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Tnt1 Retrotransposon Mutagenesis in Soybean

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Tnt1 Retrotransposon Mutagenesis: A Tool for Soybean [Glycine max (L.) Merr.] Functional Genomics

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ABSTRACT

Insertional mutagenesis is a powerful tool for determining gene function in both model and crop plant species. *Tnt1*, originally identified in tobacco, is a retrotransposon that replicates via an RNA copy that is reverse transcribed and integrated elsewhere in the plant genome. Based on studies in a variety of plants, *Tnt1* appears to be inactive in normal plant tissue but can be reactivated by tissue culture. Our goal was to evaluate the utility of the *Tnt1* retrotransposon as a mutagenesis strategy in soybean. Experiments showed that the *Tnt1* element was stably transformed into soybean plants by *A. tumefaciens*-mediated transformation. Twenty-seven independent transgenic lines carrying *Tnt1* insertions were generated. Southern blot analysis revealed that the copy number of transposed *Tnt1* elements ranged from 4-19 insertions, with an average of ~8 copies per line. These insertions showed Mendelian segregation and did not transpose under normal growth conditions. Analysis of 99 *Tnt1* flanking sequences revealed insertions into 62 (62%) annotated genes, indicating that the element preferentially inserts into protein coding regions. *Tnt1* insertions were found in all 20 soybean chromosomes, indicating that *Tnt1* transposed throughout the soybean genome. Furthermore, FISH (Fluorescence In Situ Hybridization) experiments validated that *Tnt1* inserted into multiple chromosomes. Passage of transgenic lines through two different tissue culture treatments resulted in *Tnt1* transposition, significantly increasing the number of insertions per line. Thus, our data demonstrate the *Tnt1* retrotransposon to be a powerful system that can be used for effective large-scale insertional mutagenesis in soybean.
INTRODUCTION

Soybean (*Glycine max* L.) is a major commodity crop that offers a wealth of resources, including proteins, oils, mineral nutrients, and natural products that impact human health and nutrition. The products of soybean are widely used as vegetable oil and protein sources for human consumption, and are valuable feedstock for the livestock industry (Gepts et al. 2005; O’Brian and Vance 2007). Research on soybean is driven by its importance as a food crop worldwide. In recent years, considerable progress has been made in developing genomic resources for soybean, including the complete sequencing of the genome, which predicts 46,430 high confidence protein encoding genes (Schmutz et al., 2010). Utilizing the Illumina Solexa sequencing platform, a gene expression atlas of the soybean genome was developed that documented transcription of up to 55,616 annotated genes (Libault et al., 2010). One remaining major challenge is the elucidation of the function of these genes, especially those encoding important agronomic traits. This challenge can be met, in part, by the development of insertional mutagenesis tools to investigate soybean gene function.

Insertional mutagenesis is an effective method for functional genomics studies. Mutagenesis can modulate gene expression and create very useful loss-of-function mutants, whose phenotypes can validate and explore gene function. Insertional mutagenesis has been successfully used to study gene function in both model and crop plant species (Cowperthwaite et al., 2002; Alnoso et al., 2003; An et al., 2003; Fladung et al., 2004; Tadege et al., 2008; Mathieu et al., 2009). A clear example is the use of T-DNA tagging to create large mutant populations of *Arabidopsis thaliana* (Alonso et al., 2003). However, although development of a T-DNA insertional mutant repository in soybean is technically possible, it would require a tremendous amount of labor since each mutant line would require an independent transformation event. Thus, a transposon-tagging strategy, where many mutations could be derived from one primary transformation event, is an attractive approach for a plant such as soybean in which transformation requires a much longer time-frame (roughly one year, from seed to seed) (Parrot and Clemente, 2004; Mathieu et al., 2009).

Transposon tagging has been used successfully in soybean. For example, Mathieu et al. (2009) utilized the well-characterized maize transposon, *Ac/Ds*, to identify a soybean male sterile line. The *Ac/Ds* is a class II transposon, which transposes into new locations in plant genomes.
via a ‘cut-and-paste’ mechanism (Wessler, 2006). However, similar to the situation with T-DNA
insertions, the use of this transposon requires many independent transformation events to create a
library sufficiently large to target the entire soybean genome because it tends to transpose to
linked sites (Ito et al., 1999; Jones et al., 1990; Parinov and Sundaresan, 2000). Perhaps a more
promising alternative is \( mPing \), a class II element originally isolated in rice (Jiang et al., 2003;
Kikuchi et al., 2003; Nakazaki et al., 2003) where it transposes at a high frequency and can
reach a high copy number in some cultivars (Naito et al., 2006). Recently, Hancock et al. (2011)
reported that \( mPing \) can successfully transpose in soybean and generated stable, heritable
insertions. However, one possible limitation to the utility of the \( mPing \) element is that the
element continues to transpose, even under normal plant growth conditions, thereby creating
somatic mutations that could complicate both phenotypic and genetic analysis.

Compared to the class II transposons, the class I retrotransposons present some
advantages for use as an insertional mutagenic tool (Kumar and Hirochika, 2001).
Retrotransposons transpose in “copy and paste” manner via a RNA intermediate (Kumar and
Benntezen, 1999). Several retrotransposons have already been used effectively as mutagens in
plants. For example, \( Tos17 \), an endogenous retrotransposon of rice, is active in the rice genome
during tissue culture and has been used for gene tagging in rice (Piffanelli et al., 2007). \( LORE1 \),
an exon-targeting endogenous retrotransposon in \( Lotus japonicus \), was recently used to develop a
medium-size mutagenized population composed of 2450 plant lines (Fukai et al., 2012). The
\( Tto1 \) element, from tobacco, has also been used for mutagenesis in \( A. thaliana \) (Okamoto and
Hirochika, 2000) and rice (Hirochika et al., 1996). \( Tnt1 \), originally isolated from tobacco, has
been successfully used in several heterologous hosts including \( Medicago truncatula \) (d’Erfurth et
al., 2003; Tadege et al., 2005; Tadege et al., 2008; Iantcheva et al., 2009), \( A. thaliana \) (Lucas et
al., 1995; Courtial et al., 2001) and lettuce (\( Lactuca sativa \), Mazier et al., 2007). Collectively,
these studies demonstrate that retrotransposons transpose preferentially into gene-rich regions,
thus making them highly mutagenic. While retrotransposons are activated in tissue culture, they
appear to be stable in mature, transgenic plants. Therefore, relatively few primary transgenic
lines can lead to large populations of mutants by repeated transfer through tissue culture. Indeed,
the retrotransposon \( Tnt1 \) has been used successfully in the model legume plant \( M. truncatula \) to
build useful mutant populations (d’Erfurth et al., 2003; Tadege et al., 2005; Tadege et al., 2008;
Iantcheva et al., 2009). The published \( M. truncatula Tnt1 \) population contains nearly 12,000

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insertion lines, representing over 300,000 insertions, and has been used successfully in both forward and reverse genetics studies (Tadege et al., 2008; Cheng et al., 2010). However, reactivation of retrotransposon transposition does not occur in every plant species examined. For example, Ishizaki and Kato (2005) failed to detect tissue culture reactivation of the \textit{Tto1} retrotransposon in transgenic potato plants.

