers (Kumar et al., 2005). In this study, we developed an activation tagging *Ac-Ds in cis* system in which the advantage of GFP and RFP double fluorescence markers was utilized. The system was shown to be effective for distributing a large number of activation-tagging *Ds* elements in the rice genome. As the *Ac-Ds* has been shown to transpose widely in the plant kingdom, we anticipate the applicability of our activation vector system for studies in monocot plants including other cereals.

RESULTS

Vector Construction and Production of Starter Lines

The T-DNA vector pSQ5 carrying a non-autonomous *Ds* element along with an immobilized *Ac* element *in cis* is shown in Figure 1. The immobilized *Ac* contains the *Ac* transposase gene under the control of cauliflower mosaic virus (CaMV) 35S promoter (Kolesnik et al., 2004). The *Ds* element has the 1785-bp 5’ terminus and 222-bp 3’ terminus of the wildtype *Ac* element (Sundaresan et al., 1995). A tetramer of the transcriptional enhancer of CaMV 35S promoter and the *Discosoma sp*. red fluorescence protein (DsRed) gene were cloned between the 5’ and 3’*Ds* termini. The DsRed (or RFP) gene, encoding a red fluorescence protein (Clontech, CA; Baird et al., 2000), is driven by the maize ubiquitin 1 promoter (Christensen and Quail, 1996). The vector carries a hygromycin phosphotransferase gene for plant transformation selection. A synthetic green fluorescent protein (sGFP) gene was cloned next to the immobilized *Ac* transposase source in the T-DNA. The GFP and RFP fluorescence markers make it possible to visually track the *Ds* element and the immobilized *Ac* element, respectively, in transgenic progeny.

The pSQ5 *Ac*-*Ds* vector was introduced into *Oryza sativa* ssp. cv. Nipponbare via *Agrobacterium tumefaciens*-mediated rice transformation. Eighty fertile transformants that were double fluorescent (GFP^+ and RFP^+) were produced. In the T_2 generation, fifty seeds of each transformant were germinated and assayed for GFP and RFP. For GFP segregation, T_2 plants of fifty-eight transformants showed 3:1 ratio and therefore carried single T-DNA locus in the rice genome. Eight transformants had multiple T-DNA integration loci based on the GFP segregation ratios (>3:1) of T_2 plants. T_2 generation of fourteen transformants did not have any plants showing GFP or RFP possibly because of *Ds* transposition into the GFP gene or due to transgene silencing (see Discussion).

High Frequency of Germinal Transposition in T_1 and T_2 Transgenic Plants

Because the activation-tagging *Ds* element and the T-DNA-based *Ac* transposase can be tracked by RFP and GFP, respectively, we determined the GFP and RFP phenotype of each T_2 plant from 53 single-T-DNA-locus populations (Table I). An average of 48 T_2 plants in each population were assayed for GFP and RFP fluorescence. The phenotype of individual plants was determined as shown in Figure 2A. Four different phenotypes were observed in these T_2 populations. Because each T_2 population was generated from a single primary transformant (T_1) and in the T_1 generation the *Ds* element carrying the RFP gene can transpose, the RFP gene and the T-DNA-anchored GFP gene may have different chromosomal locations and may segregate in the T_2 plants (Fig. 2B). Therefore, it is likely that the GFP^-RFP^+ or GFP^+RFP^- plants in T_2 generation were derived from transposition events in the T_1 generation. In GFP and RFP assays of T_2 plants of 53 transformants (Table I), GFP^-RFP^+ plants were identified from T_2 populations of 23 transformants (No. 19-41). These GFP^-RFP^+ plants indicated that 43.4% (23/53) of the T_1 transformants carried transposed *Ds* elements that were germinally transmitted to the T_2 generation. Among the 53 T_2 populations, 12 populations (No. 42-53) had GFP^+RFP^- plants but no GFP^-RFP^+ plants, which suggested that a fraction of excised *Ds* elements did not reinsert in the rice genome (see Discussion).

To detect transposition in T_2 generation, we investigated T_3 populations of 37 transformants. For the 37 transformants, 18 transformants (Table I, No.1-18) did not show transposition in T_2 generation, 14 transformants (Table I, No.19-21, 30-32, 35, 42-43, 46, 48-51) showed transposition, and T_2 families of 5 transformants (No.54-58) were not analyzed. The T_3 families of the 37 transformants were obtained by selfing heterozygous T_2 plants. As GFP-heterozygous T_2 seedlings gave a lower level of GFP fluorescence than GFP-homozygous seedlings (Kumar et al., 2005), we selected the heterozygous T_2 plants based on their lower GFP intensity. In this way we were able to reduce the T_2 homozygotes to 8.2% according to the results of GFP assays of T_3 generation (data not shown). In each T_3 family, 200 to 400 plants were assayed to identify GFP^-RFP^+ transposant plants.

For the No.1 to 18 transformants, at least one GFP^-RFP^+ plant was identified in T_3 families of 12 transformants (Table II, No.1-3, 6, 8-12, 14-15, 18). We were able to identify transposition events in the T_3 generation but not in their T_2 families because just 42 to 50 plants from each T_2 family were analyzed (Table I, No.1-3, 6, 8-12, 14-15, 18). For the 14 transformants that showed transposition in T_2 generation, at least one germinal transposition event was identified from T_3 families of 13 transformants (Table II, No.19-21, 30-32, 35, 42-43, 46, 48-51). In a total of 905 T_3 families derived from 37 primary transformants, germinal transposition events were identified in 295 (32.6%) families (Table II). These transposition events in the T_3 generation were derived from 30 (81.1%) of the transformants examined. Higher germinal transposition frequencies (20-83.3%) were detected in the T_3 families of 19 single-T-DNA-locus transformants (Table II), which were among one third (32.7%) of all 58 single-T-DNA-locus transformants.

