Tnt1 Retrotransposon Mutagenesis: A Tool for Soybean Functional Genomics[1][W][OA]

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Insertional mutagenesis is a powerful tool for determining gene function in both model and crop plant species. *Tnt1*, the transposable element of tobacco (*Nicotiana tabacum*) cell type 1, 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 (*Glycine max*). Experiments showed that the *Tnt1* element was stably transformed into soybean plants by *Agrobacterium 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 four to 19 insertions, with an average of approximately eight 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, 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.

Soybean (*Glycine max*) 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 the 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; Alonso 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 (*Arabidopsis thaliana*; Alonso et al., 2003). However, although the 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 1 year, from seed to seed; Parrott 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 (*Zea mays*) transposon, *Ac/Ds*,

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to identify a soybean male-sterile line. *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 (Jones et al., 1990; Ito et al., 1999; Parinov and Sundaresan, 2000). Perhaps a more promising alternative is *mPing*, a class II element originally isolated in rice (*Oryza sativa*; 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 analyses.

Compared with 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 a "copy-and-paste" manner via an RNA intermediate (Kumar and Bennetzen, 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-sized mutagenized population composed of 2,450 plant lines (Fukai et al., 2012). The *Tto1* element, from tobacco (*Nicotiana tabacum*), has also been used for mutagenesis in Arabidopsis (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, 2008; Iantcheva et al., 2009), Arabidopsis (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, 2008; Iantcheva et al., 2009). The published *M. truncatula Tnt1* population contains nearly 12,000 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., 2011). However, reactivation of retrotransposon transposition does not occur in every plant species examined. For example, Ishizaki and Kato (2005) failed to detected tissue culture reactivation of the *Tto1* retrotransposon in transgenic potato (*Solanum tuberosum*) plants.

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

RESULTS AND DISCUSSION

Generation of *Tnt1* Retrotransposon-Containing Soybean Lines

Although *Tnt1* transposes very efficiently in the legume model plant *M. truncatula* and several other plants, including tobacco, Arabidopsis, and lettuce (Courtial et al., 2001; d'Erfurth et al., 2003; Tadege et al., 2005, 2008; Mazier et al., 2007; 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 *Tto1* retrotransposon from tobacco transposes in tobacco, Arabidopsis, 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 *M. truncatula*, the reactivation protocol of *Tnt1* optimized to cv R108 is not applicable for cv Jemalong (Iantcheva et al., 2009). Therefore, it is necessary to determine if *Tnt1* transposes in soybean and also to optimize the methodology to induce its transposition.

To investigate whether the *Tnt1* element can transpose in soybean during tissue culture, *Agrobacterium tumefaciens*-mediated transformation was performed using a modified soybean cotyledonary node transformation protocol (Zeng et al., 2004). The plasmid *pSH-Tnt1* containing the *Tnt1* element was constructed by inserting the *Tnt1* DNA into the binary vector pZY101, which carries a selectable *bar* gene marker for glufosinate resistance (Fig. 1A). Transformations were performed in cv Maverick, a genotype that is susceptible to glufosinate. cv Maverick is an elite soybean genotype that is resistant to stress conditions and shows a consistently higher transformation frequency when compared with other genotypes (Z.Y. 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 Figure 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