The goal of our study thus was to explore the utility of the \textit{Tnt1} retrotransposon as a mutagenesis strategy in soybean (\textit{Glycine max}). Our findings demonstrate that the \textit{Tnt1} is an attractive and efficient system that can now be used for large-scale insertion mutagenesis in soybean.

\textbf{RESULTS AND DISCUSSION}

\textbf{Generation of \textit{Tnt1} retrotransposon containing soybean lines.}

Although \textit{Tnt1} transposes very efficiently in the legume model plant \textit{M. truncatula} and several other plants including tobacco, \textit{A. thaliana} and lettuce (Courtial et al., 2001; d’Erfurth et al., 2003; Tadege et al., 2005; Mazier et al., 2007; Tadege et al., 2008; Iantcheva et al., 2009), it was important to evaluate its utility in soybean for two reasons. First, due to the economic importance of soybean, it is critical to develop improved gene discovery tools. Second, it is known that some retrotransposons are genotype-specific for transposition or exhibit high efficiency only on specific genotypes. For example, the \textit{Tio1} retrotransposon from tobacco transposes in tobacco, \textit{A. thaliana} and rice (Hirochika, 1993; Hirochika et al., 1996; Okamoto and Hirochika 2000), but does not transpose in potato tissue culture (Ishizaki and Kato, 2005). In \textit{M. truncatula}, the reactivation protocol of \textit{Tnt1} optimized to cultivar R108 is not applicable for cultivar Jemalong (Iantcheva et al., 2009). Therefore, it is necessary to determine if \textit{Tnt1} transposes in soybean and also to optimize the methodology to induce its transposition.

To investigate whether the \textit{Tnt1} element can transpose in soybean during tissue culture, \textit{Agrobacterium tumefaciens}-mediated transformation was performed using a modified soybean cotyledonary-node transformation protocol (Zeng et al., 2004). The plasmid \textit{pSH-Tnt1} containing the \textit{Tnt1} element was constructed by inserting the \textit{Tnt1} DNA into the binary vector pZY101, which carries selectable \textit{bar} gene marker for glufosinate resistance (Figure 1A). Transformations were performed in \textit{Glycine max} cv. Maverick, a genotype that is susceptible to
glufosinate. Genotype Maverick is an elite soybean genotype that is resistant to stress conditions and shows a consistently higher transformation frequency when compared to other genotypes (ZY Zhang, unpublished data). Twenty-seven independent glufosinate resistance plants (verified by leaf painting assay) were generated by this approach. To determine if these regenerated plants harbored Tnt1, PCR experiments were performed using three primer pairs specific for Tnt1 and one primer pair specific for the bar gene as indicated in Fig. 1A. All 27 lines gave positive PCR amplifications for all Tnt1- and bar-specific primer pairs (data not shown).

**Tnt1 transposes in regenerated soybean plants**

To verify the PCR results and to determine if Tnt1 integrated into the soybean genome and transposed during tissue culture, we performed Southern blot analysis on all 27 lines that were positive by PCR. Chromosomal DNA of these plants was extracted and digested with restriction enzyme NdeI. Southern blot analysis was performed using a 755-bp Tnt1 internal fragment as the probe, which corresponds to bases 1067 - 1822 of the retrotransposon. The same blot was then stripped and re-probed with a 480-bp bar internal fragment. The NdeI sites and probe locations are shown in Figure 1A. NdeI cuts the Tnt1 DNA once at position 1983 nt and it also cuts once within the T-DNA region (near the left border). Therefore, a line carrying Tnt1 associated with a T-DNA should show a 3.44-kb band when the above-mentioned probe is used; while most other hybridization bands would represent transposed Tnt1 copies. Southern blot analysis of 17 Tnt1 containing plants and the parent line Maverick using the Tnt1 probe is shown in Figure 1B. As expected, a 3.44-Kb band (indicated by an arrow) was present, representing non-transposed Tnt1 (i.e., T-DNA associated) in all 27 lines. This band also hybridized with the bar probe (Figure 1C), further confirming that it is T-DNA associated. We detected no plant carrying a Tnt1 element without the T-DNA, as has been reported in *M. truncatula*, where 11.2% of the regenerated plants carried only the retrotransposon (D’Erfurth et al., 2003). In addition to the 3.44-Kb band, multiple Tnt1-hybridizing bands, which did not hybridize to the bar probe, were detected in all plants tested, indicating that Tnt1 is able to transpose in soybean during the tissue culture associated with transformation (Figure 1B). Transposed Tnt1 copy numbers ranged from 4-19, with an average of ~8 copies per line. Thus, the results of these experiments confirm that the Tnt1 element was stably transformed soybean plants by *A. tumefaciens*-mediated transformation. Since the plants we analyzed were derived directly from the tissue culture of
primary transformation, the observed transposition events likely occurred during *A. tumefaciens*-mediated transformation. The copy numbers of transposed *Tnt1* elements in soybean were similarly, perhaps slightly less, than that reported for *M. truncatula* (ranging from 4 to more than 30 insertions), *A. thaliana* (ranging from 0 to 26 insertions) or lettuce (more than 30 copies) (Courtial et al., 2001; D’Erfurth et al., 2003; Mazier et al., 2007). Further optimization of the transformation method may permit generation of lines with significantly more *Tnt1* insertion events.

Southern blot analysis revealed that all *Tnt1* harboring soybean plants contained transposed *Tnt1* elements. No plant contained just a single copy of T-DNA. This result is similar to *M. truncatula*, but contrasts with reports in *A. thaliana*, where several regenerated plants contained no transposed *Tnt1* copy, or in lettuce, where four different regenerated lettuce plants were found to contain only a truncated version of the T-DNA, but no transposed copies of *Tnt1*.