Generation of Activation-Tagging *Ds* Lines of Rice and Isolation of *Ds* Flanking Sequence Tags

We grew a total of 3057 T_2 plants that were derived from 37 primary transformants. An average of 300 seeds (T_3) were produced from each T_2 plant. The GFP negative selection marker and the RFP positive selection marker were utilized to screen T_3 families to obtain putative stable transposants (GFP^-RFP^+ plants). Using the scheme as shown in

Figure 3, we initially screened 905 T_3 families that were derived from the 37 transformants (Table II). In the next screening step, we did not continue with the T_3 families derived from transformants for which transposition frequencies in the T_3 generation were less than 10%. Instead, we focused on the 1181 T_3 families of the 26 transformants which generated transposition frequencies in the T_3 of 10% to 83.3% (Table II). Among the 1181 T_3 families, 343 families were found to have at least one GFP^-RFP^+ plant per family. Taken together, a total of 2086 T_3 families were screened and GFP^-RFP^+ transposants were selected from 638 (30.6%) of those families.

The putative stable transposants were subjected to adaptor-ligation PCR to determine *Ds* flanking sequence (Fig. 3). We analyzed 559 transposants that were derived from 26 primary transformants. A total of 463 sequences were obtained after adaptor-ligation PCR, sequencing, and filtration against the T-DNA sequence (to eliminate the *Ds* donor locus). In Blast search against the Rice GE genome database RiceGE Functional Genomics Database (http://signal.salk.edu/cgi-bin/RiceGE), 311 (67.2%) sequences were mapped on the rice chromosomes (Table III). The 152 remaining sequences were 73 (15.8%) redundant FSTs from siblings carrying the same insertions, and 79 (17.1%) unmapped sequences due to insertions in rice repeat sequence regions. Our explanation for the redundant FSTs is that a small number of T_2 siblings carried the same *Ds* insertion due to transpositions in the T_1 parent (Table 1), which were propagated by T_2 siblings to the T_3 generation (see Discussion). In further analysis of the FSTs, the TIGR rice genome database (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml) was searched for gene homology between the FSTs and rice cDNA sequences. There were 186 mapped FSTs with hits to rice cDNA sequences. Such FSTs were 40.2% (186/463) among the FSTs obtained and represented *Ds* insertions in the genic regions.

We analyzed the distribution of *Ds* insertions among the rice chromosomes. For each single-T-DNA-locus transformant, in the T_3 generation, the *Ds* element was found to be distributed among different rice chromosomes (Table III). When all the 26 transformants are taken in account, the largest chromosomes (Chr.1 and Chr.3) carry the largest numbers (49 and 44, respectively) of *Ds* insertions, and the smallest chromosomes (Chr.9 and Chr.10) carry smallest numbers of *Ds* insertions (18 and 18). Based on the size of each chromosome, the *Ds* insertions appear to be evenly distributed in the rice genome.

Enhanced Expression of Rice Genes Adjacent to the Activation-Tagging *Ds*

To examine whether expression of the *Ds*-tagged rice genes was altered, reverse transcriptase (RT)-PCR was performed on transposant lines with *Ds* insertion adjacent to the rice genes. We randomly selected twenty four transposant lines and examined gene transcript levels using semi-quantitative RT-PCR method. Initially, we analyzed the closest rice gene for each *Ds* insertion, which was at distance of 1 to 7 kb upstream or downstream of the *Ds* element. RNA was extracted from the leaves or roots of 60-d-old rice plants. RT-PCR was performed to compare gene expression levels in the twenty four candidate lines to those in the wildtype rice Nipponbare. Two pairs of primers specific to each gene were tested and each RT-PCR experiment was repeated at least three times under the same condition. As shown in Figure 4A, gene expression was enhanced in the line ADS247 whose *Ds* insertion was 1609 bp upstream of Os03g15050 as compared to Nipponbare. In ADS248 whose *Ds* insertion was 1880 bp upstream of a plasma membrane-type ATPase gene (Os11g29490), the transcript level was significantly higher than in Nipponbare as suggested by the RT-PCR results (Fig. 4B).

However, the other twenty two transposant lines showed the same results as Nipponbare in semi-quantitative RT-PCR when just one gene was analyzed. We further tested eight of such transposant lines by amplifying other genes in the vicinity of the *Ds* insertion. Three overexpressed *Ds* lines were identified through RT-PCR examination of the additional genes (Fig. 4C, 4D and 4E). In ADS305 whose *Ds* insertion was 12.2 kb upstream of the gene Os09g30439, the transcript level was slightly higher than in Nipponbare as indicated in the results of both leaf and root tissues (Fig. 4C). In ADS427 where the *Ds* was inserted 1349 bp upstream of Os04g42840, the transcript level was increased significantly based on the amount of the RT-PCR product in ADS427 and wildtype rice (Fig. 4D). In the RT-PCR analysis of ADS210, we examined expression levels of four genes that are 1.8 to 11.8 kb from the *Ds* insertion (Fig. 4E). Just for the gene Os10g41410, which is 11.8 kb upstream of the *Ds* element, transcript level was significantly enhanced in ADS210 as compared to Nipponbare.

