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MONITORING DS TRANSPOSITION IN THE SOYBEAN GENOME

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MONITORING DS TRANSPOSITION IN THE SOYBEAN GENOME

by

Manmeet Singh

A Thesis

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Soybean (Glycine max (L.) Merrill) is a major oilseed commodity which partitions carbon and nitrogen flux during embryogenesis towards two primary storage reserves, protein and oil, at approximately 40% and 20%, respectively in the seed. This attribute makes soybean a valuable feedstock in many food, feed and industrial applications. Over the past decade, a wealth of genomic resources has been established for soybean that will aid in elucidating the underlying biology governing the growth and development of the crop. This in turn will foster innovative breeding and genetics approaches leading to improvements in agronomics and end-use quality. Loss- and gain-of function mutants are powerful resources that complement functional genomics programs. Here we report on the creation of a repository of transgenic soybean events carrying a constitutive activation tag delineated by the maize dissociation (Ds) element, along with transposition frequency estimates of Ds delineated activation tag and enhancer-trap element induced upon stacking with a constitutive maize activator (Ac)-transposase cassette. To meet this goal we produced approximately 587 F1 Ac-stacks with the Ds-activation tag element and 144 F1 Ac-stacks with the Ds-enhancer trap element. Among 16 F2 derived populations from Ac X Ds-activation stacks we observed 26 unique germinal transpositions with an estimated 3.15% transposition frequency. Whereas among 22 F2 derived populations from the Ac X Ds-enhancer trap stacks only six unique germinal transpositions were
detected, translating to an estimated 0.5% transposition frequency. Based on sequence data collected from junctions about the transposed $Ds$ elements it appears that in soybean, $Ds$ quite frequently re-inserts at unlinked positions respective to its corresponding launch site. Two germinal mutants characterized, a $Ds$-enhancer trap and a $Ds$-activation tag, landed in the third intron of a putative cyclic nucleotide binding domain gene, and a predicted IMP/GMP specific nucleotidase, wherein the former resulted in a reduction in tagged transcript accumulation, while the latter lead to miss-expression of the tagged gene.
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CHAPTER 1

INTRODUCTION TO TRANSPOSON TAGGING
Soybean (*Glycine max* (L.) Merrill) is a legume crop and member of the family Fabaceae. It is one of the most important crops in the world because of its unique carbon flow in the seed which goes into approximately 40% protein and 20% oil. So it is widely used for food, feed and various industrial purposes all over the world. In 2011 Soybean was grown on 75.0 million acres and the production was estimated to be 3,060 million bushels in the US (USDA, 2011). Also, there are wide genomic resources available for soybean including detailed physical and genetic maps, SNPs and SSR markers (Shultz *et al.*, 2006). The soybean genome is predicted to be 1.115 gigabases of which 975 megabases has been sequenced and assembled using a whole genome shotgun approach predicting 46,430 protein-coding genes of high confidence and another ~20,000 genes with low confidence (Schmutz *et al.*, 2010). The gene prediction is based on sequence homologies and *ab-initio* methods, and there is a need to assign function to these genes. So there is a need to develop an efficient functional genomics tool to complement the genomic resources available in soybean. Such a tool should allow us to understand the functioning of the desired genes and the data thus available can be used for improvement of soybeans for better traits and improved production. This genomics tool would be used to develop a repository of soybean mutants harboring mutants tagged for most of the genes of interest.

There are several genomic tools that have been used to study gene function in various crops. One of the most commonly used tools is T-DNA insertional mutagenesis. It involves the delivery of T-DNA into the plant genome via agro-bacterium mediated transformation (Gelvin, 2003). The T-DNA might occasionally land into a functional
gene and thus, disrupt the gene function resulting in a mutant plant. T-DNA tagged populations have been successfully developed in various crop species like Arabidopsis (Alonso et al., 2003; Azpiroz-Leehman R and Feldmann KA, 1997; Sussman et al., 2000), rice (Jeon et al., 2000; Jeong et al., 2002; Sallaud et al., 2004) and Brachypodium distachyon (Thole et al., 2012). One drawback is that sometimes there are no visible phenotypes (Goebl MG and Petes TD, 1986). To overcome this drawback gene trap and enhancer trap elements can be used. The enhancer trap element has a minimal promoter which drives the expression of the visual marker gene only when the T-DNA lands in proximity to an endogenous gene promoter (Sundaresan et al., 1995). The gene trap element does not have a promoter but has one or more splice acceptor sequences and a reporter gene usually a visible marker gene like β-glucuronidase or green fluorescent protein (Springer, 2000). When it gets inserted in to the intron of a gene, it forms a fusion transcript with the endogenous gene resulting in the expression of the reporter gene. It allows us to look at the gene expression patterns even for genes with lethal phenotypes (Springer, 2000). These elements can help in gene identification and have been used for gene tagging in various plant species like Arabidopsis (Babiychuk et al., 1997; Campisi et al., 1999) and rice (Jeon et al., 2000; Sallaud et al., 2004).

Sometimes the T-DNA would disrupt the gene function but there will be no phenotype because of the gene redundancy and this is generally the case in polyploidy plant species. On the other hand some gene knockouts maybe lethal like for genes involved in embryo development. The gain of function mutants using activation tag elements could be an
alternative to this problem (Walden et al., 1994). Activation tag elements can involve the use of multiple enhancer elements to increase the expression of nearby genes in a tissue specific manner (Jeong et al., 2002; Weigel et al., 2000) or strong promoters like CaMV 35S promoter to get a constitutive expression (Neff et al., 1999). The over-expression of a gene product might lead to obvious phenotypes revealing the function of the gene or the pathway involved. Activation tagging has been successfully used as a mutagenesis tool in Arabidopsis (Weigel et al., 2000), rice (Jeong et al., 2002), poplar (Busov et al., 2003), tomato (Mathews et al., 2003) and tobacco (Ahad et al., 2003). Since soybeans have an efficient agrobacterium mediated transformation system (Clemente and LaVallee, 2000; Zhang et al., 1999), we can develop a T-DNA tagged repository of mutants with enhancer, gene traps and activation tags. But soybean transformation is very a laborious and lengthy process and it is practically impossible to have a population of T-DNA transformed soybean events large enough to cover the whole genome. The use of T-DNA tags along with a transposon based system is a possible solution to this problem. The development of an initial T-DNA tagged population with transposons will act as initial launch sites for further mutagenesis.

Transposable elements

Transposons or transposable elements (TEs) were first discovered by Barbara McClintock in maize (McClintock, 1950). She discovered and characterized the Ac/Ds TEs while studying altered pigmentation in aleurone layer of the mutant maize kernels, and was awarded the Noble prize for her discovery in 1983. Transposons are DNA
fragments that can move from one position in the genome to another and can also duplicate in this process. They are a large component of the plant genome accounting up to 50 – 80% in some grass species like maize (Meyers et al., 2001). Even though TEs form a large portion of the plant genomes, they are mostly inactive due to transposon silencing (Lisch, 2009). There are two general classes of TEs, Class II or DNA transposons and Class I or retrotransposons. Class I TEs are further characterized into long terminal repeat (LTR) and non-LTR retrotransposons (Feschotte et al., 2002). TEs make up to ~59% of the Soybean genome consisting of ~42% LTR retrotransposons and ~17% DNA transposons (Schmutz et al., 2010).

**Class II TEs**

Class II/DNA transposons transpose by a “cut and paste” mechanism. They are characterized by terminal inverted repeat sequences (TIRs) that are required for recognition by the transposase for transposition although some sub-terminal sequences may also be necessary. There are several families of Class II TEs characterized by unique TIRs and sequence length of the target site duplication (TSD). Each family has an autonomous and non-autonomous element. The autonomous element encodes for a transposase and can transpose independently. The non-autonomous element, on the other hand, requires presence of the autonomous element encoding the transposase for its transposition (Kunze R et al., 1997). DNA TEs have been observed in many plant species like Tam3 in Antirrhinum (Martin et al., 1985), An2 in Petunia(Koes et al., 1995) but
Ac/Ds, En/Spm and Mu from Maize are the most widely studied and commonly used DNA transposons for transposon tagging.

**Activator/Dissociation**

As mentioned earlier, Ac/Ds TEs were first discovered by Barbara McClintock in maize. Ac, also called Activator, is an autonomous element, and Ds the non-autonomous element. Ds or Dissociation is a truncated version of Ac formed by internal deletions or substitutions thus losing its ability to encode the transposase (McClintock, 1950). Ac is characterized by 11bp TIRs and eight bp TSD on transposition. Since its discovery and the first successful cloning of the waxy gene locus in maize (Shure et al., 1983), the Ac/Ds system has served as a gene cloning tool across various plant species. The germinal excision frequencies of Ac are very low in maize (Brutnell, 2002) but it has been shown to actively transpose in heterologous plant systems (Baker et al., 1986; Enoki et al., 1999; Van Sluys et al., 1987; Yoder et al., 1988) with excision frequencies of 0-70% (Bancroft et al., 1992; Briza et al., 2000; Jones et al., 1990; Nakagawa et al., 2000). The frequency of unique germinal Ds re-insertions varies from 0.37% in tobacco (Biezen, 1996), 0-20% (Nakagawa et al., 2000) to 41% (Kolesnik et al., 2004) in rice, 4% in Arabidopsis (Bancroft et al., 1992), up to 6% in tomato (Briza et al., 2000; Carroll et al., 1995) and 11.8 to 17.1% in barley (Singh et al., 2006).