In order to be useful for large scale mutagenesis in soybean, it is critical that the *Tnt1* insertion pattern does not exclude any chromosomes. As one method to determine the *Tnt1* transposition pattern, two *Tnt1* containing plants were analyzed using Fluorescence In Situ Hybridization (FISH). The *Tnt1* containing line BS5-6 chromosomes were hybridized with Texas Red labeled *pSH-Tnt1* plasmid DNA. Chromosomes of untransformed Maverick served as control. As shown in Fig. 2A, the hybridization signals (red dots) were detected on most BS5-6 chromosomes; whereas no signal was detected in the parental control line (Fig. 2C). Moreover, multiple hybridization signals were observed in several chromosomes (Fig. 2A and 2B). Based on the Southern blot analysis, the line BS5-6 posses ~19 transposed copies of *Tnt1* (Fig. 1B). The FISH data showing multiple *Tnt1*-hybridizing regions is consistent with these results. We should note that although the probe DNA used for FISH experiments contained the entire *Tnt1* and T-DNA, the Southern blot analysis revealed that most of the hybridization signals observed in the chromosomes of BS5-6 were transposed *Tnt1* elements. To further clarify this, we cloned the 5.3-kb *Tnt1* DNA into vector pBluescriptSK(+) and used the resulting plasmid construct (*pBS-Tnt1*) as probe for FISH experiments to examine line BS5-6. We found that hybridization signals observed with the *pBS-Tnt1* probe (Fig. 2E and 2F) were comparable to those obtained with *pSH-Tnt1* (Fig. 2A and 2B). No hybridization signals were detected when the empty pBluescriptSK(+) vector was used as probe (data not shown). Furthermore, a FISH experiment was also performed on another *Tnt1* line BS6-19 using *pBS-Tnt1* as probe. The results (Fig. 2I...
and 2J) revealed very strong hybridization signals on several chromosomes. In addition, several weaker signals were also detected on other chromosomes. As expected, no signal was detected in the parental control line (Fig. 2G and 2H). Southern blot analysis predicted 15 Tnt1 insertions in line BS6-19. To examine the nature of the stronger FISH hybridizing signals, we performed Fiber-FISH to estimate the size of the hybridizing region. When a pBluescriptSK(+) plasmid harboring Tnt1 was labeled with biotin and used as a probe (Fig. 2K), a strong signal was detected in line BS6-19 over an estimated length of ca. 34 kb. A plausible explanation for this length is that multiple copies of Tnt1 DNA inserted into the same position on one chromosome in this line. This would suggest that the weaker FISH hybridizing signals likely represent one or at most a few inserted Tnt1 elements; whereas the stronger hybridizing bands likely represent tandemly arrayed, multiple copies of Tnt1.

Tnt1 efficiently transposes into coding regions

To identify Tnt1 insertion sites, we performed TAIL-PCR (Ratet et al., 2006) on 18 independent transgenic lines to recover Tnt1 flanking sequences. Of the 99 Tnt1 insertion sites identified, 62 were located in annotated genes (Table I). Moreover, Tnt1 insertions were found in all 20 soybean chromosomes (Fig. 3), as indicated by mapping the flanking sequences to the published soybean genome sequence (Schmutz, 2010). Therefore, consistent with the results of the FISH analysis, Tnt1 appears to transpose throughout the soybean genome.

To obtain efficient mutagenesis in plants such as soybean, which has a relatively large genome, it will be important to use a transposon system with an insertional preference for coding regions, rather than intergenic regions. One of the main advantages of using retrotransposons for mutagenesis is that they have been documented to transpose preferentially into gene-rich regions. Tnt1 does transpose preferentially into gene-rich regions in M. truncatula, A. thaliana and lettuce (Courtial et al., 2001; D’Erfurth et al., 2003; Mazier et al., 2007). Our analysis of 99 Tnt1 flanking sequences revealed that the element inserted into 62 (62%) annotated genes. If Tnt1 insertion into the soybean genome had occurred randomly, the tagging efficiency should have been 9.8 % (46,430 genes of 2 kb / 950 Mb genome; Schmutz et al., 2010). Therefore, our results suggest that Tnt1 preferentially inserts into protein coding regions in soybean.

Tnt1 insertions are stable and heritable in soybean
We examined the expression of *Tnt1* transposase using RT-PCR in young leaves of progeny lines derived from three, independent transgenic events. As shown in Figure 4, expression of the *Tnt1* was detected in the tissues of the *Tnt1* transgenic plants but not in leaves of the parent line Maverick. Comparison of three different transgenic events showed that the level of transposase expression was variable, which may be due to positional effects at the various *Tnt1* insertion sites.

To determine if *Tnt1* insertions are active in self-fertilized progeny plants, the original T₀ *Tnt1* soybean transgenic events were allowed to self-fertilize, and the locations of the *Tnt1* insertions in progeny of six lines were examined by Southern blot analysis. The Southern blot analysis results of the T1 progenies of *Tnt1* lines BS5-12-8R, BS6-19, BS5-6, BS5-12, BS12-7 and BS5-12-12C, as well as T2 progenies of line BS6-19, using the *Tnt1* probe are shown in Figure 5 and supplemental Figure S1. The locations of the *Tnt1* insertions were found to be stable in the progeny lines because no new band was observed, indicating an absence of additional germinal or somatic transpositions. Because the Southern blot analysis method was not accurate enough to resolve all of the different insertions in a transgenic event, the segregation of individual *Tnt1* insertions from one event (BS5-12-8R) was examined using PCR amplification of selected flanking regions and scored as either present, absent or heterozygous in each of the progeny lines (see Table II). For three of four tested loci, the segregation pattern was close to a ratio of 1: 2: 1 (25% wild type, 50% heterozygous and 25 % homozygous for a given *Tnt1* insertion). These results indicate that *Tnt1* insertions do follow Mendelian segregation. The segregation results for insertion #3 (Table II) indicated that no homozygous mutant locus was detected, which could be attributable to lethality associated with insertion or the population tested (18 plants) was too small.