It was observed that the CaMV enhancers in *Ds* in ADS210 activated the farthest gene Os10g41410 but did not affect expression of the other closer genes (Fig. 4E). In ADS305, as compared to Os09g30439, Os09g30458 is closer to the *Ds* insertion but did not get activated (Fig. 4C). On the other hand, in ADS427, the CaMV enhancers in *Ds* only affected expression of Os04g42840, which is closer to the *Ds* element than the gene Os04g42860 (Fig 4D). Therefore, for the function of the CaMV enhancers, likelihood of gene activation was not directly related to the distance from the CaMV enhancers to the gene.

DISCUSSION

We have developed an *in cis* activation tagging *Ac*-*Ds* system in which a T-DNA vector carries a *Ds* element containing 4x CaMV enhancers along with the *Ac* transposase gene. In testing the vector in rice, a total of 638 stable *Ds* transposant plants were selected from seedlings (T_3) of 2086 T_2 plants that were derived from 26 primary transformants. In analysis of 559 transposant lines, 311 of the *Ds* insertions were mapped on rice chromosomes.

Enrichment of unlinked transpositions is an important step for random insertional mutagenesis using the *Ac*-*Ds* transposable elements, as these elements have a feature of preferential transposition to closely linked sites. In the *Ac*-*Ds* vector systems we developed in Arabidopsis (Sundaresan et al., 1995) and rice (Kolesnik et al., 2004), a “negative-positive” selection scheme was successfully utilized for enrichment of unlinked transpositions. The *Ac*-*Ds* vectors were designed in such a way that an *IaaH* or GFP gene functioned as a counter-selection (or negative selection) marker against the *Ac* T-DNA, the *Ds*-donor T-DNA and linked *Ds* transpositions (Sundaresan et al., 1995; Kolesnik et al., 2004). In this study, the activation vector system utilizes an improved “negative-positive” scheme that is based on dual GFP and RFP fluorescence markers for high-throughput unlinked transposition selection (Kumar et al., 2005). Additionally, our vector system combines the merits of *in cis Ac-Ds* elements that do not need genetic crossing and activation/knockout dual function in the *Ds* element.

The activation-tagging *Ds* element showed germinal transposition in the primary transformants (T_1). In analysis of 53 single-T-DNA-locus transformants, GFP^-RFP^+ plants were identified in T_2 generation of 23 (43.4%) transformants. These GFP^-RFP^+ plants represented germinal transposition events that were transmitted from the T_1 generation. For 22.6% (12/53) of the transformants, a fraction of excised *Ds* elements did not reinsert in the rice genome because the T_2 populations had GFP^+RFP^- plants but not the GFP^-RFP^+ plants. It should be noted that these results were obtained using just 42 to 50 T_2 plants per transformant. Since a rice plant produces 200 to 400 seeds, if more T_2 progeny were assayed for GFP and RFP fluorescence, the frequency of primary transformants generating germinal transposition events could be higher than 43.4%.

From analysis of the T_2 families, we also found the germinal transposition frequency varied depended on transformants. The T_3 families of one third of the single-T-DNA-locus transformants showed high transposition frequencies (20-83.3%). The result was similar to the result of Kolesnik et al. (2004) that various frequencies were observed in different cross combinations of *Ds* line and *Ac* line. Therefore, it was important to select the right starter transformants to establish tagging populations. First, when generating transgenic plants of the activation vector, we did GFP and RFP fluorescence assay of the hygromycin resistant calluses and plantlets to select transformants with normal GFP and RFP function. Transgenic rice plants carrying multiple or rearranged T-DNA copies may result in transgene silencing (Sallaud et al., 2003). Second, single-T-DNA-locus transformants were identified based on GFP segregation in T_2 seedlings and single-T-DNA-locus T_2 plants were selected for generating T_3 populations. In screen for putative stable *Ds* transposants, we initially performed pilot screen of all T_3 families and then focus on families with high germinal transposition frequencies. These strategies together with the GFP and RFP visual method highly facilitated screening of stable transposants.

In the design of a transposon tagging system, it is also essential to prevent large number of transposant siblings from entering the pipeline. In this study, an important step was to avoid using transformants whose T_2 generation exhibited several transposants

(GFP^-RFP^+ and GFP^+RFP^- plants). For each of the transformants, we performed GFP and RFP assays on a small number of T_2 plants to know about the frequency of transposants. In establishing most of the T_3 populations, we utilized transformants whose T_2 progeny contained no or a low to moderate rate of transposition events. Among the 463 *Ds* flanking sequences obtained, 73 (15.8%) sequences were redundant FSTs from siblings carrying the same *Ds* insertions. The number of sibling FSTs could be further reduced if the T_2 families for generating T_3 populations were more strictly selected.

In testing the activation tag vector system in rice, we obtained a total of 463 FSTs sequences, of which 311 (67.1%) FSTs were from mapped *Ds* insertions, 79 (17.1%) were from insertions in repeat sequences, and 73 (15.8%) were redundant sibling FSTs as discussed above. Among the 67.1% (311/463) mapped FSTs, 40.2% (186/463) had hits to rice cDNA sequences and represented *Ds* insertions in the genic regions. For the other 26.9% (125/463) FSTs that showed no homology to rice cDNA sequences, we were not able to make a consistently clear distinction between the FSTs from 5' or 3' regulatory sequences of rice genes and the FSTs from *Ds* insertions in the intergenic regions. These results confirmed the previous reports that the *Ac-Ds* elements preferentially transpose to genic regions (Enkoi et al., 1999; Kolesnik et al., 2004; van Enckevort et al., 2005). For the strategy of activation tagging, *Ds* insertions in the intergenic regions are desirable as compared to the insertions in genic regions. However, for the transposant lines where the *Ds* element is inserted in a rice gene, the CaMV enhancers in *Ds* could still activate other neighboring genes. As indicated in the semi-quantitative RT-PCR results, genes that were over 10 kb away from the *Ds* insertions were activated by the CaMV enhancers in *Ds*.