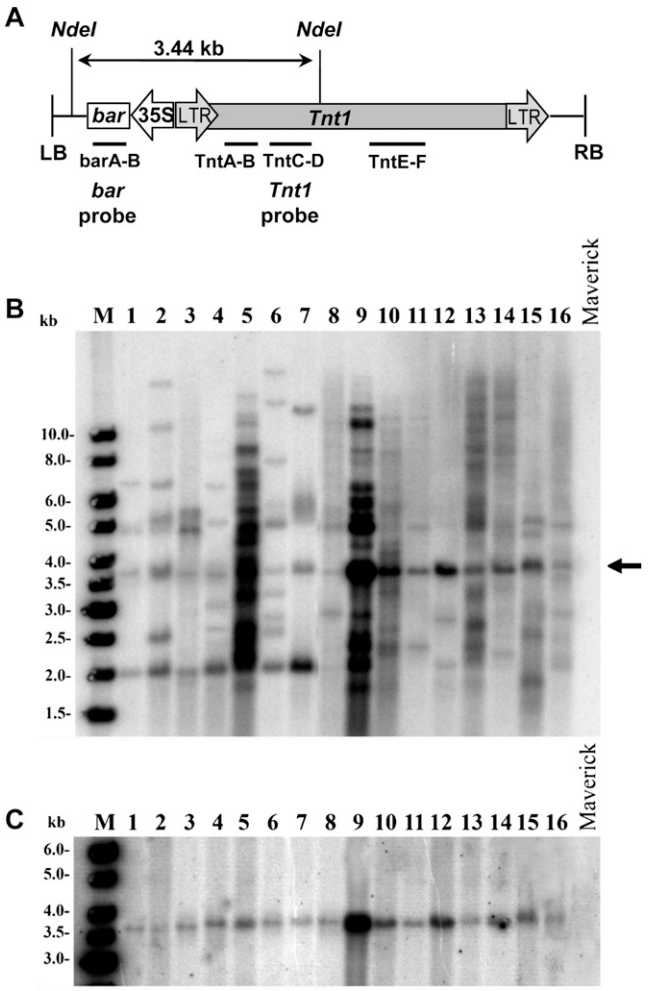

Figure 1. A, Diagram of plasmid *pSH-Tnt1* containing the *Tnt1* element in the binary vector pZY101. LB and RB, Left and right borders, respectively; *bar*, gene conferring glufosinate resistance; 35S, promoter 35S; LTR, long terminal repeat. *Nde*I restriction sites and PCR fragments for *bar* (barA-B) and *Tnt1* (TntA-B, TntC-D and TntE-F) amplification 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 *Nde*I and probed with the TntC-D PCR fragment. The arrow indicates hybridization bands representing nontransposed *Tnt1* (i.e. T-DNA associated). M, Molecular weight markers. Lanes 1 to 16 show *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. cv Maverick is the parent line. C, Southern-blot analysis of *Tnt1* transgenic lines to identify *bar*-hybridizing bands. The blot used in B was stripped and rehybridized using the barA-B PCR fragment as a probe.

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 *Nde*I. Southern-blot analysis was performed using a 755-bp *Tnt1* internal fragment as the probe, which corresponds to bases 1,067 to 1,822 of the retrotransposon. The same blot was then stripped and reprobed with a 480-bp *bar* internal fragment. The *Nde*I sites and probe locations are shown in Figure 1A. *Nde*I cuts the *Tnt1* DNA once at position 1,983 and 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 16 *Tnt1*-containing plants and the parent line cv Maverick using the *Tnt1* probe is shown in Figure 1B. As expected, a 3.44-kb band (indicated by the arrow) was present, representing nontransposed *Tnt1* (i.e. T-DNA associated) in all 27 lines. This band also hybridized with the *bar* probe (Fig. 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 (Fig. 1B). Transposed *Tnt1* copy numbers ranged from four to 19, with an average of approximately eight copies per line. Thus, the results of these experiments confirm that the *Tnt1* element was stably transformed in 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 four to more than 30 insertions), Arabidopsis (ranging from zero 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 the 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 Arabidopsis, 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 cv Maverick served as controls. As shown in Figure 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).