In order to be useful as a mutagen, *Tnt1* insertions should remain inactive during normal plant growth and exhibit segregation consistent with a single locus. It is important for mutant analysis that new rounds of transposition do not occur in subsequent generations. Similar to our findings in soybean, studies have shown that *Tnt1* insertions are genetically independent and follow Mendelian segregation in *A. thaliana*, *M. truncatula* and lettuce (Courtial et al., 2001; D’Erfurth et al., 2003; Mazier et al., 2007). In *A. thaliana* and *M. truncatula*, while reverse transcription products of *Tnt1* are detectable, they did not result in the integration of new germinal *Tnt1* copies in the progeny of transformed plants (Courtial et al., 2001; D’Erfurth et al.,
Although expression of the \textit{Tnt1} transposase could be detected in the vegetative tissue of transgenic plants, there was no evidence that the \textit{Tnt1} element was able to transpose in mature plants and in subsequent generations under normal growth conditions. These stable insertion events were heritable and segregated in a Mendelian fashion.

\textbf{\textit{Tnt1} transposition can be re-activated in soybean by in vitro culture}

Given the time needed to produce independent soybean transgenic lines, practical use of the \textit{Tnt1} transposon in soybean would require that a few initial transgenic lines be used to reactivate the transposon through tissue culture in order to generate populations with large numbers of independent insertions. To investigate the feasibility of this approach, we tested the two published methods for soybean regeneration to gauge their ability to reactivate \textit{Tnt1} transposition. The cotyledons of the \textit{Tnt1} containing T1 plant seeds were used as explants for the first approach (Zeng et al., 2004). The explants were treated by wounding or wounding followed by 12 hrs shaking in a 1M sucrose solution. The latter treatment was tested since it was reported to significantly increase the frequency of \textit{Tnt1} transposition in \textit{M. truncatula} cv. Jemalong (Iantcheva et al., 2009). Over 40 plants were regenerated from wounded cotyledons of seven \textit{Tnt1} T0 lines with or without sucrose treatment. Those plants were examined by Southern blot analysis. The results (Fig 6) revealed that one line, BS5-12, showed a significant number of new \textit{Tnt1} transposition events (up to 20 copies). The original T0 parental line contained only 4 \textit{Tnt1} insertion sites. The regenerated plants from other lines tested by this approach produced 0 to 5 new \textit{Tnt1} insertions in the genome. However, the sucrose treatment did not enhance the frequency of \textit{Tnt1} transposition in these experiments.

In order to verify that the new bands observed were novel \textit{Tnt1} insertions sites, TAIL-PCR was performed to recover the flanking soybean sequence from four plants generated from reactivation of event BS5-12. Twenty \textit{Tnt1} flanking sequences were obtained from those plants. Ten specific primers were designed from the \textit{Tnt1} flanking sequences of reactivated line BS5-12-8R and 5 primers were designed from the \textit{Tnt1} flanking sequences of reactivated line BS5-12-12C. Those primers were paired with a \textit{Tnt1}-specific primer LTR7 and used for PCR reactions. PCR results revealed that all of the BS5-12-8R primers (paired with LTR7 primer) produced PCR products with BS5-12-8R chromosomal DNA. Similarly, all five BS5-12-12C primers produced PCR products with genomic DNA of BS5-12-12C. No PCR product was produced...
using the genomic DNA of the T0 plant BS5-12 or the parent plant as templates. These results confirm that the new hybridization bands observed by Southern blot analysis were indeed novel Tnt1 insertions. Thus, our results clearly demonstrate that the cotyledon approach does reactivate Tnt1 transposition and generate additional insertion sites. However, the fact that only one line exhibited a high frequency of transposition suggests that the original site of Tnt1 insertion may affect the ability to transpose. These results are similar to the case of M. truncatula, where only a few lines were shown to transpose at a high frequency by repeated transfer in tissue culture. However, these ‘starter lines’ were sufficient to generate a large, insertional mutant population (D’Erfurth et al., 2003; Iantcheva et al., 2009). In the case of soybean, our results suggest that an extended period in tissue culture, perhaps with repeated wounding, enhanced the frequency of transposition in the BS5-12 line. Consistent with the previous results, analysis the Tnt1 flanking sequences obtained from the reactivated plants showed that Tnt1 inserted preferentially into annotated genes in 12 (60%) of the isolated integration sites.

In addition to the use of cotyledonary nodes, soybean can also be regenerated from somatic embryos (Trick et al., 1997). Somatic embryos were generated from immature embryos collected from Tnt1-transformed T1 plants. Individual plants from five independent Tnt1 containing lines were selected for passage through somatic embryogenesis. During the tissue culture treatment, seven mature embryos were selected at the end of a 5 week histodifferentiation step for transposon display analysis (van den Broeck, Maes et al. 1998; Hancock et al. 2011) using Tnt1 specific primers. This allowed for comparison of the Tnt1 insertions in the original plant and the resulting somatic embryos. As expected, the Tnt1 insertions present in the parent plant were found to segregate in a Mendelian fashion in the somatic embryos. In addition, the somatic embryos of four of the genotypes tested showed a small number (1-5) of novel bands that were not present in the parent (i.e. BS8-5 and BS5-6, Figure 7). However, the somatic embryos produced from the BS5-13 line showed a large increase in the total number of novel bands (up to 20) in the somatic embryos (Figure 7). Some of these novel insertions were shared between embryos, indicating that they occurred early in production of embryogenic tissue. Some bands were also unique to single embryos suggesting that they occurred later in embryo development. We should note that Tnt1 containing line BS5-12 was also tested for reactivation by this approach but showed only limited reactivation. Similarly, reactivation experiments performed using the cotyledon-node approach with event BS5-13 produced only 1 or 2 new Tnt1 insertions.
copies (data not shown). These results suggest that the efficiency of reactivation approaches are related to the genotypes of original \textit{Tnt1} containing lines used.

To verify that novel insertion sites arose during somatic embryogenesis, we excised three novel bands from the transposon display gel, performed PCR amplification with appropriate primers, and sequenced the PCR products. This resulted in four sequences that included both the end of the \textit{Tnt1} element and soybean genomic sequence. A homology search allowed for the insertion sites to be located in the soybean genome (Table III). Using primers that flank the \textit{Tnt1} insertion sites, we were able to verify the presence of three of these \textit{Tnt1} insertions (data not shown). Of these, one was found to be present in the original BS5-13 plant, but the remaining two \textit{Tnt1} insertions were confirmed to be novel insertions that occurred during tissue culture treatment. This analysis confirms that the majority of the bands observed in transposon display represent true transposition events.