A unique feature of these transposable elements is that Ac/Ds transpose preferentially to linked sites in maize (~60%) (Dooner and Belachew, 1989; Greenblatt, 1984) and the same phenomenon has been observed in other crops like tobacco (58-70%)(Biezen, 1996;
Dooner et al., 1991; Jones et al., 1990), Arabidopsis (68%) (Bancroft and Dean, 1993b),
tomato (~50%) (Carroll et al., 1995; Osborne et al., 1991) and rice (~80%) (Nakagawa et
al., 2000). Whereas in tomato, the preferential linked transposition was not as distinct;
the frequency of Ac transposition to linked and unlinked sites was found to be similar
(Healy et al., 1993; Osborne et al., 1991). In rice, the Ac/Ds population was screened for
only unlinked transposition and a germinal transposition frequency of 41% was observed
(Kolesnik et al., 2004). Even in maize, some studies show that Ac transpositions to un-
linked sites is up to 50% (Dooner and Belachew, 1989) and this property of Ac/Ds to
transpose to unlinked sites along with linked transposition has been used for genome-
wide mutagenesis (Vollbrecht et al., 2010). This suggests that the Ac/Ds transposon
system can be used as a great tool for local as well as whole genome mutagenesis.
Moreover, the Ds transposition has been observed to be preferential to the gene rich
regions in rice (72%) (Kolesnik et al., 2004), Arabidopsis (55%) (Parinov et al., 1999)
and barley (86%) (Singh et al., 2006). A high copy number of Ac has been correlated to
low excision frequency in maize but it was observed to have a positive correlation in
Arabidopsis (Bancroft and Dean, 1993a), tobacco (Jones et al., 1989). In Arabidopsis,
high expression of Ac was also correlated to higher excision frequencies (Long et al.,
1993b). Ac/Ds TEs can be used along with gene trap and enhancer trap elements as a tool
for functional genomics (Chin et al., 1999; Jeon et al., 2000; Sundaresan et al., 1995).
Gain of function mutants can also be obtained by using an activation tagged transposon
system (Jeong et al., 2002; Suzuki et al., 2001).
This system has been successfully used for gene tagging in tobacco (Briza et al., 2000; Fitzmaurice et al., 1999), Arabidopsis (Bancroft et al., 1992), rice (Chin et al., 1999; Nakagawa et al., 2000), barley (Cooper et al., 2004), and tomato (Meissner et al., 2000). The Ac/Ds system has been very successful in gene cloning in these plants like the albino mutant gene ALB3 in Arabidopsis (Long et al., 1993a), the TMV resistance gene N in tobacco (Dinesh-Kumar et al., 1995), the ABA2 gene involved in ABA biosynthesis in Nicotiana plurnbaginifolia (Marin et al., 1996), and the Cf-9 gene in tomato for resistance to Cladosporium fulvurn (Jones et al., 1994) to name a few. In rice, more than 60,000 Ds harboring lines have been developed use gene traps and enhancer traps providing a valuable resource for rice functional genomics (An et al., 2005). A large database of over 28,000 Ds insertion lines is available in Arabidopsis (Ito et al., 2005; Kuromori et al., 2004). Similarly, a database has been developed for maize and other crops and the aim is to generate Ds insertion lines that would serve as initial launch pads for further mutagenesis.

**Enhancer/Suppressor-mutator**

Enhancer/Suppressor-mutator (En/Spm) is another DNA TE that has been widely used for transposon tagging as a mutagenesis tool. It is a member of the CACTA transposable element superfamily characterized by 13 bp TIRs terminating with the sequence CACTA and three bp TSD. It was discovered in maize independently by Peterson (Peterson, 1953) and McClintock (McClintock, 1954) who named the autonomous element as Enhancer and Suppressor-mutator, respectively. Inhibitor/defective Spm (I/dSpm) represents the
non-autonomous element. The Spm transcript is alternatively spliced to give four transcripts designated \textit{tnpA-D}, where \textit{tnp-A} and \textit{tnp-D} are necessary for transposition (Kunze R \textit{et al.}, 1997). The transposition of En/I has also been shown to linked sites in maize. En/Spm TEs are mobile in heterologous plant systems like tobacco (Masson and Fedoroff, 1989), potato (Frey \textit{et al.}, 1989), and Arabidopsis (Cardon \textit{et al.}, 1993). The two component En/I system has been successfully used for transposon tagging in Arabidopsis (Aarts \textit{et al.}, 1995). It has also been used as an activation tagging tool in Arabidopsis (Marsch-Martinez, 2011; Marsch-Martinez \textit{et al.}, 2002). A number of maize gene have been cloned using this system and also in other plants like the \textit{MS2} male sterility gene in Arabidopsis (Aarts \textit{et al.}, 1993).

\textit{Mutator}

The maize \textit{Mutator (Mu)} transposable element is the most active and mutagenic transposon among the plant transposons. The transposition frequencies of \textit{Mu} can be as high as 100\% (Alleman and Freeling, 1986) and it has been used to clone many maize genes (Bensen \textit{et al.}, 1995; Walbot, 2000). It was discovered by Robertson in maize in 1978 (Robertson, 1978) and since then has been used for cloning several genes in maize like the \textit{bz2} allele (McLaughlin and Walbot, 1987) and the \textit{Ae1} locus (Stinard \textit{et al.}, 1993). It is a DNA transposon and has more than eight classes of transposons characterized by \textasciitilde220bp TIRs each class with unique internal sequences with a nine bp TSD (Chomet \textit{et al.}, 1991). The autonomous mobile elements are called \textit{MuDR} which has two genes \textit{mudrA} and \textit{mudrB} (Lisch, 2002). The non-autonomous \textit{Mu} elements are
formed by deletions or re-arrangements of the internal gene sequences. One of the features of Mutator is the duplicative transposition, so when an active Mutator plant is crossed with a non-Mutator plant up to three fold increase in Mu elements can occur for a low copy number stock and almost same numbers of Mu elements are present in the progeny for a high copy number stock (Kunze R et al., 1997). When a low copy number of Mu is used, the transposition frequencies are similar as in Ac (Walbot, 2000). The high copy number and transposition frequency is desirable for mutagenesis but it might pose a problem for analyses like assignment of phenotypes to a mutant gene. Unlike Ac/Ds and En/I, Mutator transposes to unlinked sites, so it can be used for whole genome mutagenesis. The Mutator element has been found to be transcriptionally inactive when transformed in rice. Thus, the epigenetic regulation of Mutator elements needs to be studied further before it can be used in any heterologous plant system (Diao and Lisch, 2006).

Class I TEs

Class I TEs or retrotransposons are abundant in plant genomes and have played a huge role in the evolution process (Bennetzen, 1996). These elements are different from Class II TEs in that they transpose via a RNA intermediate and thus “copy and paste”. They are classified into two categories based on their structure and mechanism as LTR and non-LTR retrotransposons. LTR retrotransposons are characterized by long terminal repeats in direct orientation. They are sub-divided into Ty1-copia and the Ty3-gypsy groups. Like DNA TEs, they have an autonomous element which has two genes gag and
pol; and non-autonomous elements which lack these genes. The gag gene codes for a capsid-like protein and the pol gene codes a polyprotein which is broken down into a protease, reverse transcriptase, RNase H and integrase enzymes (Feschotte et al., 2002). The non-LTR transposons are further classified into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). The LINE element is autonomous and encodes for an ORF1, a gag-like protein, an endonuclease and a reverse transcriptase. SINEs are non-autonomous and act as parasites on the transposition machinery of LINEs (Feschotte et al., 2002).