We analyzed interchromosomal distribution of the 311 *Ds* insertions that were mapped. It appeared that the insertions were evenly distributed in the rice genome and the insertion number of each chromosome was proportional to the chromosome size. This is different from the previous report (Kolesnik et al., 2004), in which *Ds* transposition showed a bias toward chromosomes 1. The different results can be explained by the numbers of *Ds* starter lines that were used in generation of the *Ds* transposant collections. We used a total of 26 *Ds* starter lines while Kolesnik et al. (2004) used just six starter lines. A single *Ds* launching pad might have preferential transposition on some chromosomes, but utilization of more starter lines might have reduced such bias. We did not analyze intrachromosomal distribution of *Ds* insertions due to the limited number of *Ds* insertions on each chromosome. The *Ds* element in our system may have preferential transposition regions like the hot spot of *Ds* insertion (Konlesnik et al., 2004), because a similar scheme of enrichment of unlinked transposition was used in this study.

We performed semi-quantitative RT-PCR of rice genes in twenty four transposant lines and observed enhanced expression of *Ds*-tagged genes in five lines. The RT-PCR results were confirmed in repeated experiments using the same condition. We never had a result that the RT-PCR product of a transposant line was less than that of wildtype rice. Therefore, the CaMV enhancers in *Ds* element were capable to activate rice genes adjacent to the *Ds* insertion, which was similar to the result of T-DNA-based activation tagging in rice (Jeong et al., 2002, 2006). However, the frequency of activation-tagged lines was 20.8% (5/24) in this study and the frequency is lower than the frequency of 52.7 % in the previous report using T-DNA in rice (Jeong et al., 2006). One reason might be that we analyzed just one gene in semi-quantitative RT-PCR for most (16 among 24) of the candidate lines. For the other eight lines, two to four rice genes in each line that are close to the *Ds* insertion were examined in RT-PCR and enhanced expression of *Ds*-tagged genes was observed in three lines. Therefore, our rate of activation-tagged lines could be increased if more genes in the candidate lines were analyzed. For the function of the CaMV enhancers, it was reported that no good relationship was found between frequency of activation and distance from the CaMV enhancers to the gene and that no correlation was observed between degree of activation and distance (Jeong et al., 2006). We also observed the similar results in RT-PCR analysis of three activation tagged lines.

Although large T-DNA insertion libraries have been generated in rice, efficient T-DNA transformation system is available just for the *japonica* subspecies. For the *indica* rice subspecies that is more widely grown in rice farming areas around the world, T-DNA transformation is still difficult. Also, most of the T-DNA transformation methods involve tissue culture step that generates high frequency of somaclonal variation, which disturbs the process of forward mutant screens. Therefore, transposon mutagenesis is an effective approach for plant functional genomics, and can serve as an alternative to T-DNA when transformation through tissue culture is inefficient as is the case for the *indica* rice species as well as many other crops. Transposable elements can be mobilized or immobilized on demand and the approach requires only a few primary transformants to generate large collection of independent transposon insertions within the genome. For this reason, generation of insertions in heterologous plants mainly depends on propagation as in the case of transposon mutagenesis in maize.

The *Ac*-*Ds* elements have features like small size of *cis*-required sequence (~600 bp of minimal *Ds*), large cargo-insert capacity, and reasonable high transposition frequency, which have made the *Ac*-*Ds* elements particularly amenable for genetic studies. The maize *Ac*-*Ds* elements can efficiently transpose in many heterologous plant species. Also, the *Ac*-*Ds* showed trans-kingdom transposition in yeast and animal species (Weil and Kunze, 2000; Emelyanov et al., 2006), which suggested that the *Ac*-*Ds* elements do not rely on host-specific factors for transposition and that host factors involved in their mobility mechanism are widely conserved. Activation tagging strategies based on maize transposable elements have been developed in tobacco (Suzuki et al., 2001) and Arabidopsis (Marsch-Martinez et al., 2002; Schneider et al., 2005). The activation tagging *Ac*-*Ds* system developed in this study was aimed for rice and other monocot plants. This *in cis* 2-element system eliminates the need for crossing and utilizes RFP and GFP for tracking *Ds* element and counter selection against the *Ac* transposase, respectively, to stabilize transposed *Ds* elements. The use of fluorescent markers instead of antibiotic or herbicide resistance increases the applicability to other plants and eliminates problems with escapes. As the dual fluorescence markers were under control of the maize ubiquitin 1 promoter (Christensen et al., 1996) that is functional in maize, rice, barley, wheat and many grasses, the principles developed here are applicable to many other monocot plants. We find that the *Ds* element preferentially transposes into genic regions, which is similar to previous reports about the maize transposable elements (Enkoi et al., 1999; Cowperthwaite et al., 2002; Kolesnik et al., 2004; van Enckevort et al., 2005). When utilized as the carrier of the CaMV enhancers, the *Ds* element offers the advantage of both activation taggging and knockout mutations. These features make transposon-based activation tagging particularly useful for large genomes with many duplicated genes such as maize, as well as polyploid plant crops. The *Ac*-*Ds* based activation vector system developed in this study is publicly available without IP restrictions, and should be applicable for the functional genomics of a range of plants and especially for that of cereals and monocot plants.