In summary, we compared the ability of two different tissue culture methods to reactivate \textit{Tnt1} transposition. In both methods, the majority of the lines tested showed modest transposition, but, in each case, a single line showed a much higher frequency of transposition. An interesting finding was that different lines were optimal for the two methods, event BS5-12 showed a higher frequency of transposition using the cotyledonary-node approach, while line BS5-13 showed higher transposition during somatic embryogenesis. Therefore, these two lines represent promising starter lines for the construction of large, mutant populations of soybean.

\textbf{\textit{Tnt1}: an insertion mutagen in \textit{Glycine max}?}

Practical use of \textit{Tnt1} for mutagenesis in soybean requires generation of several initial transgenic lines for subsequent reactivation by repeated tissue culture regeneration. This approach is especially well suited for a plant such as soybean, in which generation of the original transgenic events is laborious and time consuming. In this strategy, the higher the number of insertions per line allows for a lower number of individual plants to be maintained in order to create a population suitable for mutant screening. The flanking sequences in this population can be readily identified using high-throughput sequencing methods to create a searchable database of insertion sites, comparable to those currently available for model species (Williams-Carrier et al., 2010; Urbański et al., 2012). Clearly, this approach has advantages over T-DNA or \textit{Ac/Ds} mutagenesis of soybean, in which a large number of independent transgenic lines would be
needed (Scholte et al., 2002; Wessler, 2006; Mathieu et al., 2009). The Ac/Ds system has the added limitation that most insertions occur within a short distance (within a few cM) of the original insertion site (Ito et al., 1999; Jones et al., 1990; Parinov and Sundaresan, 2000). Unlike the mPing transposable element (Hancock et al., 2011), Tnt1 appears to be stable in mature plants with no evidence of additional germinal or somatic insertions. Consistent with findings in other plants species, such as M. truncatula, A. thaliana and lettuce (Courtial et al., 2001; D’Erfurth et al., 2003; Mazier et al., 2007), Tnt1 transposition in soybean targets gene-rich regions preferentially, which makes it highly effective for mutagenic gene function studies. Our data revealed that Tnt1 transposition generates from 4 to up to 20 insertions per plant in soybean. These insertions are stable during the life cycle of soybean and they are genetically independent and can be separated by recombination. Therefore, unwanted insertions can be removed through serial back-crossing to the parental line. If one wants to work with a line with a clean single Tnt1 insertion, a couple of rounds of back crossing will be required.

Soybean is an ancient tetraploid whose genome has undergone at least two rounds of whole genome duplication (Schmutz et al., 2010). This raises the possibility that gene functional redundancy due to the presence of homeologous gene copies could limit the ability to obtain informative phenotypes for single transposon insertions. However, clearly phenotypes can be obtained by chemical or radiation mutagenesis (Cooper et al., 2008; Bolon et al., 2011). Moreover, we previously identified a male sterile mutant of soybean using Ac/Ds mutagenesis in soybean (Mathieu et al., 2009). Even in the model plant A. thaliana, the presence of multigene families can limit the ability to obtain phenotypes by mutating a single member of the family (Stacey et al., 2006). Hence, it remains to be seen whether the paleotetraploid nature of the soybean genome would create any significant limitations to the use of large-scale transposon mutagenesis for gene functional studies.

CONCLUSION
We successfully introduced the Tnt1 retrotransposon into stably-transformed soybean plants by A. tumefaciens-mediated transformation. The inserted Tnt1 elements appear to be inactive in somatic plant tissues and were inherited in a Mendalian fashion. However, the activity of these elements could be reactivated by two different tissue culture treatments. Analysis of the sequences flanking the Tnt1 insertion sites showed that the element preferentially inserts into
protein-coding regions. Two Tnt1 lines, originally containing only a few copies of Tnt1 element, were shown to be highly efficient for transposition upon passage through tissue culture; they therefore represent highly promising lines for the development of large, mutant populations in soybean. The development and characterization of such a population would create an extremely useful resource for both basic and applied studies of this important crop plant.

MATERIALS AND METHODS

Plant material and plant growth conditions

*Glycine max* cultivar Maverick was used for all plant transformation experiments. Soybean plants were grown in soil in the greenhouse and watered alternatively with de-ionized water and a nutrient solution (Miracle Grow) with a cycle of 18 hours light at 29°C and 6 hours dark at 24°C.

Bacterial Strains and T-DNA vectors

The *E. coli* strain DH5α (Sambrook et al., 1989) was used for cloning and the propagation of the different vectors. *A. tumefaciens* strain AGL1 was used in all plant transformation experiments. Plasmids were introduced into AGL1 by direct DNA transfer (An et al., 1988). An EcoRI fragment containing the entire Tnt1 element from plasmid pHLV4909 (gift from Helene Lucas, INRA Versailles) was cloned into the binary vector pZY101 (Vega et al., 2008) to yield pSH-Tnt1. The vector pZY101 carries the bar gene for glufosinate resistance. The resulting plasmid pSH-Tnt1 was used for all transformations. The *Agrobacterium* strain was grown in YEP medium containing rifamycin (30mg/L), spectinomycin (100mg/L) and kept at 250 rpm shaking for overnight at 28°C. Cotyledonary explants derived from 5 day old seedlings of genotype Maverick were used for the cocultivation.

Plant transformation and selection

All T0 transgenic soybean events were developed following the protocol as described previously (Zeng et al., 2004), except that antioxidants dithiothreitol and sodium thiosulfate were added to the cocultivation medium at the concentrations of 3.3mM and 1.0mM, respectively (Olhoft et al., 2003); also, 0, 10, and 5mg/L glufosinate was added to the first and second shoot induction media, as well as shoot elongation medium. Each regenerated plant was screened three
times from plantlet to plant stage using herbicide leaf-painting to assess the functional expression of the *bar* gene. All of the plant transformations were performed in the MU-Plant transformation Core facility.

**Examining the occurrence of *Tnt1* element and T-DNA in transformed plants**

Chromosomal DNA was isolated from plants according to Dellaporta et al. (1983). PCR experiments were performed to examine the *Tnt1* insertions and T-DNA in regenerated plants. Three pairs of *Tnt1* specific primers and one pair of bar gene specific primers (Fig. 1) were used:

- TntA, 5’-TGGTATCAGACAGGTTCTGCT-3’; TntB, 5’-AAATGTGACAAAAATTCGTACCT-3’; TntC, 5’-AACGGACTAACACAGCTTGCC-3’; TntD, 5’-ATAACTCTCTGATCCATCTCGGTC-3’; TntE, 5’-TTGATTTTGACGAAATTTTTCTCCC-3’; TntF, 5’-CCTGCCATATCAGCATCTGTATAG; barA, 5’-TACCATGAGCCCAGGCCC-3’ and barB, GGCTGAAGTCCAGCTGGCC-3’.