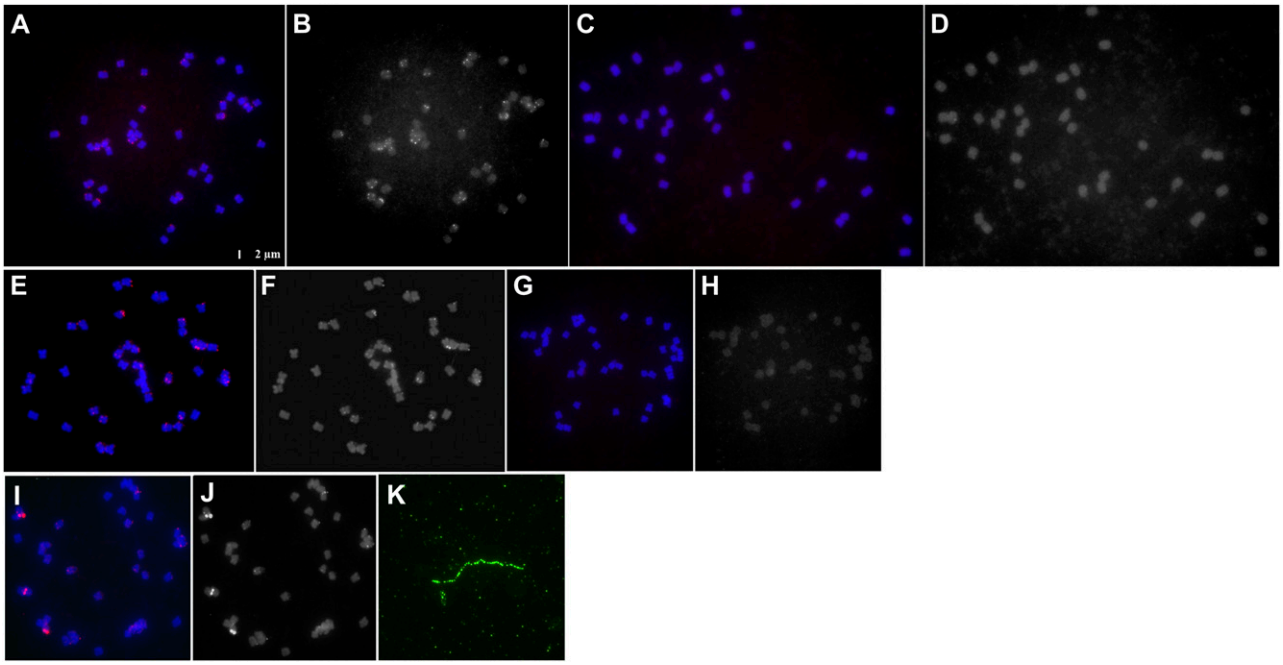

Figure 2. FISH-based characterization of *Tnt1* mutant lines BS5-6 and BS6-19. A and C, Chromosomes of line BS5-6 or the parent line cv Maverick were hybridized with Texas Red-labeled *pSH-Tnt1* plasmid DNA, respectively. E and G, Chromosomes of line BS5-6 or cv Maverick were hybridized with Texas Red-labeled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

Moreover, multiple hybridization signals were observed in several chromosomes (Fig. 2, A and B). Based on the Southern-blot analysis, line BS5-6 possesses approximately 19 transposed copies of *Tnt1* (Fig. 1B). The FISH data showing multiple *Tnt1*-hybridizing regions are 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 pBluescript SK+ and used the resulting plasmid construct (*pBS-Tnt1*) as a probe for FISH experiments to examine line BS5-6. We found that hybridization signals observed with the *pBS-Tnt1* probe (Fig. 2, E and F) were comparable to those obtained with *pSH-Tnt1* (Fig. 2, A and B). No hybridization signals were detected when the empty pBluescript SK+ vector was used as a probe (data not shown). Furthermore, a FISH experiment was also performed on another *Tnt1* line, BS6-19, using *pBS-Tnt1* as a probe. The results (Fig. 2, I and J) 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. 2, G and H). 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 pBluescript SK+ 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 approximately 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 thermal asymmetric interlaced (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 et al., 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

Table I. *Tnt1 hit genes identified by TAIL-PCR*

Gene	Gene Location	*Tnt1* Location
Glyma01g01090	Gm01:746970.0.750314	749,413
Glyma01g01300	Gm01:937365.0.939890	940,048
Glyma01g01560	Gm01:1160245.0.1164111	1,162,180
Glyma01g38870	Gm01:50865317.0.50867505	50,866,204
Glyma01g43460	Gm01:54468406.0.54469790	54,468,673
Glyma01g44730	Gm01:55288549.0.55291102	55,288,466
Glyma02g04770	Gm02:3902268.0.3906661	3,905,465
Glyma02g06890	Gm02:5533736.0.5541825	5,540,590
Glyma02g37830	Gm02:43141981.0.43145206	43,142,581
Glyma03g38120	Gm03:44522550.0.44526793	44,523,593
Glyma04g02350	Gm04:1631557.0.1634732	1,633,312
Glyma04g05960	Gm04:4543997.0.4549817	4,549,348
Glyma04g06710	Gm04:5178279.0.5181655	5,180,888
Glyma05g28120	Gm05:33994151.0.33996925	33,994,629
Glyma05g36000	Gm05:39924225.0.39927013	39,925,842
Glyma06g45450	Gm06:48160130.0.48165969	48,165,167
Glyma07g00500	Gm07:224177.0.230842	225,571
Glyma07g06950	Gm07:5607639.0.5610266	5,608,653
Glyma07g10570	Gm07:8857596.0.8860631	8,860,452
Glyma07g15940	Gm07:15658959.0.15662391	15,658,960
Glyma07g38720	Gm07:43421544.0.43422862	43,422,239
Glyma08g10350	Gm08:7484480.0.7485874	7,484,671
Glyma08g43240	Gm08:43081527.0.43082006	43,081,605
Glyma08g45860	Gm08:45121245.0.45131117	45,121,559
Glyma09g29810	Gm09:36663252.0.36666676	36,664,035
Glyma09g35670	Gm09:41618503.0.41620799	41619901
Glyma10g00260	Gm10:75667.0.78986	78,077
Glyma10g40240	Gm10:47715528.0.47720018	47,719,673
Glyma11g02780	Gm11:1804302.0.1806201	1,804,729
Glyma11g04750	Gm11:3266510.0.3268970	3,268,423
Glyma11g36880	Gm11:38179557.0.38181293	38,179,954
Glyma11g37500	Gm11:38592441.0.38597663	38,593,222
Glyma12g04480	Gm12:2982711.0.2987078	2,983,780
Glyma12g28980	Gm12:32372131.0.32373204	32,372,213
Glyma12g32280	Gm12:35785488.0.35787769	35,787,066
Glyma12g35440	Gm12:38564240.0.38567572	38,566,080
Glyma13g20420	Gm13:23889695.0.23897572	23,890,533
Glyma13g32090	Gm13:34359721.0.34361227	34,361,073
Glyma13g35520	Gm13:36943648.0.36950770	36,944,632
Glyma13g39540	Gm13:40114057.0.40114640	40,114,263
Glyma14g04190	Gm14:2812976.0.2815950	2,812,976
Glyma14g04200	Gm14:2818106.0.2821324	2,820,386
Glyma14g17330	Gm14:19105307.0.19109002	19,108,051
Glyma14g35580	Gm14:44570215.0.44571936	44,570,215
Glyma14g40090	Gm14:49077959.0.49084565	49,081,971
Glyma14g40250	Gm14:49220022.0.49221998	49,221,894
Glyma15g08070	Gm15:5675190.0.5675401	5,675,305
Glyma15g08350	Gm15:5892318.0.5898492	5,892,294
Glyma16g25770	Gm16:29864908.0.29872224	29,864,835
Glyma17g03940	Gm17:2586560.0.2590026	2,586,583
Glyma17g35300	Gm17:39275298.0.39276346	39,276,103
Glyma18g00440	Gm18:148373.0.150712	149,539
Glyma18g43100	Gm18:52435074.0.52438457	52,435,987
Glyma18g53850	Gm18:62121982.0.62123472	62,122,569
Glyma19g02320	Gm19:2042249.0.2046314	2,043,219
Glyma19g07410	Gm19:8755326.0.8759630	8,758,960
Glyma19g29690	Gm19:37423341.0.37424387	37,424,167
Glyma19g37430	Gm19:44580492.0.44583090	44,580,764
Glyma20g25660	Gm20:35302630.0.35306802	35,302,638
Glyma20g28620	Gm20:37540008.0.37541602	37,540,335
Glyma20g30490	Gm20:39122592.0.39129208	39,127,429
Glyma20g34300	Gm20:42690498.0.42692467	42,690,537