MATERIALS AND METHODS

Vector Construction

In construction of the activation tagging *Ac-Ds* vector pSQ5, the *Ubi-DsRed-Nos* cassette was from pSK62 (Kumar et al., 2005), the 1785-bp 5'*Ds* and 222-bp 3'*Ds* were from pWS32 (Sundaresan et al., 1995), and the 4x CaMV 35S enhancers was from the AcREH construct (Suzuki et al., 1999). First, pWS32 was digested with *Nco*I/*Eco*RI and ligated to an *Nco*I/*Hin*dIII/*Eco*RI linker to generate pSQ1. Second, the 4x CaMV 35S enhancers was released as a 1.4-kb *Hin*dIII fragment from the AcREH construct and cloned at the pSQ1 *Hin*dIII site to make pSQ2. At the third step, multi-DNA ligation was performed using (i) the 1.7-kb 3'*Ds*/4x enhancers fragment from *Sac*I/*Eco*RI digested pSQ2, (ii) the 1785-bp 5'*Ds* from *Sac*I/*Hin*dIII digested pWS32, (iii) a 3.3-kb *Ubi-DsRed-Nos* cassette from *Hin*dIII/*Eco*RI digested pSK62. The multi-ligation products were digested with *Sac*I and 6.8-kb product (i.e. the activation-tagging *Ds* element) was purified and cloned into *Sac*I site of pCAMBIA-1300. The activation-tagging *Ds* element was partially sequenced to confirm junctions of fragments of the multi-ligation. The *Ds* element was finally cloned at the *Sac*I site of the pSSZ40 construct (Kolesnik et al., 2004) carrying the *CaMV35S-Ac* and *Ubi-sGFP-Nos* cassettes. Backbone of the pSQ5 vector was from pCAMBIA-1300 (Genbank Accession AF234296). The vector pSQ5 is in the public domain and will be made available by the corresponding author upon request.

Plant Transformation

Mature seeds of *O. sativa* ssp. *japonica* cv. Nipponbare were cultured on NB medium (Li et al., 1993) for 3 to 4 weeks to induce embryogenic callus. The callus was co-cultivated with *Agrobacterium* strain LBA4404 containing the pSQ5 *Ac-Ds* vector and selected on NB medium containing 50 µg L^{-1} of hygromycin B (Roche Diagnostics, IN) as described

(Yin and Wang, 2000; Sallaud et al., 2003). GFP and RFP positive (GFP^+RFP^+) calluses were transferred to pre-regeneration medium (Yin and Wang, 2000) and cultured at 25 ˚C and in dark for 10 to 15 days. The GFP^+RFP^+ calluses were further cultured on regeneration medium (Yin and Wang, 2000) under light for 2 to 3 weeks. GFP^+RFP^+ transformant plantlets were finally transferred to greenhouse.

GFP/RFP Fluorescence Assays

Transformed rice callus and plantlets were examined under a Zeiss SV11 fluorescence microscope (Zeiss, Hamburg, Germany) using appropriate filters of GFP and RFP fluorescence. Mature seeds of transgenic plants were germinated at 25 or 30 ˚C and in dark for 4 to 6 days. The emerging seedlings were screened for GFP fluorescence using the Green Fluorescence Protein Macro Detector Set (Model: GFP-MDS-20BB, BLS, Hungary). RFP fluorescence visualization of seedlings was under the Zeiss SV11 fluorescence microscope.

Cloning of *Ds* Flanking Sequences Using Adaptor-Ligation PCR

Rice DNA of stable *Ds* transposants was isolated using the DNeasy 96 Plants Kit (Qiagen, MD). In adaptor-ligation PCR, about 100 ng of rice genomic DNA was added to digestion-ligation reaction mixture, which contained the *Hin*dIII and *Eco*RI adaptors, the enzymes of *Eco*RI, *Hin*dIII, and T4 DNA ligase (Alonso *et al.*, 2003). PCR primers of the first-round PCR were Ds5-1A (5'-acggtcgggaaactagctctacc-3') and AP1 (5'-gtaatacgactcactatagggc-3', Alonso *et al.*, 2003). Products of the first round PCR were diluted 10 times with H_2O. The second round PCR was performed using primers Ds5-4 (5'-ctcgggttcgaaatcgatcgggat-3') and AP2-C (5'-tggtcgacggcccgggctgc-3'). PCR products were sequenced using ABI Biosystems BigDye v3.1 and ABI 3730x1 DNA sequencers.

Reverse Transcriptase–PCR

Total RNA was extracted from leaves or roots of *Ds* insertion lines and wild-type rice using the Trizol Regent (Invitrogen, Cat. No. 15596-018). One microgram of RNA was reverse transcribed in a total volume of 25 μL that contained 0.4 nmol of oligo(dT)$_{18}$ primer, 5 mM dNTPs, 20 units of SUPERase-in (Ambion, Cat. No. AM2694), and 200 units of M-MLV reverse transcriptase (Promega, Cat. No. M1701) in the MLV reaction buffer. PCR was performed in 25 μL of solution containing 1x TaKaRa Ex Taq polymerase buffer, 0.2 μM gene specific primers, 2.5 mM dNTPs, 0.5 unit of TaKaRa Ex Taq polymerase, and 1 μL cDNA reaction product. The reaction condition included denaturing template DNA at 95 ˚C for 5 min, 24 to 27 cycles of PCR (94 ˚C, 30 sec; 56 ˚C, 40 sec; 72 ˚C, 50 sec), and a final incubation step (72 ˚C, 5 min).

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FIGURE LEGENDS

Figure 1. T-DNA of the activation tagging *Ac-Ds* vector pSQ5. RB and LB, right and left borders of the T-DNA; Ubi, maize ubiqutin 1 promoter; GFP, green fluorescence protein gene as a negative selection marker; RFP, red fluorescence protein (or DsRed) gene as selection marker for the *Ds* element; 4xEn, a tetramer of CaMV 35S enhancers; HPH, hygromycin phosphotransferase gene as plant transformation selection marker. The T-DNA is in the backbone of pCAMBIA-1300.