**Molecular Analysis**

Standard procedures were used in the isolation of plasmid DNA, gel electrophoresis, PCR reactions, DNA ligation, transformation and electroporation (Sambrook *et al.* 1989). Restriction and modification enzymes were obtained from Promega Biotech (Madison, WI). Soybean plant chromosomal DNA were extracted from young leaves according to the procedures described by Dellaporte *et al.* (1983). Fifteen µg of RNase A treated genomic DNA for each line was digested with *NdeI* and separated on a 0.8% agarose TAE gel running at 30V overnight. DNAs were transferred to Zeta Probe GT Nylon membrane (Bio-Rad Laboratories) and used for Southern blot analysis. Southern blot hybridizations were carried out following the procedures of Klein-Lankhorst *et al.* (1991). A 755-bp *Tnt1* internal fragment corresponding to bases 1067nt-1822nt of the retrotransposon was used as probe (Fig. 1A). Prime-a-Gene DNA labeling system (Promega Biotec, WI) was used for labeling DNA probes. The $\alpha^{32}$P-dATP (3000 Ci/mol)-labeled probes were used for hybridization. After hybridizing with the *Tnt1* probe, the blots were stripped according to the instruction of manufacturer and re-probed with a 480-bp *bar* internal fragment. After washing, the membrane was exposed to a phospoimager screen and then visualized using a FujiFilm Fluorescent Imager Analyzer FLA 3000.
RNA isolation and Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from young leaves was isolated using TriZol® Reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, California). The isolated RNA was further purified and treated with DNase TURBO DNA-free according to the manufacturer’s instructions (Ambion Inc, Austin, Texas). The 1st strand cDNA was synthesized using Promega AMV reverse transcriptase and used as input in PCR reactions using Promega Taq polymerase according to the manufacturer’s instructions (Promega Madison WI) with the following PCR conditions: 94°C for 5 min, 94°C for 30 sec, 57°C for 1 min, 72°C for 2 min, 35 cycles, and 72°C for 5 min. The Tnt1 gene-specific forward and reverse primers used were as follows: 5’-TGGTATCAGAGCACAGGTTCTGCT-3’ (forward primer) and 5’-AAATGTGACAAAAAATTCGTACCT–3’ (reverse primer). The Cons 6 primers [Libault et al., 2010; 5’-AGATAGGGAAATTGTGCAGGT–3’ (forward primer) and 5’-CTAATGGCAATTGCAGCTCTC –3’ (reverse primer)] designed from the sequence of gene Glyma12g05510 were used as internal control.

FISH and fiber-FISH analyses

Sample preparation, FISH and fiber-FISH experiments, and image processing were performed precisely as described in Gill et al. (2009). The plasmid DNAs of pSH-Tnt1 or pSH-Tnt1-SK [Tnt1 DNA clone into pBluescript SK(+)] were labeled with Texas Red and used as probes for FISH experiments. The biotin labeled pSH-Tnt1-SK DNA was used as a probe for fiber-FISH experiments.

Genetic analysis

Genomic DNA was isolated from plants according to Dellaporta et al. (1983). The segregation of different Tnt1 insertions in a randomly chosen line BS5-12-8R was examined by PCR using the following genes-specific forward and reverse primers on genomic DNA: 5’-CGAACATTACACCACTAAGATGTC’ (Glyma20g34300F) and 5’-TGACATCTCAAATTACTTTCATTG-3’ (Glyma20g34300R); 5’-TAAGTGTCGTACGCTAATGCGATC-3’ (Glyma17g35300F) and 5’-TCAATTCTTCGCCATCGTTACAC-3’ (Glyma17g35300R); 5’-
ACCAAGCTTTTGTGACTGCATCCAC-3’ (Glyma01g01300F) and 5’-
TATATCTTCTTTGTGGACTACAAGG-3’ (Glyma01g01300R); 5’-
GCCAAGCTTGTATAGGGAGGAGTTA-3’ (Glyma19g07410F) and 5’-
TGTGTCTGTATAGGGACGATAA-3’ (Glyma19g07410R) in combination with the Tnt1
right border primer: 5’- TATTATCCGCTTTATTACCCTGTA-3’ (LR7). PCR reactions were
conducted using Takara Ex Taq Polymerase (Takara, Madison, WI) under the following
conditions: 94°C for 5 min, 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, 35 cycles, and 72°C for
5 min.

Tnt1 flanking sequences isolation and sequencing
The Tnt1 flanking sequences were recovered by Thermal Asymmetric Inter Laced PCR
(TAIL-PCR) described by Ratet et al. (2006). The arbitrary primers used were AD1
[NCTGA(G/C)T(A/T)T(G/C)G(A/T)GTT], AD2 [NGTCGA(G/C)(A/T_GANA(A/T)GAA] and
AD3 [(A/T)GTGNAG(A/T)ANCANAGA]. Three Tnt1 specific primers Tntail3
(TCTGGATGAATGAGACTGGAG, corresponded to bases 4696-4717nt of Tnt1), LTR4
(TACCGTATCTCGGTGCTACA, corresponded to bases 534-553nt/5258-5277nt of Tnt1) and
LTR7 (TATTATCCCGCTTTATTACCCTGTA, corresponded to bases 555-578nt/5279-5302nt of
Tnt1) were used for primary, secondary and tertiary PCR reactions, respectively. The PCR
products were cloned into pGem-T Easy vector (Promega Biotec Madison, WI) and sequenced.
DNA sequencing was performed in DNA Core facility of University of Missouri.

Homology searching
The flanking sequences of the tagged loci were compared to the sequences of the
database using the BLAST program in websites: http://blast.ncbi.nlm.nih.gov, the Phytozome
(http://www.phytozome.net/soybean.php) and Soybase (http://soybase.org).