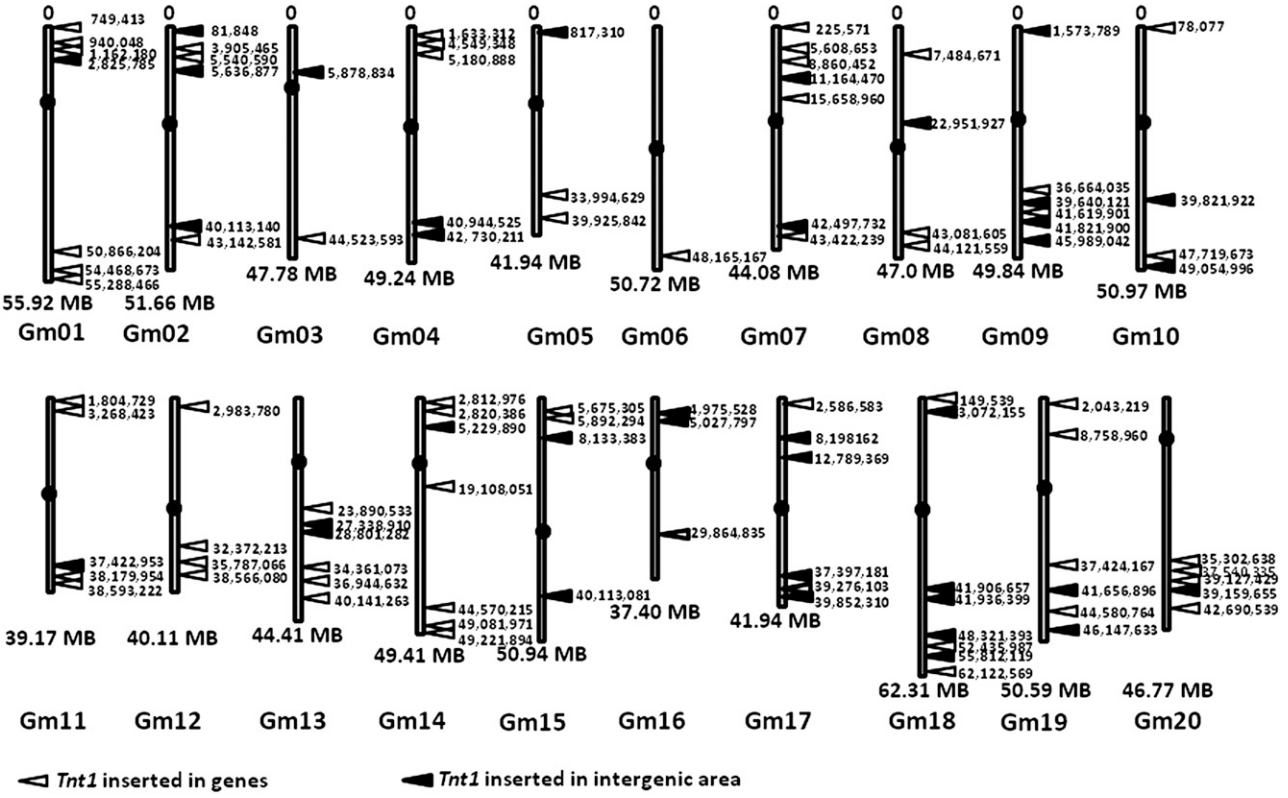

Figure 3. Locations of *Tnt1* insertion sites in the soybean genome. *Tnt1* flanking sequences were identified in 18 *Tnt1* lines by TAIL-PCR. White arrowheads, *Tnt1* inserted in coding regions; black arrowheads, *Tnt1* inserted in intergenic regions; black circles, centromeres.