Figure 2. GFP and RFP fluorescence assay of young seedlings (T_2) derived from pSQ5 transgenic rice plants. A, GFP and RFP assays and the scheme to determine GFP and RFP phenotype of each T_2 plant. B, a diagram showing a transposition event in the primary transformant (T_1) and its germinal transmission to T_2 population.

Figure 3. Selection of stable *Ds* insertion lines and generation of *Ds* flanking sequence tags (FSTs).

Figure 4. Semi-quantitative RT-PCR analysis of five *Ds* insertion lines. Left, schematic representation of the *Ds* insertion and the CaMV 35S enhancers (4xEn) relative to the nearby genes. The map of each chromosomal region was based on the Rice GE genome database RiceGE Functional Genomics Database (http://signal.salk.edu/cgi-bin/RiceGE). Genes that were examined in semi-quantitative RT-PCR are marked with dotted-line boxes. Right, RT-PCR analysis of activation-tagged genes. Rice *Act1* transcript was amplified as control. WT, the wildtype rice Nipponbare. RT-PCR using leaf or root RNA was indicated. A, Line ADS247 was tagged by the activation-tagging *Ds* element 1609 bp upstream of Os03g15050, which encodes the phosphenolpyruvate carboxykinase. B, Line ADS248 was tagged by a *Ds* 1880 bp upstream of Os11g29490, which encodes a plasma membrane-type ATPase. C, Line ADS305 was tagged by a *Ds* 12.2 kb upstream of Os09g30439, which encodes a heat shock protein. D, Line ADS427 was tagged by a *Ds* 1349 bp upstream of Os04g42840, which encodes a HEAT repeat family protein. E, Line ADS210 was tagged by a *Ds* 11.8 kb upstream of Os10g41410, which encodes the

nucleoside diphosphate kinase 1. For each gene, the RT-PCR result was confirmed using two different pairs of specific primers and the result from using one pair of primers was shown. Each PCR experiment was repeated at least three times to confirm the result.

Table I. *GFP and RFP assays of 53 T_2 families*

No.	Transformant	No. of T_2 plants	GFP^+RFP^+	GFP^-RFP^-	GFP^+RFP^-	GFP^-RFP^+
1	A3	50	38	12	0	0
2	A5	48	34	14	0	0
3	A14	50	39	11	0	0
4	A15	47	36	11	0	0
5	A28	57	42	15	0	0
6	A30	50	35	15	0	0
7	A39	50	33	17	0	0
8	A51	50	37	13	0	0
9	A52	49	34	15	0	0
10	A72	45	25	20	0	0
11	B4	49	36	13	0	0
12	B16	48	34	14	0	0
13	B30	49	33	16	0	0
14	B46	42	28	14	0	0
15	B49	45	28	17	0	0
16	B50	45	37	8	0	0
17	B52	50	38	12	0	0
18	B53	43	31	12	0	0
19	A35	50	36	11	2	1
20	A56	50	32	12	5	1
21	A57	47	29	11	6	1
22	A66	48	26	3	9	10
23	A68	50	24	9	13	4
24	A70	50	30	2	9	9
25	B9	50	26	11	10	3
26	B15	47	32	3	8	4
27	B17	48	28	9	4	7
28	B18	45	24	8	7	6
29	B23	49	29	12	6	2
30	B28	45	28	13	2	2
31	B31	49	36	12	0	1
32	B45	50	27	10	12	1
33	B47	47	29	7	3	8
34	B54	48	21	5	13	9
35	B62	47	30	10	6	1
36	B65	46	30	5	4	7
37	A13	31	23	1	1	6
38	A36	51	40	3	0	8
39	A37	78	56	9	0	13
40	A50	50	14	15	20	1
41	B36	49	38	5	0	6
42	A8	41	30	10	1	0
43	A34	48	37	10	1	0
44	A74	50	27	13	10	0
45	A83	45	23	11	11	0
46	B10	49	39	9	1	0
47	B12	49	27	15	7	0
48	B13	49	32	14	3	0
49	B24	47	34	12	1	0
50	B41	49	36	9	4	0
51	B48	19	13	5	1	0
52	B69	49	26	13	10	0
53	A55	49	10	9	30	0

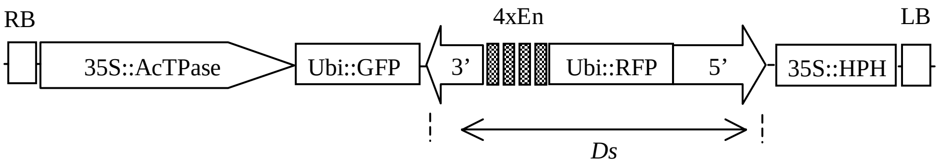
Table II. *GFP and RFP assays of 905 T_3 families*