Several retrotransposons have been reported in various plant species like Tal in Arabidopsis (Voytas and Ausubel, 1988), BARE1 in barley (Manninen and Schulman, 1993), and PDR1 and Tps12 in petunia (Pearce et al., 2000). Tnt1, a LTR retrotransposon, was the first active retrotransposon discovered and isolated from tobacco (Grandbastien et al., 1989). The Tnt1 transposition was found to be activated by fungal extracts in tissue culture (Melayah et al., 2001). It has been used for tagging in Arabidopsis (Lucas et al., 1995), Medicago (Tadege et al., 2008) and lettuce (Mazier et al., 2007). New retrotransposons Tto1, 2 and 3 belonging to the copia-like group of LTR retrotransposons were discovered in tobacco by Hirochika and were found to be activated in tissue culture conditions (Hirochika, 1993). Tto1 was found to transpose actively in rice plants (Hirochika et al., 1996). In addition; Tos10, Tos17 and Tos19 are the most active retrotransposons from rice and have also been shown to be activated under tissue culture conditions (Hirochika, 1997). Most of the retrotransposons transpose to unlinked sites
with a preference for certain genes, the transposon insertions are at a high frequency but stable and a large number of insertions can be obtained; all these factors make them suitable for gene tagging (Kumar and Bennetzen, 1999; Kumar and Hirochika, 2001). Moreover, they have been shown to be activated by certain stress responses like tissue culture or viral infection which can be used to control their transposition activity (Wessler, 1996). A repository of Tos17 insertion lines has developed for mutagenesis in rice and a large scale characterization has been done (Piffanelli et al., 2007). A miniature inverted repeat transposable element mPing from rice has been recently used for mutagenesis in soybean (Hancock et al., 2011). mPing has been shown to transpose preferentially to the 5’ untranslated region of the genes in rice and was shown to enhance the expression of certain stress inducible genes (Naito et al., 2009).

A wide range of Class I and Class II TEs are present in plant genomes. Their unique feature of transposition to a different location in the genome makes them suitable to use as a gene tagging tool and to cut down the labor involved in T-DNA insertional gene tagging. The transposition activity has been tested for most of them in native and heterologous plant genomes and they have been used to tag genes and generate stable mutations. Retrotransposons can be difficult to monitor because of high copy number and the requirement of specific stress conditions like tissue culture for transposition. So we decided to use the two element Ac/Ds transposon system for mutagenesis in soybean since it has been widely studied and successful in other plant species, the simplicity of the “cut and paste” mechanism, and its tendency for both local and whole genome
mutagenesis. A repository of transgenic soybean plants carrying the Ds-delineated enhancer traps and activation tags were developed to serve as initial launch pads for transposition and further mutagenesis. The long term goal of this project is to develop a repository of mapped germinal Ds transposition events covering the whole soybean genome to serve as a functional genomics tool for soybeans.
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CHAPTER 2

MONITORING DS TRANSPOSITION IN THE SOYBEAN GENOME
ABSTRACT

Soybean (Glycine max (L.) Merrill) is a major oilseed commodity which partitions carbon and nitrogen flux during embryogenesis towards two primary storage reserves, protein and oil, at approximately 40% and 20%, respectively in the seed. This attribute makes soybean a valuable feedstock in many food, feed and industrial applications. Over the past decade, a wealth of genomic resources has been established for soybean that will aid in elucidating the underlying biology governing the growth and development of the crop. This in turn will foster innovative breeding and genetics approaches leading to improvements in agronomics and end-use quality. Loss- and gain-of function mutants are powerful resources that complement functional genomics programs. Here we report on the creation of a repository of transgenic soybean events carrying a constitutive activation tag delineated by the maize dissociation (Ds) element, along with transposition frequency estimates of Ds delineated activation tag and enhancer-trap element induced upon stacking with a constitutive maize activator (Ac)-transposase cassette. To meet this goal we produced approximately 587 F₁ Ac-stacks with the Ds-activation tag element and 144 F₁ Ac-stacks with the Ds-enhancer trap element. Among 16 F₂ derived populations from Ac X Ds-activation stacks we observed 26 unique germinal transpositions with an estimated 3.15% transposition frequency. Whereas among 22 F₂ derived populations from the Ac X Ds-enhancer trap stacks only six unique germinal transpositions were detected, translating to an estimated 0.5% transposition frequency. Based on sequence data collected from junctions about the transposed Ds elements it appears that in soybean, Ds quite frequently re-inserts at unlinked positions respective to its corresponding launch
site. Two germinal mutants characterized, a $Ds$-enhancer trap and a $Ds$-activation tag, landed in the third intron of a putative cyclic nucleotide binding domain gene, and a predicted IMP/GMP specific nucleotidase, wherein the former resulted in a reduction in tagged transcript accumulation, while the latter lead to miss-expression of the tagged gene.
INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is major global feedstock for protein and oil that are incorporated in a multitude of food, feed and industrial applications. In addition to its value as a commodity, soybean is an attractive legume model due to the wealth of genomics resources developed over the past decade, including over 50,000 SNP and 33,000 SSR markers (Soybase.org), complementing genetic maps (Choi et al., 2007; Shoemaker et al., 2008), along with a fully sequenced genome (Schmutz et al., 2010) and a reliable transformation system (Parrott and Clemente, 2004).

The soybean genome is predicted to encode for 46,430 genes with relative high confidence, with an approximate 20,000 gene predictions at a reduced level of confidence (Schmutz et al., 2010). Assigning function to the respective gene predictions will greatly enhance our understanding of the biology underlying soybean, and aid in translating this information to application through soybean breeding and genetics approaches targeting agronomic and composition improvements.

Loss- and gain- of function mutants are powerful tools in functional genomics programs. Loss-of-function mutants can be achieved through chemical means such as exposure to EMS, and fast neutrons, or via T-DNA insertional mutagenesis (Kuromori et al., 2009). Two limitations associated with loss-of-function approaches are; due to gene redundancy a phenotype may be masked, and/or homozygousity of a mutant allele of a critical gene may lead to lethality. On the other hand, gain-of-function approaches (Tani et al., 2004; Kondou et al., 2010) are designed to induce miss-expression of a tagged gene, that often
result in dominant mutations thereby permitting display of a phenotype when the tagged allele is heterozygous.

Development of large a repository of T-DNA tagged soybean mutants that mirrors the insertional collection established for Arabidopsis (Feldmann, 1991; Walden, 2002) or rice (An et al., 2003) would be a challenge to create due to the current state-of-the-art of soybean transformation (Parrott and Clemente, 2004). To circumvent the need to produce large numbers of transgenic events an alternative approach is to couple tagging elements with transposons. To this end the two component maize activator (Ac)/dissociation (Ds) system has been exploited in a number of heterologous plant systems including tobacco (Fitzmaurice et al., 1999), Arabidopsis (Bancroft et al., 1992; Ito et al., 2005), rice (Chin et al., 1999; Nakagawa et al., 2000; Kolesnik et al., 2004), barley (Cooper et al., 2004; Singh et al., 2006) and tomato (Meissner et al., 2000).

In heterologous plants germinal transposition of the maize Ds element has varied, for example, reports have communicated 0.37% in tobacco (Van Der Biezen et al., 1996), up to 41% in rice (Nakagawa et al., 2000; Kolesnik et al., 2004), 4% in Arabidopsis (Bancroft et al., 1992), 6% in tomato (Carroll et al., 1995; Briza et al., 2000) and up to 17% in barley (Singh et al., 2006). In its native genomic context the Ds element tends to transpose to linked positions relative to its point of origin (Dooner and Belachew, 1989), this tendency can be exploited as a regional mutagenesis tool (Knapp et al., 1994; Ahem et al., 2009). As similar pattern of linked transposition of Ds is also observed in heterologous systems (Jones et al., 1990; Osborne et al., 1991; Bancroft and Dean, 1993; Nakagawa et al., 2000). However, the Ds element also transposes to unlinked positions,
both in its native context (Dooner and Belachew, 1989; Vollbrecht et al., 2010) and heterologous systems (Healy et al., 1993; Kolesnik et al., 2004). Therefore, the \( Ac/Ds \) transposon system can provide a route to create stocks of regional and global genetic mutants in plants. Moreover, unlike the other approaches being explored to create populations of legume mutants such as chemical mutagens (Bolon et al., 2011), the retrotransposon Tnt1 (d'Erfurth et al., 2003), and the miniature inverted repeat element \( mPing \) (Hancock et al., 2011), \( Ds \) will transpose at a reduce frequency per genome, therefore, simplifying hypothesis testing due to the lower number of insertions per genome, which may require backcrossing to “clean-up” potential confounding effects imparted by non-target insertions. Conversely, \( Ds \) repositories will require significantly larger numbers than collections established from other mutagen approaches, with similar numbers of tag alleles.

In the study communicated herein, we monitored the transposition frequencies of two \( Ds \) elements, one that harbors an activation tag, and a second that carries enhancer trap element (Sundaresan et al., 1995), in soybean with the aim to access the impact of insert size within the \( Ds \) termini on frequency of transposition along with pattern of reinsertion in the soybean genome.
RESULTS

Soybean transformations with CsVMV activation tag (pPTN999)

We have established approximately 500 transgenic soybean events harboring the T-DNA element carrying the Ds-delineated CsVMV activation tag, and collected corresponding T2 populations. Junction fragments have only been captured on a small subset of these events to date (Table 1). Given the selection steps during the transformation process requires expression of the herbicide resistance trait, i.e. bar gene; it is likely that the majority of the launch sites for the Ds-activation tag reside in a transcriptionally active region of the chromosome.