been documented to transpose preferentially into gene-rich regions. *Tnt1* does transpose preferentially into gene-rich regions in *M. truncatula*, Arabidopsis, 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 per 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 reverse transcription (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 cv 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 progeny of *Tnt1* lines BS5-12-8R, BS6-19, BS5-6, BS5-12, BS12-7, and BS5-12-12C, as well as T2 progeny 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

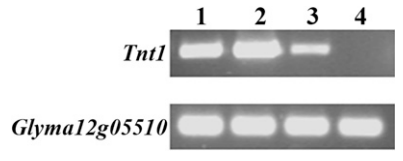

Figure 4. Analysis of *Tnt1* expression in young leaves of *Tnt1* transgenic plants by RT-PCR. As a control, expression of the constitutively expressed *Glyma12g05510* gene (Libault et al., 2010) was also determined. Lane 1, BS5-12-8R; lane 2, BS5-12-12C; lane 3, BS6-19; lane 4, cv Maverick (parent line).

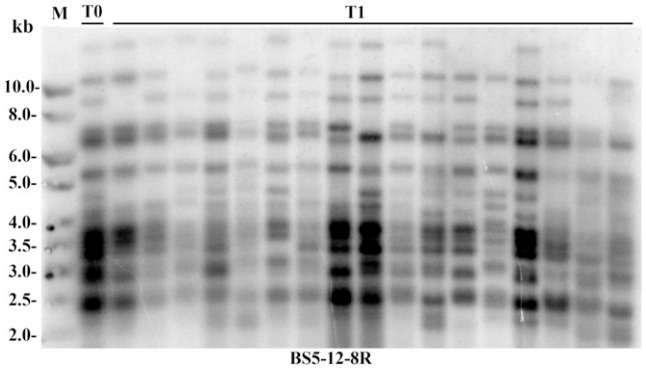

Figure 5. Southern-blot analysis of the *Tnt1* line BS5-12-8R and its T1 progeny obtained by self-pollination. Fifteen micrograms of chromosomal DNA from different plants was digested with *Nde*I and hybridized with *Tnt1* probe. M, Molecular weight markers; T0, BS5-12-8R; T1, progeny of BS5-12-8R.

amplification of selected flanking regions and scored as present, absent, or heterozygous in each of the progeny lines (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 that 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 Arabidopsis, *M. truncatula*, and lettuce (Courtial et al., 2001; d'Erfurth et al., 2003; Mazier et al., 2007). In Arabidopsis and *M. truncatula*, while RT 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., 2003). Although expression of the *Tnt1* transposase could be detected in the vegetative tissue of transgenic plants, there was no evidence that the *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.

Tnt1 Transposition Can Be Reactivated in Soybean by in Vitro Culture

Given the time needed to produce independent soybean transgenic lines, practical use of the *Tnt1* transposon in soybean would require that a few initial

Table II. *Segregation of Tnt1 insertions in line BS5-12-8R*

PCR analysis results are shown for the segregation of *Tnt1* insertions in the progeny of line BS5-12-8R. Insertions 1, 2, 3, and 4 were inserted into *Glyma20g34300*, *Glyma17g35300*, *Glyma01g01300*, and *Glyma19g07410*, respectively. +/+, Wild-type homozygous plants; +/−, heterozygous plants; −/−, homozygous plants for a given *Tnt1* insertion.

BS5-12-8R	Insertion No. 1	Insertion No. 2	Insertion No. 3	Insertion No. 4
T0	+/−	+/−	+/−	+/−
Plant 1	−/−	+/−	+/−	+/−
Plant 2	+/−	+/−	+/+	+/−
Plant 3	+/−	+/+	+/−	+/+
Plant 4	+/−	+/+	+/−	+/−
Plant 5	+/−	+/−	+/−	−/−
Plant 6	+/−	+/−	+/+	+/−
Plant 7	+/−	−/−	+/−	+/−
Plant 8	−/−	+/+	+/−	+/−
Plant 9	−/−	+/−	+/−	+/−
Plant 10	+/+	+/+	+/−	+/−
Plant 11	+/−	+/−	+/−	+/−
Plant 12	+/+	+/−	+/−	−/−
Plant 13	+/−	+/−	+/+	+/−
Plant 14	+/−	+/−	+/−	+/+
Plant 15	+/−	+/+	+/−	+/−
Plant 16	+/−	+/−	+/−	+/−
Plant 17	−/−	−/−	+/−	+/−
Plant 18	+/+	+/+	+/−	+/−
cv Maverick	+/+	+/+	+/+	+/+
Ratio				
+/+	3	6	3	2
+/−	11	10	15	14
−/−	4	2	0	2