Reactivation of Tnt1 transposition
The T0 transgenic Tnt1 events were reactivated using two different tissue culture
approaches. The first approach used cotyledons as explants through organogenesis-based in vitro
tissue culture. All the steps and media followed the protocol described by Zeng et al. (2004) with
modifications and no Agrobacterium inoculation was involved. The major modifications
included the replacement of MS-based medium (Murashige and Skoog, 1962) with B5-based medium (Gamborg et al., 1968) for all culture stages and the use of 0.2mg/L IAA (indoleacetic acid) and 2mg/L zeatin riboside for the shoot elongation stage, as well as the deployment of a step-up selection strategy. Briefly, seeds of primary transgenic Tnt1 events were germinated for 5 days on B5-based germination medium. The cotyledonary node explants were prepared by wounding with a razor blade with or without sucrose solution treatment. Sucrose treatment was performed by shaking (120 rpm) the wounded explants in 1M sucrose solution for 12 hours. Treated explants were then cultured on B5-based shoot induction medium for the first two weeks and followed by an additional two weeks of subculture on the same fresh medium amended with 5mg/L glufosinate. Explants were transferred biweekly onto fresh B5-based shoot elongation medium amended with 10mg/L glufosinate. Shoots longer than 3-cm were excised and cultured in B5-based rooting medium without glufosinate selection. Each plantlet (with a shoot and roots) was transferred to Metro-mix 200 soil (Hummert International, Earth City, MO) in a jiffy pot inside a Magenta culture vessel for acclimatization. Hardened plantlets were transferred to 3-gallon pots containing Pro-mix soil mixed with Peters 20-20-20 (Hummert International, Earth City, MO) in greenhouse. Plants were watered as needed. Each event was screened three times from plantlet to plant stage using herbicide leaf-painting for the functional expression of the bar gene.

For the second approach, seeds of Tnt1 containing T0 plants were germinated and grown in the greenhouse. Somatic embryogenesis and plant regeneration was performed on the immature embryos collected from these plants. The production of somatic embryos was performed as previously described (Trick et al. 1997), excluding bombardment and antibiotic selection. DNA purification of the parent plant and differentiated embryos were performed using the CTAB method (Murray and Thompson 1980). The transposon display protocol was essentially the same as described in Hancock et al. (2011) except using Tnt1 specific primers (Tnt1 P3 [primary amplification] 5’-CCAACCAAACCAAGTCAACA-3’, Tnt1 P4 [secondary amplification] 5’-GGTTGGCTACCAAACCAAAG-3’). Excised transposon display bands were PCR amplified with the appropriate primers and cloned into pJET1.2 (Fermentas) for sequencing.

ACKNOWLEDGMENTS
We are grateful to Helene Lucas, INRA Versailles for providing the Tnt1 plasmid pHLV4909. Thanks to Neng Wan (University of Missouri) for taking care of the transgenic plants in the greenhouse.
LITERATURE CITED


Murray, M. G. and W. F. Thompson (1980) Rapid isolation of high molecular-weight plant
DNA. Nucleic Acids Research 8: 4321-4325.


FIGURE LEGENDS

Figure 1. A, Diagram of plasmid pSH-Tnt1 containing the Tnt1 element in the binary vector pZY101. LB and RB, left and right borders, respectively; bar, gene conferring glufosinate resistance; 35S, promoter 35S; LTR, Long Terminal Repeat. NdeI restriction sites and PCR fragments for bar (barA-B) and Tnt1 amplification (TntA-B, TntC-D and TntE-F) are shown. PCR fragment barA-B or TntC-D was used in Southern blot analysis to probe for bar or Tnt1, respectively.
B, Southern blot analysis of Tnt1 primary transgenic lines to identify Tnt1-hybridizing bands. Chromosomal DNA (15 - 20 µg) from each transgenic line was digested with NdeI and probed with TntC-D PCR fragment. The solid arrow indicates hybridization bands representing non-transposed Tnt1 (i.e., T-DNA associated). M: molecular weight markers. Lanes 1-16: Tnt1 mutants BS2-5, BS2-6, BS3-5, BS5-4, BS5-6, BS5-10, BS5-12, BS5-13, BS6-19, BS6-20, BS7-5, BS7-7, BS7-8, BS7-10, BS8-5 and BS8-7, respectively. Maverick: the parent line.
C, Southern blot analysis of Tnt1 transgenic lines to identify bar-hybridizing bands. The blot used in Figure 1(B) was stripped and re-hybridized using barA-B PCR fragment as probe.

Figure 2. FISH-based characterization of Tnt1 mutant lines BS5-6 and BS6-19. A and C, chromosomes of line BS5-6 or the parent line Maverick were hybridized with Texas Red labeled pSH-Tnt1 plasmid DNA, respectively; E and G, chromosomes of line BS5-6 or Maverick were hybridized with Texas Red labeled pBS-Tnt1 plasmid DNA, respectively; I, chromosomes of line BS6-19 were hybridized with pBS-Tnt1 probe; B, D, F, H and J: Gray-scale images of the chromosomes in A, C, E, G and I, respectively, K, Fiber-FISH shows a ca. 34 kb Tnt1 DNA fiber in line BS6-19.

Figure 3. Location of Tnt1 insertion sites in the soybean genome. Tnt1 flanking sequences were identified in eighteen Tnt1 lines by TAIL-PCR. Empty arrow heads, Tnt1 inserted in coding regions; solid arrow heads, Tnt1 inserted in intergenic regions; Dark circles, centromeres.
Figure 4. Analysis of \textit{Tnt1} expression in young leaves of \textit{Tnt1} transgenic plants by RT-PCR. As control, expression of the constitutively expressed \textit{Glyma12g05510} gene (Libault et al., 2010) was also determined. Lane 1, BS5-12-8R; lane 2, BS5-12-12C; lane 3, BS6-19 and lane 4, Maverick (parent line).

Figure 5. Southern blot analysis of the \textit{Tnt1} line BS5-12-8R and its T\textsubscript{1} progenies obtained by self-pollination. Fifteen µg of chromosomal DNA from different plants were digested with \textit{NdeI} and hybridized with \textit{Tnt1} probe. T0: BS5-12-8R; T1: progenies of BS5-12-8R. M: molecular weight markers.

Figure 6. Remobilization of \textit{Tnt1} transposition by tissue culture using cotyledon as explants with wound or wounding plus sucrose treatments. Southern blot analysis were performed by using \textit{NdeI} digested chromosomal DNAs of regenerated plants from \textit{Tnt1} lines BS5-14 and BS5-12 with the \textit{Tnt1} probe. The hybridization bands which presents in parent lines were marked with an asterisk (*) and the unmarked bands were potential novel insertions in regenerated plants. P1 and P2, \textit{Tnt1} primary transgenic lines BS5-14 and BS5-12, respectively. RA1: plants regenerated from line BS5-14 and RA2: plants regenerated from line BS5-12. W: plants regenerated from wound treated cotyledons and S: plants regenerated from wound plus 1M sucrose treated explants. M: molecular weight standards.