Monitoring Ds transposition in soybean

Three soybean events harboring Ds-delineated enhancer trap elements (pPTN335) (Mathieu et al., 2009) were selected for stacking with Ac-transposase (pPTN398). The first event designated 456-1, has a single locus with the T-DNA element carrying the Ds-delineated enhancer trap residing on chromosome 15, upstream of the seed hydrophobic protein HPS1.5 (Gijzen et al., 1999). The second Ds-delineated enhancer trap event is referred to as 457-10, and carries two T-DNA loci one on chromosome 7, and the other on chromosome 8, with the latter residing within a lypoxygenase gene (glyma08g20190). Based on the molecular data obtained from this event it appears that the transgenic locus on chromosome 7 is missing a region proximal to the right border (RB), including a portion of the GUS gene while the enhancer trap T-DNA in glyma08g20190 is intact (Data not shown). The third is referred to as 455-5, where junction information has not been ascertained.
A *Ds*-delineated activation tag (pPTN999) event designated 770-4 carries a T-DNA launch site on chromosome 11, tagging a putative type III effector *avr* factor (Table 1). The selected *Ds* parents, 456-1, 457-10, 455-5 and 770-4 (or its genetic clone 770-3) were crossed with *Ac*-transposase event 545-19 or 543-14 (only enhancer trap events). A total of 16 F2 populations were genotyped from the 770-4 (or its genetic clone 770-3) (Table 2) along with 22 F2 populations from 455-5, 457-10 or 456-1 (Table 3). PCR primer set 1/2 (Supplemental Table S1, Supplemental Fig S1) was used to monitor for transposition, in which the absence of a product, due to product size limitation >7.0 kb, would imply no *Ds* transposition in enhancer trap stacks; while the amplification of an approximately 1.1 kb product would indicate movement, and the PCR product capturing the footprint. With respect to *Ds*-activation tag *Ac* stack, employing primer set 11/12 (Supplemental Table S1 and Figure S1) in the PCR screen would result in either 793 bp product or an approximately 2.0 kb product reflecting a transposition, or lack thereof, respectively (Supplemental Fig S2).

As can be seen in Table 3, among the 22 F1 plants screened, a footprint was observed in 19 of the 22 based on observed PCR product with primer set 1/2 (Table S1, Supplemental Fig S2). Among the 1155 F2 individuals screened footprints were observed in 304 (26%), with only 6 unique germinal transpositions observed (Table 3). A confirmatory Southern blot analysis on two of the six germinal transpositions is shown in Fig 1. The parental event 456-1 displays a GUS hybridizing band, with the 5’ GUS probe, at approximately 6.5 kb (Fig 1A), and the same size signal in F2 individuals, except F2-80 where the hybridization band resides at approximately 2.7 kb. When re-probed with the bar ORF,
the expected 3.0 kb fragment (Fig 1B) is observed in the parent and non-transposed alleles reflecting the Eco RV fragment within the binary vector pPTN335 (Supplemental Fig S1), along with that contained in the Ac-transposase binary pPTN398. Sequence analysis on the junction fragment captured from the re-insertion in F1-11/F2-80 (Fig 1A) revealed that the transposed enhancer trap landed in a non-coding region on chromosome 13 (Table 4), reflecting an unlinked transposition, which can explain the absence of a second bar hybridizing signal (Fig 1B).

Figures 1C&D show the clonal germinal transpositions in five of 10 F2 individuals derived from 457-10 X Ac stack, wherein the parent used in the cross 457-10 carries two transgenic loci; one that resides on chromosome 8, in a lipoxygenase (glyma08g20190) and the other in chromosome 7 in a non-coding region, that is missing a region proximal to the RB including one of the Ds termini, along with a portion of the GUS ORF, hence, a non-transposable element. Figure 1C displays the hybridization signals observed using the 5’ GUS probe. The approximate 1.5 kb fragment represents the locus residing in glyma08g20190, while that present above the 13 kb the transposed Ds-enhancer trap. In Fig 1D the membrane is re-probed with the bar ORF. The bar signal associated with the Ac T-DNA element is highlighted. The bar signal that resides at 2.0 kb represents the footprint of the transposition out from the launch site in glyma08g20190, while the F2 individuals that display the 3 kb signal carry the truncated enhancer trap element in chromosome 7. For example, individual F2-47 still has Ac, carries a re-insertion of the enhancer trap that transposed from chromosome 8, and harbors the truncated enhancer trap T-DNA on chromosome 7, along with a footprint. In
the case of F2-41, the Ac T-DNA is present, as is the re-insertion of the Ds-enhancer trap element, and the corresponding footprint, but the truncated T-DNA on chromosome 7 segregated away. Sequence information gathered from the junction fragment captured from F2-24, carrying the same clonal insertion as shown in Fig 1C revealed the Ds-enhancer trap transposed from chromosome 8 to chromosome 6 and landed in the third intron of a potassium gated ion channel (glyma06g08110) (Table 4).

A summary of the Ds transpositions monitored from the Ac stacks with the Ds-delineated activation tag (pPTN999; Fig S1B) is shown in Table 2. Among the 16 F1 individuals all had a footprint present based on the amplification of the 793 bp PCR product using primer set 11/12 (Supplementary Table S1 and Figure S1). From the 16 F1 plants a total of 826 F2 individuals were screened, of which 437 (53%) displayed a Ds excision, based on presence of a footprint (Table 2). Among these, 26 unique re-insertions were confirmed based on Southern blot analysis. This corresponds to an estimated 3.15% germinal transposition frequency.

Figure 2 shows an example of F2 individuals derived from three F1 plants that were created by crossing event 770-4, in which Ds-delineated activation tag T-DNA (pPTN999; Fig S1B) resides on chromosome 11 (Table 1), with Ac-transposase (pPTN398) event 545-19. Fig 2A displays the hybridization signals when the membrane is probed with the CsVMV promoter; wherein two distinct germinal re-insertions are observed in F2 individuals derived from F1-1 including an identical one seen in three F2 plants (F2-13, F2-16 and F2-32), with a differing size signal in F2-34 (Fig 2A). Likewise among 5 F2 individuals shown derived from the F1-6 plant, there are three unique signals
Re-probing the membrane with the bar ORF the Ac-transposase can be seen in all the F2 individuals screened except for F2-20, derived from F1-4 (Fig 2B). Moreover a footprint can be seen in only six of the 10 F2 individuals genotyped, suggestive of an unlinked transposition. Indeed sequence data gathered from the re-insertion observed in F1-6/F2-31 (Fig 2) reveals that the transposition launched from chromosome 11 (Table 1) and re-inserted in chromosome 5 tagging glyma05g08840 (Table 5), thereby confirming the unlinked transposition.

We sequenced 16 footprints left following transposition from the T-DNA element of the enhancer trap (pPTN335) (Fig 3). The data revealed that the excision was not always precise with a few base deletions/additions observed (Fig 3). Moreover, among four sequenced Ds re-insertions we observed 8 bp target site duplications, but with varying sequences (Fig 4).

**Characterization of Ds-delineated enhancer trap re-insertion into a putative potassium gated ion channel (glyma06g08110)**

F3 individuals from a Ds stable lineage, i.e. not harboring the Ac-cassette, derived from Ds-delineated enhancer trap stack with Ac (457-10 x 545-19 F1-14/F2-35) in which the Ds element transposed from chromosome 8, launch site within a lypoxygenase (glyma08g20190), and re-inserted into chromosome 6 within the third intron of glyma06g08110 (Table 4) were further characterized. A Southern blot analysis was carried out on seven F3 individuals carrying the stabilized Ds-enhancer trap element in glyma06g08110 (Fig 5A), using as a probe the junction fragment captured in from the TAIL PCR reaction amplified by primers 17/18 (Supplemental Table S1). As can be
seen in Fig 5A, the hybridization signal shifts from 2.7 kb in control lanes, expected size based genomic sequence data, to 9.5 kb in the tagged F3 plants. This approximate 6.5 kb up shift corresponds to the size of the Ds-delineated enhancer trap present in pPTN335 (Supplemental Fig S1A). We subsequently conducted targeted PCR reactions using primer sets specific for the corresponding junctions, further confirming the location of the Ds element in this locus (Data not shown).

We monitored expression of glyma06g08110 via RT-PCR in leaves, roots and flowers in both controls and tagged individuals (Fig 5B) using the primer set 19/20 (Supplemental Table S1); wherein expression was only observed in the latter in control plants, and highly reduced in flowers of Ds tagged individuals (Fig 5B). The reduction in expression of glyma06g08110 in flowers in the homozygous lineages carrying the Ds-enhancer trap was confirmed via northern blot analysis (Fig 5C). This gene, glyma06g08110 was cloned from soybean genome for further analysis using primer set 21/22 (Supplemental Table S1).