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 *Tnt1* transposition. The cotyledons of the *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 h of shaking in a 1 M Suc solution. The latter treatment was tested since it was reported to significantly increase the frequency of *Tnt1* transposition in *M. truncatula* 'Jemalong' (Iantcheva et al., 2009). Over 40 plants were regenerated from wounded cotyledons of seven *Tnt1* T0 lines with or without Suc 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 *Tnt1* transposition events (up to 20 copies). The original T0 parental line contained only four *Tnt1* insertion sites. The regenerated plants from other lines tested by this approach produced zero to five new *Tnt1* insertions in the genome. However, the Suc treatment did not enhance the frequency of *Tnt1* transposition in these experiments.

In order to verify that the new bands observed were novel *Tnt1* insertion sites, TAIL-PCR was performed to recover the flanking soybean sequence from four plants generated from reactivation of event BS5-12. Twenty *Tnt1* flanking sequences were obtained from those plants. Ten specific primers were designed from the *Tnt1* flanking sequences of reactivated line BS5-12-8R, and five primers were designed from the *Tnt1* flanking sequences of reactivated line BS5-12-12C. Those primers were paired with a *Tnt1*-specific primer, LTR7, and used for PCR. PCR results revealed that all of the BS5-12-8R primers (paired with the 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 template. 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 of 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.

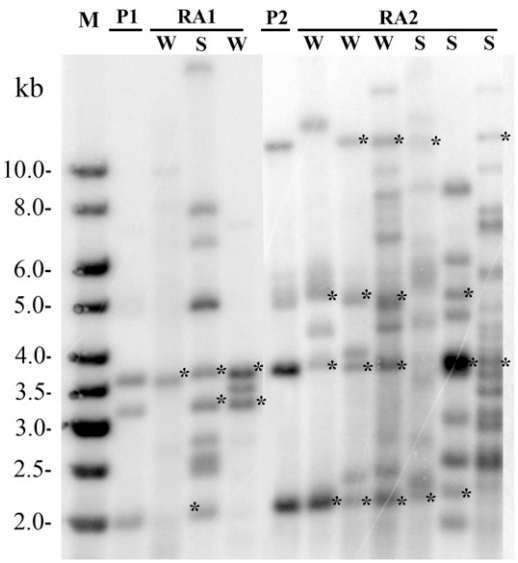

Figure 6. Remobilization of *Tnt1* transposition by tissue culture using cotyledons as explants with wound or wounding plus Suc treatment. Southern-blot analysis was performed by using *Nde*I-digested chromosomal DNA of regenerated plants from *Tnt1* lines BS5-14 and BS5-12 with the *Tnt1* probe. The hybridization bands that presented in parent lines are marked with asterisks, and the unmarked bands were potential novel insertions in regenerated plants. M, molecular weight standards; P1 and P2, *Tnt1* primary transgenic lines BS5-14 and BS5-12, respectively; RA1, plants regenerated from line BS5-14; RA2, plants regenerated from line BS5-12; W, plants regenerated from wound-treated cotyledons; S, plants regenerated from wound plus 1 M Suc-treated explants.

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 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 (one to five) of novel bands that were not present in the parent (i.e. BS8-5 and BS5-6; Fig. 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 (Fig. 7). Some of these novel insertions were shared between

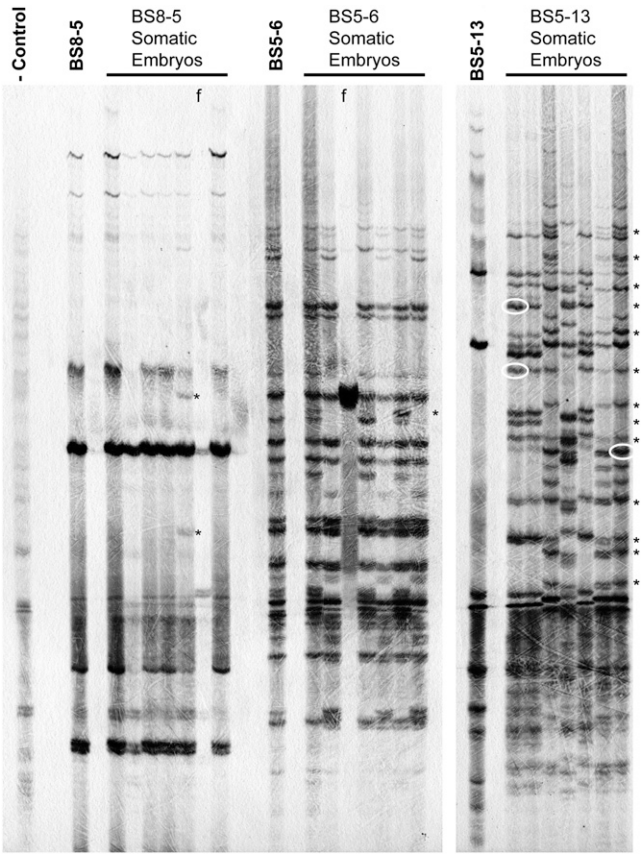

Figure 7. Autoradiograph of *Tnt1* transposon display analysis of somatic embryos produced from three *Tnt1*-containing lines. Potential novel insertions (not present in the parent plant) are marked with asterisks. White ovals indicate the bands that were excised from the gel for sequence analysis. Untransformed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