No.	Transformant	T_3 families tested	T_3 families with germinal transposition[a]
1	A3	18	4 (22.2%)
2	A5	20	1 (5%)
3	A14	20	12 (60%)
4	A15	30	0
5	A28	10	0
6	A30	10	3 (30%)
7	A39	20	0
8	A51	20	3 (15%)
9	A52	20	1 (5%)
10	A72	6	5 (83.3%)
11	B4	30	10 (33.3%)
12	B16	10	2 (20%)
13	B30	20	0
14	B46	10	5 (50%)
15	B49	9	4 (44.4%)
16	B50	10	0
17	B52	10	0
18	B53	20	3 (15%)
19	A35	55	27 (49.1%)
20	A56	74	49 (66.2%)
21	A57	99	36 (36.4%)
30	B28	30	5 (16.7%)
31	B31	30	13 (43.3%)
32	B45	72	42 (58.3%)
35	B62	10	5 (50%)
42	A8	34	23 (67.6%)
43	A34	20	2 (10%)
46	B10	20	3 (15%)
48	B13	20	3 (15%)
49	B24	20	0
50	B41	24	13 (54.1%)
51	B48	10	3 (30%)
54	A29	21	1 (4.7%)
55	A62	10	5 (50%)
56	A73	30	10 (33.3%)
57	B3	23	1 (4.5%)
58	B76	10	1 (10%)
Total		905	295 (32.6%)

[a] Germinal transposition was based on identification of ≥1 of GFP^- RFP^+ plant. In brackets is % of T_3 families containing germinal transposition

Table III. *Interchromosomal distribution of Ds insertions derived from 26 transformants*

Transformant	Rice chromosomes												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
A62	9	7	9	4	5	3	3	4	4	3	3	7	61
A73	4	2	6	3	5	3	3	2	1	3	8	2	42
A14	8	3	5		6	3	2	1	1	3	2	2	36
A57	6	1	3	1	2	3	2	1	4	2	3	1	29
A35	4	2	1		2	2	3	1	1		1	3	20
B62	3		3	3			1	1	1	3	2	2	19
B28	5	1	2	2	3		1	3		1			18
A8	1	2	3	1	6		1	1	1	1			17
B4	1	3	4		2		1		3			1	15
A56		3	1	1	1	1		1					8
B31	2	1	2	1		1		1					8
B41	1	1		2	1			1	1				7
B49	1		1			1		1	1				5
B45			2			1						1	4
A72	1		1					1					3
B13		1				1	1						3
B53	1			1		1							3
A3			1	1									2
A34	1						1						2
B16	1						1						2
B46					1	1							2
A51					1								1
A52												1	1
A29										1			1
A30										1			1
B10				1									1
Total	49	27	44	21	35	21	20	19	18	18	19	20	311
%	15.8	8.7	14.1	6.8	11.3	6.8	6.4	6.1	5.8	5.8	6.1	6.4	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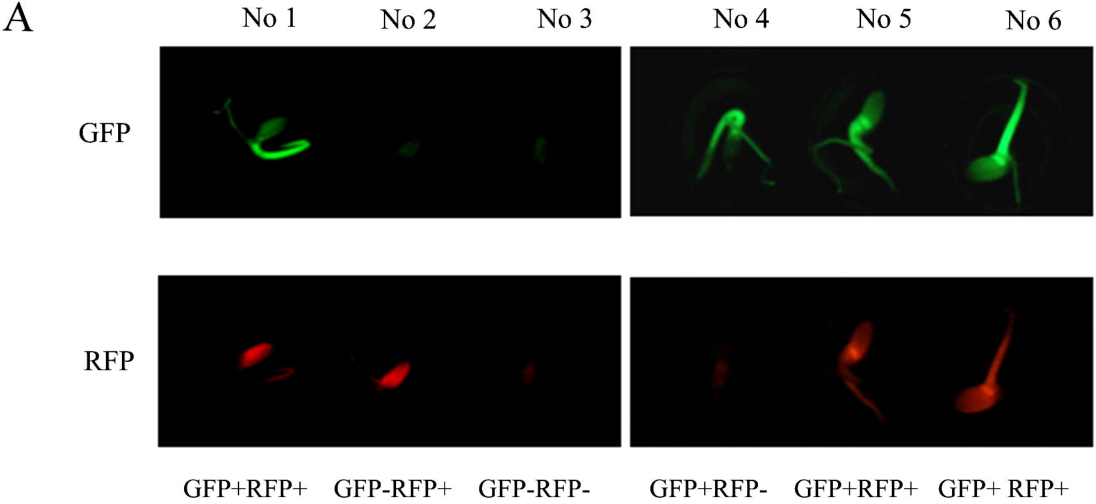
Ubi::GFP
4xEn
3’
Ubi::RFP
5’
35S::HPH
LB
Ds


A
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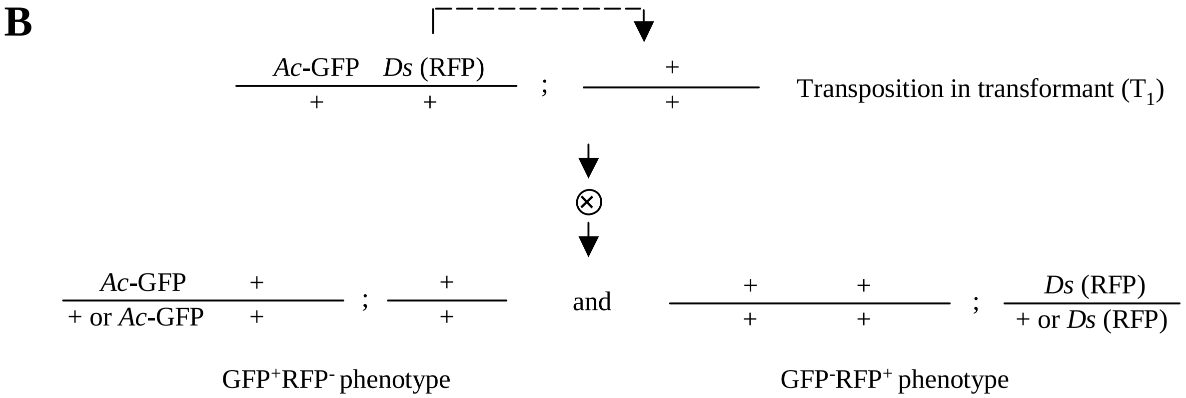
No 2
No 3
No 4
No 5
No 6
GFP
RFP
GFP+RFP+
GFP-RFP+
GFP-RFP-
GFP+RFP-
GFP+RFP+
GFP+ RFP+