The closest homology found to glyma06g08110 in Arabidopsis is the cyclic nucleotide gated ion channel 18 (Frietsch et al., 2007), which was implicated to play a role in pollen tube growth. In order to see if this phenotype translated to soybean we monitored pollen germination in Ds-tagged lineages along with controls (Fig 5D/E). As can be seen in the images (Fig 5D/E) both pollen tube germination and growth were drastically retarded in the tagged lines. We monitored this phenotype across varying levels of Ca++, K+ and Na++ and the only pattern observed was differential germination and growth in the tagged lines (Data not shown).
Miss-expression induced by a re-insertion of a Ds-delineated activation tag

Soybean event 770-4 (clone of 770-3) harbors the T-DNA element carrying the Ds-delineated activation tag (pPTN999) on chromosome 11 (Table 2). A lineage derived from a stack with Ac event 545-19, designated 770-3 x 545-19 F$_1$-6/F$_2$42 (Table 5) was found with the Ds-activation element re-inserted on chromosome 15 just upstream of glyma15g21240.1 a putative cytosolic purine 5’ nucleotidase an enzyme highly active in root nodules (Christensen and Jochimsen, 1983). We monitored expression of glyma15g21240.1 in pods, leaves, and root tissues via RT-PCR and northern blot (Fig 6) using primer set 15/16 (Supplemental Table S1), with no expression observed in controls, and miss-expression in all tissues sampled, demonstrating the predicted constitutive expression pattern based on the genetic location of the activation tag (Fig 6).
DISCUSSION

A repository of approximately 500 soybean events that harbor the Ds-delineated activation tag element harbored in the binary vector pPTN999 has been established. The mapping of a small subset of these (Table 1) reveals that they are positioned across the genome and the repository is of sufficient size that there is a reasonable probability that there a multiple events positioned per chromosome. A pattern of re-insertion of Ds elements is that it often remobilizes a short distance from its point of origin (Jones et al., 1990; Osborne et al., 1991; Ahem et al., 2009), hence, with a proper design a Ds-based transposon system can be an effective regional mutagen (Knapp et al., 1994; Gidoni et al., 2003). However, among the set of Ds re-insertions characterized to date all have been to unlinked positions in the soybean genome (Tables 4 & 5). Therefore, assembling a repository of soybean events carrying Ds-launch sites spaced throughout a specific chromosome for targeted regional mutagenesis in and by itself will not suffice.

In addition, based on the estimated transposition frequencies monitored with the two Ds elements evaluated in soybean, the Ds-delineated enhancer trap and Ds-delineated activation tag, the size of the genetic element being carried by the transposon impacts both excision and re-insertion. The former harbors an approximately 6.5 kb element within the Ds termini, while the latter is approximately 500 bp between the Ds-termini. With the excisions and re-insertions occurring at higher frequencies with the smaller element, 0.5% and 3.15%, for the Ds-delineated enhancer trap and Ds-delineated activation tag, respectively.
A number of the F$_2$ individuals genotyped with $Ds$ germinal transpositions carried clonal insertions, suggestive of insertions early in development that hit the cell lineage to the germline. We recently monitored F$_3$ and F$_4$ seed derived from lines carrying both Ac and $Ds$-activation tags, from both lower and upper portions of the plants; while transposition was still occurring, germinal transposition frequencies were comparable for clonal and independent insertions observed from seeds harvested across the various nodes of the plant (Data not shown).

The analysis of a number of $Ds$ excision sites reveal that the region flanking original $Ds$ sites is not intact and there are deletions/additions of a few base pairs at the site of $Ds$ excision called ‘footprints’ (Baran et al., 1992). In addition, we observed 8bp target site duplication at the site of $Ds$ re-insertions as observed in maize (Muller-Neumann et al., 1984; Du et al., 2011). Both these features are highly characteristic of $Ds$ transposition in its native context, thus suggesting a similar mechanism for transposition in soybean.

Approximately 58% of the $Ds$ re-insertions mapped were in genic regions, either up-stream or within the gene, demonstrating the tendency of $Ds$ to transpose to gene rich regions (Parinov et al., 1999; Kolesnik et al., 2004). We characterized one such insertion in which the $Ds$-enhancer element landed in the third intron of glyma06g08110, a putative potassium gated ion channel, wherein we observed translation of the phenotype similar to a T-DNA mutant of a highly homologous gene in Arabidopsis (Frietsch et al., 2007) in the $Ds$-tag glyma0608810 (Fig 5), thereby confirming the in silico gene call.

A second characterized $Ds$-tag was a re-insertion of the activation tag upstream of a putative cytosolic purine 5’ nucleotidase, in this case constitutive expression of the gene
(glyma15g21240.1) was induced (Fig 6). This activation tag event is currently being phenotyped. Moreover, we have identified additional $Ds$-activation tags in which the re-insertion is positioned within the genic region in orientations such that expression of either negative sense or truncated transcripts would be predicted. These are currently being characterized further to monitor for a dominant silencing phenotype of the tagged gene and/or gene family (Data not shown).

From the $Ds$-delineated activation tag soybean events created, we have established a total of 587 $F_2$ populations derived from crosses with the 545-19 event carrying the $Ac$-cassette with 45 different pPTN999 events. Populations from this collection will be sown under field conditions to monitor the germinal transposition frequencies induced outside a greenhouse environment, which in turn will permit better estimates on, cost and size of building a $Ds$-activation tag soybean repository as a tool in functional genomics programs for the crop. To this end two critical components will need to be addressed, a reliable high throughput method to map the $Ds$-insertions and the development of an identity-preserved storage and distribution system linked to a searchable database. The former can theoretically be achieved by coupling a strategic DNA pooling system with next generation sequencing technologies, while the latter will require buy in by the plant community.
MATERIALS AND METHODS

Genetic Constructs

Binary vectors carrying a constitutive \textit{Ac}-transposase cassette and enhancer trap elements, designated pPTN398 and pPTN335, respectively, were previously described (Mathieu et al., 2009). A \textit{Ds} delineated constitutive activation tag element was assembled by sub-cloning the cassava vein mosaic virus promoter (CsVMV) (Verdaguer et al., 1998) between \textit{Ds} termini that reside in vector pBBDs/Xho (gift from Cliff Weil, Purdue University). The resultant \textit{Ds}-delineated CsVMV promoter was subsequently cloned into the binary vector pPTN200 (Sato et al., 2004), which carries a \textit{bar} gene (Thompson et al., 1987) cassette for selection of transgenic events. The final \textit{Ds}-delineated activation tag binary vector is referred to as pPTN999.

Soybean Transformations

Soybean transformations were carried out using the cotyledonary-node explant coupled with \textit{Agrobacterium tumefaciens} as previously described (Zhang et al., 1999). Soybean genotype ‘Thorne’ (McBlain et al., 1993) was used for transformation.

Characterization of transgenic events

Transgenic soybean events harboring the respective \textit{Ds}-delineated CsVMV activation tag (pPTN999), enhancer trap (pPTN335) along with corresponding \textit{Ac} (pPTN398) stack populations, were grown under greenhouse conditions. Southern and northern blot analyses were carried out as previously described (Buhr et al., 2002; Eckert et al., 2006). All probes were generated through random prime synthesis incorporating dCT\textsuperscript{32}P, using Prime-It II kit following the manufacturer’s protocol (Agilent technologies Cat# 300385).
Stacks, F₁ along with subsequently derived populations, of selected pPTN999 events (Ds-delineated CsVMV activation tag) and pPTN335 (Ds-delineated enhancer trap) were genotyped using primer sets 5/6; 7/8; and 9/10 (Supplemental Table S1) to monitor for presence of Ac and the corresponding Ds element. Somatic Ds transpositions were monitored in F₁ and derived populations from the stacks using primer sets 1/2 and 11/12 (Supplemental Table S1).

Germinal transpositions in subsequent generations derived from the selected stacks were monitored by PCR utilizing primer sets 3/4 and 13/14 (Supplemental Table S1) for pPTN335 and pPTN99 stacks, respectively. While primer sets 5/6 and 9/10 (Supplemental Table S1) were used to look for re-insertion Ds-delineated elements from pPTN335 and pPTN999, respectively. Southern blot analysis was used to confirm the PCR predicted germinal/re-insertion of Ds elements in F₂ and F₃ individuals. In the confirmatory Southern analysis, total genomic DNA from pPTN335 stacks were digested with Eco RV, while the pPTN999 stacks were digested with Eco RI to aid in the genotyping of the germinal transpositions, relative to the corresponding launch (i.e. parental pPTN335 or pPTN999). The resultant membranes were hybridized with either CsVMV promoter (pPTN999) or GUS, 5’ end of ORF, (pPTN335) to monitor for germinal re-insertions. The membranes were stripped and re-probed with the bar ORF which would provide insight on linkage with the original launch allele (i.e. “footprint”) along with presence of the Ac-transposase in the individual.