embryos, indicating that they occurred early in the 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 one or two new *Tnt1* copies (data not shown). These results suggest that the efficiency of reactivation approaches are related to the genotypes of the original *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 *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 *Tnt1* insertion sites, we were able to verify the presence of three of these *Tnt1* insertions (data not shown). Of these, one was found to be present in the original BS5-13 plant, but the remaining two *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 *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.

Tnt1: An Insertion Mutagen in Soybean?

Practical use of *Tnt1* for mutagenesis in soybean requires the 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, a higher 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 *Ac/Ds* mutagensis 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 centimorgan) of the original insertion site (Jones et al., 1990; Ito et al., 1999; 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 plant species,

Table III. *Tnt1 insertions identified in somatic embryos*
Asterisks indicate insertions that could be verified by PCR.

Tnt1 Location	Type of Insertion
GM10:48222768	Intergenic
GM08:16425209	Potential promoter region*
GM03:40433849	Exon*
GM09:895535	Approximately 2.5-kb downstream of a coding region* (present in BS5-13)

such as *M. truncatula*, Arabidopsis, 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 four 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 backcrossing to the parental line. If one wants to work with a line with a clean single *Tnt1* insertion, a couple of rounds of backcrossing 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 (Mathieu et al., 2009). Even in the model plant Arabidopsis, 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 Mendelian 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 the *Tnt1* element, were shown to be highly efficient for transposition upon passage through tissue culture; therefore, they 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

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

Bacterial Strains and T-DNA Vectors

The *Escherichia coli* strain DH5α (Sambrook et al., 1989) was used for cloning and the propagation of the different vectors. *Agrobacterium tumefaciens* strain AGL1 was used in all plant transformation experiments. Plasmids were introduced into AGL1 by direct DNA transfer (An et al., 1988). An *Eco*RI fragment containing the entire *Tnt1* element from plasmid pHLV4909 (a gift from Helene Lucas) 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 *A. tumefaciens* strain was grown in yeast extract peptone medium containing rifampicin (30 mg L^{-1}) and spectinomycin (100 mg L^{-1}) and kept at 250-rpm shaking overnight at 28°C. Cotyledonary explants derived from 5-d-old seedlings of genotype cv 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 3.3 and 1.0 mM, respectively (Olhoft et al., 2003); also, 0, 10, and 5 mg L^{-1} glufosinate was added to the first and second shoot induction media as well as the shoot elongation medium, respectively. 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 at the University of Missouri Plant Transformation Core facility.

Examining the Occurrence of the *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*-specific primers (Fig. 1) were used: TntA, 5′-TGGTATCAGAGCACAGGTTCTGCT-3′; TntB, 5-AAATGTGACAAAAAATTCGTACCT-3′; TntC, 5′-AACGGACTAATCACACAGCTTGCC-3′; TntD, 5′-ATAACTCTCGTATCCATCTCGGTC-3′; TntE, 5′-TTGATTTTGACGAAATTTTCTCCC-3′; TntF, 5′-CCTGCCATATCAGCATCTGTATAG; barA, 5-TACCATGAGCCCAGAACGCCC-3′; and barB, GGCTGAAGTCCAGCTGCCAGAAAG-3′.

Molecular Analysis

Standard procedures were used in the isolation of plasmid DNA, gel electrophoresis, PCR, DNA ligation, transformation, and electroporation (Sambrook et al., 1989). Restriction and modification enzymes were obtained from Promega. Soybean plant chromosomal DNA was extracted from young leaves according to the procedures described by Dellaporta et al. (1983). Fifteen micrograms of RNase A-treated genomic DNA for each line was digested with *Nde*I and separated on a 0.8% agarose Tris-acetate EDTA gel running at 30 V 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 1,067 to 1,822 of the retrotransposon was used as a probe (Fig. 1A). The Prime-a-Gene DNA labeling system (Promega) was used for labeling DNA probes. The [α-^{32}P]dATP (3,000 Ci mol^{-1})-labeled probes were used for hybridization. After hybridizing with the *Tnt1* probe, the blots were stripped according to the instructions of the manufacturer and reprobed with a 480-bp *bar* internal fragment. After washing, the membrane was exposed to a phosphor imager screen and then visualized using the FujiFilm Fluorescent Imager Analyzer FLA 3000.