B
Ac-GFP
Ds (RFP)
+
+
;
+
+
Transposition in transformant (T1)
⊗
Ac-GFP
+ or Ac-GFP
+
+
;
+
+
and
+
+
+
+
;
Ds (RFP)
+ or Ds (RFP)
GFP+RFP- phenotype
GFP-RFP+ phenotype

Grow 3057 heterozygous T_2 plants of 37 primary (T_1) transformants

↓

GFP and RFP screens of 905 T_3 familes of the 37 transformants; 295 putative GFP^-RFP^+ plants (stable transposants) selected (Table II)

↓

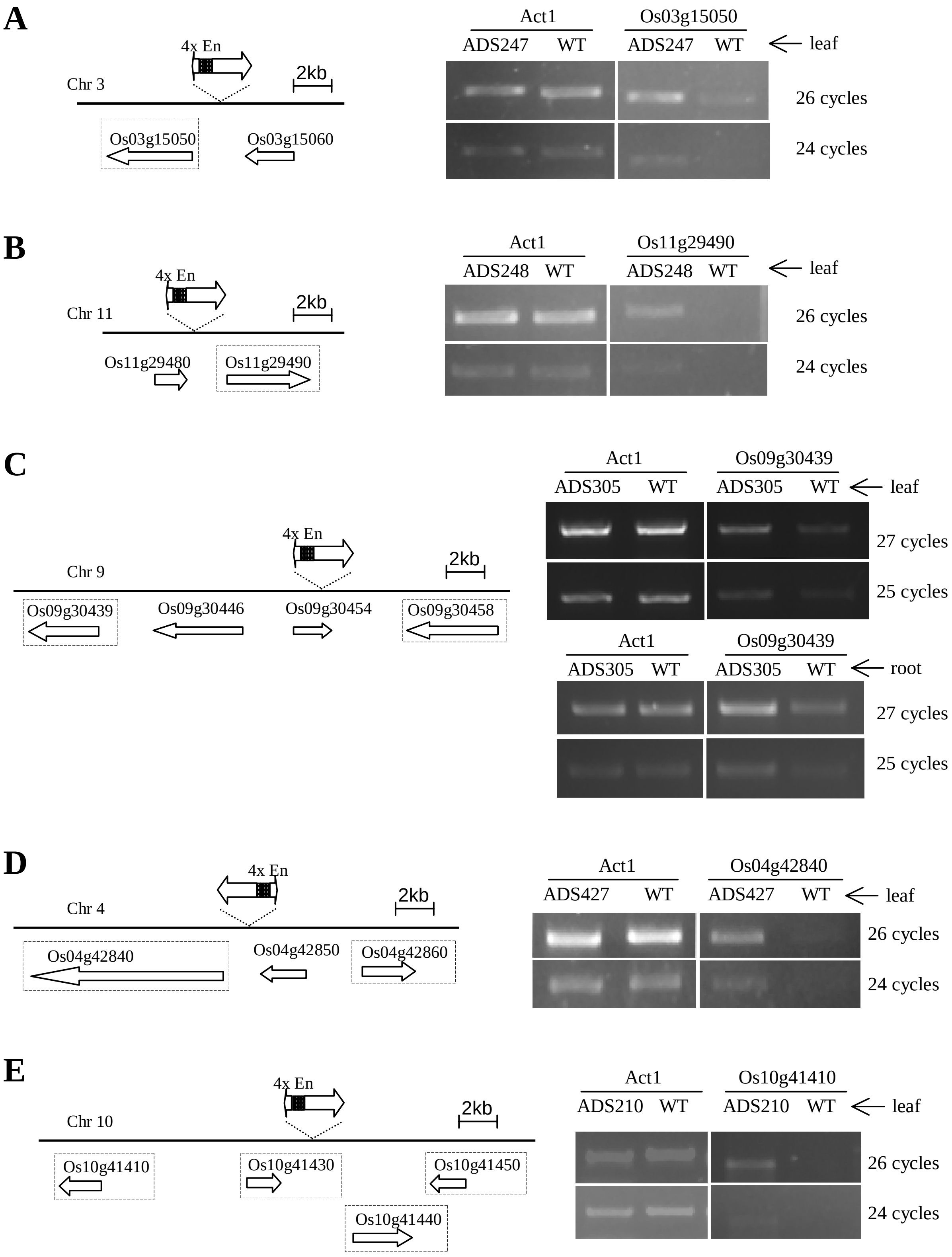
26 transformants with transposition frequency between 10% and 83.3% were identified; additional 343 GFP^-RFP^+ plants were selected from 1181 T_3 families of those transformants

↓

559 GFP^-RFP^+ transposants were subject to adaptor-ligation PCR and sequencing analysis to determine *Ds* flanking sequences

↓

463 sequences were obtained and 311 unique FSTs were mapped on the rice genome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WT
ADS247
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24 cycles
B
4x En
2kb
Chr 11
Os11g29480
Os11g29490
Act1
Os11g29490
ADS248
WT
ADS248
WT
leaf
26 cycles
24 cycles
C
4x En
2kb
Chr 9
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ADS305
WT
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WT
leaf
27 cycles
25 cycles
Act1
Os09g30439
ADS305
WT
ADS305
WT
root
27 cycles
25 cycles
D
4x En
2kb
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24 cycles