Capturing of junction fragments
DNA sequences about the parental T-DNA launch site, and Ds re-insertion lines were amplified from genomic DNA by TAIL-PCR (Liu et al., 1995). Specific and nested primers for first and second round PCR used for amplification of junction fragments about pPTN335, and pPTN999 T-DNA borders, along with corresponding Ds-insertions are listed in Supplemental Table S2.

An inverse PCR strategy was also employed to capture junction fragments about the Ds-re-insertion sites for crosses derived from pPTN999 (CsVMV activation tag). In these cases genomic DNA was digested with Alu I, with re-circulation and PCR conditions carried as described by Ochman et al. (Ochman et al., 1988). The first PCR reaction was carried out with primer set CsVMV-F: TACGGGAAAAACTATGGAAGTATTATG and Ds-RB1b: CGTCCGATTTGCACCTTTAACC. The resultant PCR product was diluted 50X and a 2µl aliquot was used as a template in a second round of PCR, using Primer set CsVMV-F and RBne1b: ATCGTATCGGGTTTCGATTA.

The derived PCR products from either TAIL-PCR or inverse PCR were gel purified, and subsequently cloned into pCR2.1-TOPO (Invitrogen Cat# 45-0641), and sequenced (Eurofins MWG Operon). The sequence junction fragments were blast searched to both soybean genome (www.phytozome.net) and Genbank.

**Phenotyping of putative cyclic nucleotide binding domain gene Ds tag line**

A Ds-delineated enhancer trap germinal transposition found within the third intron of a putative gated ion channel (glyma06g08110). Due to its similarity to previously phenotype T-DNA tagged Arabidopsis allele (Frietsch et al., 2007), we monitored pollen germination of the Ds-enhancer trap tagged soybean line. The medium used to monitor
pollen germination consisted 0.01% H$_3$BO$_3$, 5 mM CaCl$_2$, 5 mM KCl, and 1 mM MgSO$_4$, supplemented with 10% sucrose and solidified with 0.01% agar (Boavida and McCormick, 2007). Partially opened soybean flowers were harvested from greenhouse grown plants in the early morning. Petals and sepals were removed, and anthers gently blotted onto germination medium to release pollen. Pollen from 10-15 flowers was blotted per plate. Plates were subsequently placed in a box with moistened paper towels to maintain high relative humidity, and incubated at 28°C for 4-6 hours, prior to monitoring germination and length.

Germination and estimated pollen lengths were ascertained using a ZEISS Axioplan 2 Imaging system, and measurements taken with ZEISS Axiovision 3.0 software. Total pollen counts were ascertained using a Nikon Eclipse Ti inverted microscope across ten random fields at 20x magnification.
REFERENCES


Van Der Biezen EA, Cardol EF, Chung HY, Nijkamp HJJ, Hille J (1996) Frequency and distance of transposition of a modified dissociation element in transgenic tobacco. Transgenic Res. 5: 343-357


FIGURES

Figure 1. Southern Blots showing germinal transposition in the F2 generation from pPTN335 x pPTN398.

The genomic DNA was digested with EcoRV. (A) Southern blot on F2 plants from 456-1x545-19 F1-10 and F1-11. The germinal transposition events, in bold, show a different size band than the parent 456-1 when hybridized with the ‘GUS’ probe. (B) The same blot was stripped and hybridized with the ‘bar’ probe; the events with transposition do not hybridize suggesting unlinked transposition. (C) Southern blot on F2 plants from 457-10x545-19 F1-14. Seven events, in bold, show Ds germinal transposition when hybridized with the ‘GUS’ probe. (D) The same blot was stripped and hybridized with the ‘bar’ probe, the events with transposition show a ‘footprint’ of expected size when hybridized with ‘bar’.

![Southern Blots](image)
**Figure 2. Southern blots on F2 plants from pPTN999 x pPTN398.**

The genomic DNA from the crosses 770-4 x 545-19 was digested with EcoRI and Southern blots were probed with either ‘CsVMV’ or ‘bar’. (A) The germinal transposition events, in bold, show a different size band than the parent 770-4 when hybridized with the ‘CsVMV’ probe. (B) The events with transposition show a footprint of expected size when hybridized with ‘bar’. Some events do not have a footprint suggesting unlinked transposition.
Figure 3. Analysis of Ds ‘footprints’.

When the Ds element is excised from its original position, the excision in not always precise and some base pair deletions are observed as shown in the sequencing data below.
Figure 4. Target site duplications at Ds re-insertion sites.

Characteristic 8bp target site duplications (TSD) when the Ds element gets re-inserted into a new position in the soybean genome.

457-10x545-19 F1-10 F2-28: 5' caaggccagaggctgACGGGGGCGACGGGGGCG ggtagttaggggtgac 3'
457-10x545-19 F1-14 F2-24: 5' gactaggtatggtacgGTTCTATGTA tgtcTGAAGATcgatcafftccac 3'
770-4x545-19 F1-6 F2-35: 5' cgtgfsdfstcaastGTACTGAAAGGCTGTgatggsaagggc 3'

8bp Target site duplications
Same length but not identical
Figure 5. Characterization of *Ds*-delineated enhancer trap re-insertion into a putative potassium gated ion channel (glyma06g08110), 457-10x545-19 F1-14 F2-35.

(A) Southern blot for F3 plant probed with the junction fragment from Glyma06g08110 proximal to *Ds* re-insertion site. There is a ~6-5kb shift in hybridizing band in the F3 plants due to re-insertion of the *Ds* element into the third intron of Glyma06g08110. (B) RT-PCR results showing no expression of Glyma06g08110 in the roots and leaf tissue; a reduced expression is observed in the GT event. (C) Northern blot on the mRNA of Glyma06g08110 depicting reduced gene expression in the GT event. (D) Pollen germination picture for the Thorne/Control set with normal pollen growth. (E) Pollen germination in the GT event showing reduced pollen germination, pollen abortion, short and kinky pollen tubes with occasional normal growing pollen grains.
Figure 6. Characterization of *Ds*-delineated activation tag re-insertion into a putative cytosolic purine 5' nucleotidase, 770-3 x 545-19 F1-6 F2-42.

Characterization of activation tagged germinal transposition event 770-3x545-19 F1-6 F2-42 (Mutant) with *Ds* re-insertion ~500bp upstream of Glyma15g21240. RT-PCR results showing enhanced expression of Glyma15g21240 in green pods (A), roots (B) and leaf tissue (C). (D) Northern blot for Glyma15g21240 in the leaf tissue showing an enhanced expression due to activation tagging.
SUPPLEMENTAL FIGURES

Figure S1. T-DNA constructs used in the study.

(A) pPTN335. *Ds*-delineated enhancer trap element with GUS visual marker gene and bar selection marker. (B) pPTN999. *Ds*-delineated activation tag element harboring constitutive *Cassava* vein mosaic virus promoter and bar selection marker. (C) pPTN398. *Ac* transposase gene driven by the constitutive Cauliflower mosaic virus 35S promoter and the bar selection marker. E-V and E-I represent restriction sites for EcoRV and EcoRI, respectively. Probe a and b represent the probes used for Southern blot analysis to screen for *Ds* germinal transposition in events carrying *Ds* element from pPTN335 and pPTN999, respectively. Probe c was used to screen for linked/unlinked transpositions and presence of *Ac*.
Figure S2. Diagrammatic representation of primers used for screening Ds transposition.

If a PCR product is amplified by primers 1 and 2 or 11 and 12, it suggests Ds excision.
If there is no PCR amplification by primers 3 and 4 or 13 and 14, it suggests germinal Ds transposition. Finally PCR amplification by primers 5 and 6 or 9 and 10 confirm Ds re-insertion. Primers 7 and 8 are used to check the presence of Ac. The table provides the details for the primers used. Tm of 50°C was used for all the primer sets.