RNA Isolation and RT-PCR

Total RNA from young leaves was isolated using Trizol Reagent according to the manufacturer's instructions (Invitrogen). The isolated RNA was further purified and treated with DNase TURBO DNA-free according to the manufacturer's instructions (Ambion) The first-strand complementary DNA was synthesized using avian

myeloblastosis virus reverse transcriptase (Promega) and used as input in PCR using Taq polymerase (Promega) according to the manufacturer's instructions with the following PCR conditions: 94°C for 5 min, then 35 cycles of 94°C for 30 s, 57°C for 1 min, and 72°C for 2 min, followed by 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 controls.

FISH and Fiber-FISH Analyses

Sample preparation, FISH and fiber-FISH experiments, and image processing were performed precisely as described by Gill et al. (2009). The plasmid DNAs of *pSH-Tnt1* or *pBS-Tnt1* (*Tnt1* DNA clone into pBluescript SK+) were labeled with Texas Red and used as probes for FISH experiments. The biotin-labeled *pBS-Tnt1* 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 gene-specific forward and reverse primers on genomic DNA: 5′-CGAACATTACACCACTAAGATGTC-3′ (Glyma20g34300F) and 5′-TGACATCTCAAATTACTTTCATTG-3′ (Glyma20g34300R); 5′-TAAGGTCGTCAGCTAATGCCGATC-3′ (Glyma17g35300F) and 5′-TCAATTCTTCCCGATCGTTTACAC-3′ (Glyma17g35300R); 5′-ACCAAGCTTTGTGACTGCATCCAC-3′ (Glyma01g01300F) and 5′-TATATCTTCTTGTGGACTACAAGG-3′ (Glyma01g01300R); 5′-GCCAAGCTTGATTCCAGGGAGATA-3′ (Glyma19g07410F) and 5′-TGTTTCTGTATGGTCAGACATAAC-3′ (Glyma19g07410R); in combination with the *Tnt1* right border primer 5′-TATTATTCCGCTTTATTACCGTGA-3′ (LTR7). PCR was conducted using ExTaq Polymerase (Takara) under the following conditions: 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, followed by 72°C for 5 min.

Tnt1 Flanking Sequence Isolation and Sequencing

The *Tnt1* flanking sequences were recovered by TAIL-PCR as described by Ratet et al. (2006). The arbitrary primers used were AD1 [5′-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3′], AD2 [5′-NGTCGA(G/C)(A/T_GANA(A/T)GAA-3′], and AD3 [5′-(A/T)GTGNAG(A/T)ANCANAGA-3′]. Three *Tnt1*-specific primers, Tntail3 (5′-TCTGGATGAATGAGACTGGAGG-3′, corresponding to bases 4,696–4,717 of *Tnt1*), LTR4 (5′-TACCGTATCTCGGTGCTACA-3′, corresponding to bases 534–553/5,258–5,277 of *Tnt1*), and LTR7 (5′-TATTATTCCGCTTTATTACCGTGA-3′, corresponding to bases 555–578/5,279–5,302 of *Tnt1*) were used for primary, secondary, and tertiary PCR, respectively. The PCR products were cloned into pGem-T Easy vector (Promega) and sequenced. DNA sequencing was performed at the DNA Core Facility of the University of Missouri.

Homology Searching

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

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 *A. tumefaciens* inoculation was involved. The major modifications included the replacement of Murashige and Skoog-based medium (Murashige and Skoog, 1962) with B5-based medium (Gamborg et al., 1968) for all culture stages, the use of 0.2 mg L^{-1} indoleacetic acid and 2 mg L^{-1} 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 d on B5-based germination medium. The cotyledonary node explants were prepared by wounding with a razor blade with or without Suc solution treatment. Suc treatment was performed by shaking (120 rpm) the wounded explants in 1 M Suc solution for 12 h. Treated explants were then cultured on B5-based shoot induction medium for the first 2 weeks and followed by an additional 2 weeks of subculture on the same fresh medium amended with 5 mg L^{-1} glufosinate. Explants were transferred biweekly onto fresh B5-based shoot elongation medium amended with 10 mg L^{-1} 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) in a Jiffy pot inside a Magenta culture vessel for acclimatization. Hardened plantlets were transferred to 3-gallon pots containing Promix soil mixed with Peters 20-20-20 (Hummert International) in a 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 were performed on the immature embryos collected from these plants. The production of somatic embryos was performed as described previously (Trick et al., 1997), excluding bombardment and antibiotic selection. DNA purification of the parent plant and differentiated embryos was performed using the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). The transposon display protocol was essentially the same as described by 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.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Southern-blot analysis of the T1 progeny of *Tnt1* lines BS6-19, BS5-6, BS5-12, BS12-7, and BS5-12-12C, as well as the T2 progeny of line BS6-19, obtained by self-pollination.

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