Figure 7. Autoradiograph of \textit{Tnt1} transposon display analysis of somatic embryos produced from three \textit{Tnt1} containing lines. Potential novel insertions (not present in the parent plant) are marked with an asterisk (*). White ovals indicate the bands that were excised from the gel for sequence analysis. Untransformed soybean DNA was used as the negative control. f = failed lanes.

Supplemental figure S1. Southern blot analysis of the T\textsubscript{1} progenies of \textit{Tnt1} lines BS6-19, BS5-6, BS5-12, BS12-7 and BS5-12-12C, as well as the T\textsubscript{2} progenies of line BS6-19 obtained by self-pollination. Fifteen µg of chromosomal DNA from different plants were digested with \textit{NdeI} and hybridized with the \textit{Tnt1} probe. T0: \textit{Tnt1} primary transgenic lines; T1 and T2: T1 and T2 progenies, respectively. M: molecular weight markers.
Table I. *Tnt1* hit genes identified by TAIL-PCR.

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PCR analysis results of the segregation of *Tnt1* insertions in the progeny of line BS5-12-8R.

Insertion #1, #2, #3 and #4 inserted into *Glyma20g34300*, *Glyma17g35300*, *Glyma01g01300* and
Glyma19g07410, respectively. +/+ correspond to wild-type homozygous plants, +/− correspond to heterozygous plants and −/− corresponds to homozygous plants for a given Tnt1 insertion.
Table III. *Tnt1* insertions identified in somatic embryo’s (asterisks indicates insertions that could be verified by PCR)

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<th><em>Tnt1</em> location</th>
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<tr>
<td>GM10:48222768</td>
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<td>GM08:16425209</td>
<td>Potential promoter region*</td>
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<td>GM03:40433849</td>
<td>Exon*</td>
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<td>GM09:895535</td>
<td>~2.5kb downstream of a coding region* (present in BS5-13)</td>
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Figure 1. A, Diagram of plasmid pSH-Tnt1 containing the Tnt1 element in the binary vector pZY101. LB and RB, left and right borders, respectively; bar, gene conferring glufosinate resistance; 35S, promoter 35S; LTR, Long Terminal Repeat. Ndel restriction sites and PCR fragments for bar (barA-B) and Tnt1 amplification (TntA-B, TntC-D and TntE-F) are shown. PCR fragment barA-B or TntC-D was used in Southern blot analysis to probe for bar or Tnt1, respectively.

B, Southern blot analysis of Tnt1 primary transgenic lines to identify Tnt1-hybridizing bands. Chromosomal DNA (15 - 20 µg) from each transgenic line was digested with Ndel and probed with TntC-D PCR fragment. The solid arrow indicates hybridization bands representing non-transposed Tnt1 (i.e., T-DNA associated). M: molecular weight markers. Lanes 1-16: Tnt1 mutants BS2-5, BS2-6, BS3-5, BS5-4, BS5-6, BS5-10, BS5-12, BS5-13, BS6-19, BS6-20, BS7-5, BS7-7, BS7-8, BS7-10, BS8-5 and BS8-7, respectively. Maverick: the parent line.

C, Southern blot analysis of Tnt1 transgenic lines to identify bar-hybridizing bands. The blot used in Figure 1(B) was stripped and re-hybridized using barA-B PCR fragment as probe.
Figure 2. FISH-based characterization of *Tnt1* mutant lines BS5-6 and BS6-19. A and C, chromosomes of line BS5-6 or the parent line Maverick were hybridized with Texas Red labeled *pSH-Tnt1* plasmid DNA, respectively; E and G, chromosomes of line BS5-6 or Maverick were hybridized with Texas Red labeled *pBS-Tnt1* plasmid DNA, respectively; I, chromosomes of line BS6-19 were hybridized with *pBS-Tnt1* probe; B, D, F, H and J, Gray-scale images of the chromosomes in A, C, E, G and I, respectively; K, Fiber-FISH shows a ca. 34 kb *Tnt1* DNA fiber in line BS6-19.
Figure 3. Location of Tnt1 insertion sites in the soybean genome. Tnt1 flanking sequences were identified in eighteen Tnt1 lines by TAIL-PCR. Empty arrow heads, Tnt1 inserted in coding regions; solid arrow heads, Tnt1 inserted in intergenic regions; Dark circles, centromeres.
Figure 4. Analysis of Tnt1 expression in young leaves of Tnt1 transgenic plants by RT-PCR. As control, expression of the constitutively expressed Glyma12g05510 gene (Libault et al., 2010) was also determined. Lane 1, BS5-12-8R; lane 2, BS5-12-12C; lane 3, BS6-19 and lane 4, Maverick (parent line).
Figure 5. Southern blot analysis of the Tnt1 line BS5-12-8R and its T1 progenies obtained by self-pollination. Fifteen μg of chromosomal DNA from different plants were digested with NdeI and hybridized with the Tnt1 probe. T0: BS5-12-8R; T1: progenies of BS5-12-8R. M: molecular weight markers.
Figure 6. Remobilization of *Tnt1* transposition by tissue culture using cotyledon as explants with wound or wounding plus sucrose treatments. Southern blot analysis were performed by using *NdeI* digested chromosomal DNAs of regenerated plants from *Tnt1* lines BS5-14 and BS5-12 with the *Tnt1* probe. The hybridization bands which presents in parent lines were marked with an asterisk (*) and the unmarked bands were potential novel insertions in regenerated plants. P1 and P2, *Tnt1* primary transgenic lines BS5-14 and BS5-12, respectively. RA1: plants regenerated from line BS5-14 and RA2: plants regenerated from line BS5-12. W: plants regenerated from wound treated cotyledons and S: plants regenerated from wound plus 1M sucrose treated explants. M: molecular weight standards.
Figure 7. Autoradiograph of Tnt1 transposon display analysis of somatic embryos produced from three Tnt1 containing lines. Potential novel insertions (not present in the parent plant) are marked with an asterisk (*). White ovals indicate the bands that were excised from the gel for sequence analysis. Untransformed soybean DNA was used as the negative control. f = failed lanes.