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<th>S.No.</th>
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### Table 1. Summary of mapped T-DNA events for pPTN999

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<td>5</td>
<td>772-3</td>
<td>18,832,080</td>
<td>Gm18</td>
</tr>
<tr>
<td>6</td>
<td>773-4</td>
<td>4,883,699</td>
<td>Gm07</td>
</tr>
<tr>
<td>7</td>
<td>774-3</td>
<td>45,313,113</td>
<td>Gm02</td>
</tr>
<tr>
<td>8</td>
<td>777-7</td>
<td>45,313,113</td>
<td>Gm02</td>
</tr>
<tr>
<td>9</td>
<td>781-15</td>
<td>45,313,113</td>
<td>Gm02</td>
</tr>
<tr>
<td>10</td>
<td>791-1</td>
<td>49,559,996</td>
<td>Gm19</td>
</tr>
<tr>
<td>11</td>
<td>778-18</td>
<td>9,577,937</td>
<td>Gm10</td>
</tr>
<tr>
<td>12</td>
<td>787-12</td>
<td>22,379,591</td>
<td>Gm13</td>
</tr>
<tr>
<td>13</td>
<td>819-13</td>
<td>37,374,835</td>
<td>Gm13</td>
</tr>
<tr>
<td>14</td>
<td>817-23</td>
<td>2,138,156</td>
<td>Gm15</td>
</tr>
<tr>
<td>15</td>
<td>806-19</td>
<td>12,783,219</td>
<td>Gm07</td>
</tr>
<tr>
<td>16</td>
<td>806-13</td>
<td>41,860,756</td>
<td>Gm13</td>
</tr>
<tr>
<td>17</td>
<td>835-2</td>
<td>7,986,852</td>
<td>Gm04</td>
</tr>
<tr>
<td>18</td>
<td>773-7</td>
<td>271,238</td>
<td>Gm03</td>
</tr>
<tr>
<td>19</td>
<td>773-8</td>
<td>14,777,592</td>
<td>Gm02</td>
</tr>
<tr>
<td>20</td>
<td>775-3</td>
<td>21,508,479</td>
<td>Gm08</td>
</tr>
<tr>
<td>21</td>
<td>774-6</td>
<td>18,424,743</td>
<td>Gm10</td>
</tr>
<tr>
<td>22</td>
<td>781-15</td>
<td>3,662,884</td>
<td>Gm06</td>
</tr>
</tbody>
</table>
Table 2. Frequency of *Ds* excisions and germinal transpositions for pPTN999 x pPTN398

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Footprinta</th>
<th>Individual F2 plants</th>
<th><em>Ds</em> excisions</th>
<th>Unique GTb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 770-3 X 545-19 F1-6</td>
<td>+</td>
<td>91</td>
<td>41</td>
<td>3</td>
</tr>
<tr>
<td>2 770-3 X 545-19 F1-1</td>
<td>+</td>
<td>83</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>3 770-3 X 545-19 F1-2</td>
<td>+</td>
<td>38</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>4 770-3 X 545-19 F1-12</td>
<td>+</td>
<td>87</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>5 770-4 X 545-19 F1-6</td>
<td>+</td>
<td>79</td>
<td>47</td>
<td>5</td>
</tr>
<tr>
<td>6 770-4 X 545-19 F1-1</td>
<td>+</td>
<td>76</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>7 770-4 X 545-19 F1-4</td>
<td>+</td>
<td>99</td>
<td>52</td>
<td>2</td>
</tr>
<tr>
<td>8 770-4 X 545-19 F1-3</td>
<td>+</td>
<td>11</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>9 770-3 X 545-19 F1-5</td>
<td>+</td>
<td>19</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>10 770-4 X 545-19 F1-7</td>
<td>+</td>
<td>25</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>11 770-3 X 545-19 F1-19</td>
<td>+</td>
<td>28</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>12 770-4 X 545-19 F1-2</td>
<td>+</td>
<td>33</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>13 770-3 X 545-19 F1-20</td>
<td>+</td>
<td>32</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>14 770-3 X 545-19 F1-3</td>
<td>+</td>
<td>32</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>15 770-3 X 545-19 F1-13</td>
<td>+</td>
<td>28</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>16 770-3 x 545-19 F1-9</td>
<td>+</td>
<td>65</td>
<td>31</td>
<td>0</td>
</tr>
</tbody>
</table>

**TOTAL** 826 437 26

- a – *Ds* excisions observed in F1 plants
- b – Germinal Transpositions representing *Ds* re-insertion in F2 generation
Table 3. Frequency of Ds excisions and germinal transpositions for pPTN335 x pPTN398

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Footprint&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Individual F2 plants</th>
<th>Ds excisions</th>
<th>Unique GT&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 455-5 x 545-19 F1-6</td>
<td>+</td>
<td>69</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2 456-1 x 545-19 F1-2</td>
<td>+</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3 456-1 x 545-19 F1-3</td>
<td>+</td>
<td>10</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4 456-1 x 545-19 F1-8</td>
<td>+</td>
<td>67</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5 456-1 x 545-19 F1-11</td>
<td>+</td>
<td>99</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>6 456-1 x 545-19 F1-10</td>
<td>+</td>
<td>96</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>7 545-19 x 456-1 F1-8</td>
<td>+</td>
<td>40</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>8 545-19 x 456-1 F1-7</td>
<td>+</td>
<td>51</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>9 545-19 x 456-1 F1-2</td>
<td>+</td>
<td>43</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>10 545-19 x 456-1 F1-14</td>
<td>+</td>
<td>49</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>11 457-10 x 545-19 F1-1</td>
<td>+</td>
<td>10</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>12 457-10 x 545-19 F1-3</td>
<td>+</td>
<td>74</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>13 457-10 x 545-19 F1-14</td>
<td>+</td>
<td>69</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>14 457-10 x 545-19 F1-23</td>
<td>+</td>
<td>120</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>15 457-10 x 545-19 F1-10</td>
<td>+</td>
<td>28</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>16 456-1 x 545-19 F1-12</td>
<td>-</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17 457-10 x 545-19 F1-7</td>
<td>-</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18 543-14 x 455-5 F1-7</td>
<td>-</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19 543-14 x 457-10 F1-2</td>
<td>+</td>
<td>55</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>20 456-1 x 545-19 F1-10</td>
<td>+</td>
<td>37</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>21 545-19 x 457-10 F1-8</td>
<td>+</td>
<td>56</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>22 543-14 x 457-10 F1-1</td>
<td>+</td>
<td>33</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>1155</td>
<td>304</td>
<td>6</td>
</tr>
</tbody>
</table>

- a – Ds excisions observed in F1 plants
- b – Germinal Transpositions representing Ds re-insertion in F2 generation
Table 4. Summary of germinal transpositions for pPTN335 x pPTN398 stacks

<table>
<thead>
<tr>
<th>F2 plant</th>
<th>Position of the Ds re-insertion</th>
<th>Gene tagged</th>
<th>Chr #</th>
<th>Position in the gene</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 457-10(14)F2-24</td>
<td>5,972,419</td>
<td>Glyma06g08110</td>
<td>06</td>
<td>Intron 3</td>
<td>Potassium voltage ligand ion channel gene</td>
</tr>
<tr>
<td>2 456-1(11)F2-76</td>
<td>38,819,380</td>
<td>Glyma13g37890</td>
<td>13</td>
<td>Exon 1</td>
<td>Cyclin gene</td>
</tr>
<tr>
<td>3 456-1(11)F2-80</td>
<td>59754016</td>
<td>-</td>
<td>18</td>
<td>-</td>
<td>In non-genic region</td>
</tr>
<tr>
<td>4 457-10(23)F2-10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>5 457-10(10)F2-28</td>
<td>49,758,864</td>
<td>-</td>
<td>02</td>
<td>-</td>
<td>In non-genic region</td>
</tr>
<tr>
<td>6 456-1(8)F2-33</td>
<td>9,301,628</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>In non-genic region</td>
</tr>
</tbody>
</table>

- Numbers in parenthesis () refer to the F1 number.
- These F2 plants come from a population crossed with the Ac event 545-19
Table 5. Summary of germinal transpositions for pPTN999 x pPTN398 stacks

<table>
<thead>
<tr>
<th>F2 plant</th>
<th>Position of the Ds re-insertion</th>
<th>Gene tagged</th>
<th>Chr #</th>
<th>Position in the gene</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>770-4(1)F2-38</td>
<td>3761366</td>
<td>09</td>
<td>Intron 7</td>
<td>ATP-binding cassette carrier</td>
</tr>
<tr>
<td>2</td>
<td>770-4(1)F2-34</td>
<td>2,391,765</td>
<td>16</td>
<td>Exon 5</td>
<td>PHD-finger protein</td>
</tr>
<tr>
<td>3</td>
<td>770-4(4)F2-20</td>
<td>39,336,655</td>
<td>03</td>
<td>2048bp - Upstream</td>
<td>Cation-transporting ATPase</td>
</tr>
<tr>
<td>4</td>
<td>770-4(6)F2-31</td>
<td>8,720,747</td>
<td>05</td>
<td>Exon 14</td>
<td>lethal(2)denticleless protein</td>
</tr>
<tr>
<td>5</td>
<td>770-4(6)F2-38</td>
<td>36787964</td>
<td>09</td>
<td>Exon 4</td>
<td>Glutamate decarboxylase/sphingosine phosphate lyase</td>
</tr>
<tr>
<td>6</td>
<td>770-4(6)F2-4</td>
<td>4355305</td>
<td>04</td>
<td>-</td>
<td>In the non-genic region</td>
</tr>
<tr>
<td>7</td>
<td>770-3(1)F2-47</td>
<td>47114995</td>
<td>11</td>
<td>3’-UTR</td>
<td>Mitotic spindle assembly checkpoint protein MAD2</td>
</tr>
<tr>
<td>8</td>
<td>770-3(6)F2-5</td>
<td>31,785,022</td>
<td>03</td>
<td>-</td>
<td>In the non-genic region</td>
</tr>
<tr>
<td>9</td>
<td>770-3(6)F2-42</td>
<td>19,470,705</td>
<td>15</td>
<td>549bp - Upstream</td>
<td>Cytosolic purine 5'-Nucleotidase-related</td>
</tr>
<tr>
<td>10</td>
<td>770-3(12)F2-12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>11</td>
<td>770-3(12)F2-42</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>12</td>
<td>770-3(6)F2-4</td>
<td>31785022</td>
<td>03</td>
<td>-</td>
<td>Non-genic region</td>
</tr>
<tr>
<td>13</td>
<td>770-3(12)F2-16</td>
<td>7390686</td>
<td>17</td>
<td>5’ UTR</td>
<td>Unknown gene</td>
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<tr>
<td>14</td>
<td>770-3(12)F2-20</td>
<td>43143634</td>
<td>02</td>
<td>Exon 1</td>
<td>KIP1-like protein</td>
</tr>
<tr>
<td>15</td>
<td>770-4(6)F2-2</td>
<td>48556553</td>
<td>10</td>
<td>-</td>
<td>Non-genic region</td>
</tr>
<tr>
<td>16</td>
<td>770-4(6)F2-5</td>
<td>31785022</td>
<td>03</td>
<td>-</td>
<td>Non-genic region</td>
</tr>
<tr>
<td>17</td>
<td>770-4(4)F2-35</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>18</td>
<td>770-3(5)F2-13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>19</td>
<td>770-4(7)F2-11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>20</td>
<td>770-4(7)F2-14</td>
<td>17465305</td>
<td>02</td>
<td>-</td>
<td>Non-genic region</td>
</tr>
<tr>
<td>21</td>
<td>770-4(2)F2-29</td>
<td>6122262</td>
<td>10</td>
<td>3’UTR</td>
<td>CDC2-related kinase</td>
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<td>770-4(2)F2-30</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>23</td>
<td>770-3(20)F2-12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Processing</td>
</tr>
<tr>
<td>24</td>
<td>770-3(3)F2-23</td>
<td>35283492</td>
<td>19</td>
<td>200bp - Upstream</td>
<td>Chalcone and stilbene syntheses</td>
</tr>
<tr>
<td>25</td>
<td>770-3(3)F2-2</td>
<td>47114995</td>
<td>14</td>
<td>2,229bp - Upstream</td>
<td>Multicopper oxidase</td>
</tr>
<tr>
<td>26</td>
<td>770-3(13)F2-2</td>
<td>1688853</td>
<td>04</td>
<td>-</td>
<td>Non-genic region</td>
</tr>
</tbody>
</table>

- Numbers in parenthesis () refer to the F1 number.
- These F2 plants come from a population crossed with the Ac event 545-19
**SUPPLEMENTAL TABLES**

TableS1. Primers used in the study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Oligonucleotide sequence (5' - 3')</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Product size</th>
<th>Screening for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 pPTN335-RB-R</td>
<td>CAGGCTTTACACTTTATGCTTCC</td>
<td>50</td>
<td>1076bp</td>
<td>$D_s$ excision/footprint for pPTN335</td>
</tr>
<tr>
<td>2 pPTN335-LB-F</td>
<td>ACAAATAACGGAATCTCAAGCAATC</td>
<td>50</td>
<td>973bp</td>
<td>pPTN335$D_s$ germinal excision/transposition</td>
</tr>
<tr>
<td>3 PCRGUSREV</td>
<td>CAGACGCCTGTTACAGTCTTCGC</td>
<td>50</td>
<td>411bp</td>
<td>GUS sequence</td>
</tr>
<tr>
<td>4 pPTN335-RB-R</td>
<td>CAGGCTTTACACTTTATGCTTCC</td>
<td>50</td>
<td>728bp</td>
<td>Ac sequence</td>
</tr>
<tr>
<td>5 pPTN335-GusF</td>
<td>AGAAAAAGCAGTCTTACTTCCATGA</td>
<td>50</td>
<td>1420bp</td>
<td>pPTN999$D_s$ germinal excision/transposition</td>
</tr>
<tr>
<td>6 pPTN335-GusR</td>
<td>CCATCCTTTGCAACCAAAGTT</td>
<td>50</td>
<td>793bp</td>
<td>$D_s$ excision/footprint for pPTN999</td>
</tr>
<tr>
<td>7 CaVMV-pPTN999-F</td>
<td>TACGGGAAAATACTGGAAGTATTATG</td>
<td>50</td>
<td>435bp</td>
<td>CaVMV sequence</td>
</tr>
<tr>
<td>8 CaVMV-pPTN999-R</td>
<td>ACTTCAAGAAAATAGCTTACACCAA</td>
<td>50</td>
<td>793bp</td>
<td>$D_s$ excision/footprint for pPTN999</td>
</tr>
<tr>
<td>9 pPTN999 LB-F</td>
<td>GAAATATTGCTAGCTGATAGTGACC</td>
<td>50</td>
<td>476bp</td>
<td>Southern blot probe for 457-10 x 545-19 F1-14 F2-35</td>
</tr>
<tr>
<td>10 pPTN999 RB-R</td>
<td>GAAATATTGCTAGCTGATAGTGACC</td>
<td>50</td>
<td>476bp</td>
<td>Southern blot probe for 457-10 x 545-19 F1-14 F2-35</td>
</tr>
<tr>
<td>11 Glyma15g RT-F1-HN</td>
<td>GATTATACCACTTTTGCTTGGCTTCT</td>
<td>53</td>
<td>728bp</td>
<td>RT-PCR, Northern blot probe for 770-3 x 545-19 F1-6 F2-42</td>
</tr>
<tr>
<td>12 Glyma15g RT-R1-HN</td>
<td>CTGGTTCATGACGGAAGAAACAGTCAAAATCGAGGTCATC</td>
<td>50</td>
<td>463bp</td>
<td>RT-PCR, Northern blot probe for 457-10 x 545-19 F1-14 F2-35</td>
</tr>
<tr>
<td>13 Gm06F1-HN</td>
<td>GTTCCATTGTTTGTTCTTATCTTCCTTCT</td>
<td>50</td>
<td>463bp</td>
<td>Cloning full length sequence of Glyma06g08110</td>
</tr>
</tbody>
</table>
Table S2. a. Primers for amplification of the T-DNA junction sequence

<table>
<thead>
<tr>
<th>Primers for amplification of the T-DNA junction sequence</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>335-borderJF-LB 1b</td>
<td>GTCGTTTTACAACGTCGTGACTG</td>
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<td>335-borderJF-LB ne1b</td>
<td>GTTACCCAACTTAATCGCCTTG</td>
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<td>335-borderJF-RB 1</td>
<td>CTCGAATTCGTAATCATGTCATAG</td>
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<td>335-borderJF-RBne1</td>
<td>GTAATCATGGTCATAGCTGTTTCT</td>
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<tr>
<td>336-borderJF-LB 1</td>
<td>ACCTTCTTTTCCACTATCTCACA</td>
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<tr>
<td>336-borderJF-LBne1</td>
<td>AAGTGACAGATAGCTGGGCAATG</td>
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<tr>
<td>336-borderJF-RB1</td>
<td>GTAATCATGGTCATAGCTGTTTCT</td>
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<td>336-borderJF-RBne1</td>
<td>GTCATAGCTGTTTCTGTGTAAT</td>
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<td>999-borderJF-LB2</td>
<td>ATTAGAGTCCCCGCAATTATACATTTAATAAC</td>
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<td>999-borderJF-LBne2</td>
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<td>GAAAATAGCCTCTACCCGTTTCCGTTT</td>
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<tr>
<td>999-borderJF-RBne1</td>
<td>TACCGTTTTGTATATCCCGTTTCC</td>
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<tr>
<td>Adaptor-1</td>
<td>CTAATACGACTCAGCTATAGGGCTCGAGCGGCCGCCCGGGAGGT</td>
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<tr>
<td>Adaptor -2</td>
<td>/5Phos/ACCTCCCCG/3AmM/</td>
</tr>
<tr>
<td>AP1</td>
<td>GGATCCTATACTAGACTCATACTAGGCC</td>
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<tr>
<td>AP2</td>
<td>CTATAGGGCTCGAGCGGC</td>
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b. Primers for amplification of junction sequences for \( Ds \) re-insertions

<table>
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<tr>
<th>Primers for amplification of junction sequences for ( Ds ) re-insertions</th>
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<tbody>
<tr>
<td>335-Dsreinsertion LB1</td>
<td>CGTTTCGGTTTACCGTTTGT</td>
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<tr>
<td>335-Dsreinsertion LBne1</td>
<td>TACCGTTTTGTATATCCCGTTT</td>
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<tr>
<td>335-Dsreinsertion RB1a</td>
<td>CCTTATAGGAGGGGTCTT</td>
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<tr>
<td>335-Dsreinsertion RBne1a</td>
<td>AGGGTCTTTGCGGATCTGAAT</td>
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