



Commentary

Irreproducibility of the Soybean Pollen-Tube Pathway Transformation Procedure

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Abstract. The interest in developing tissue culture-independent genetic transformation methods for plants has been growing. The pollen-tube pathway transformation technique is one method; however, this method is controversial because it is difficult to duplicate and produces insufficient molecular evidence to confirm transformation. Our objective was to evaluate the robustness of the soybean pollen-tube pathway technique (*Glycine max* L. Merr.). Solutions of purified DNA constructs carrying a *bar* marker gene and a *gus* reporter gene or a gene of interest (*npk1*) were applied to severed styles of flowers 6-8 h after self-pollination. The experiment was repeated 3 summers in the field, in which 4 DNA constructs and 7 soybean genotypes were tested. A total of 4793 progeny seeds were harvested from 5590 individually treated soybean flowers. All seeds were germinated and screened for transformants with herbicide spray, histochemical GUS assay, and Southern blot analysis. Although 2% of progenies showed partial resistance to the herbicide, no positive plants were identified from GUS assay and Southern analysis. Our results indicate that soybean pollen-tube pathway transformation is not reproducible.

Key words: pollen-tube pathway, soybean, transformation

Abbreviations: RAPD, random amplified polymorphic DNA.

Introduction

Numerous gene transfer methods have been developed in the past 2 decades for a wide range of plant species (Christou, 1995; Birch, 1997). Most of these methods are tissue culture based, requiring regeneration of whole plants from transformed cells. The utility of the techniques greatly depends on the establishment of tissue culture procedures in the species (Birch, 1997).

Developing tissue culture-independent genetic transformation systems is of great interest because such systems would avoid constraints imposed by genotype specificity in transformation and regeneration and eliminate tissue culture-induced genetic variation. In addition, transgenic plants would be produced inexpensively and rapidly. The *Agrobacterium*-mediated floral dipping method developed for the

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model plant *Arabidopsis thaliana* has greatly impacted biological research of that species (Bent, 2000).

Pollen-tube pathway transformation—use of the pollen-tube pathway to deliver foreign DNA into embryo sacs—was first reported in cotton (Zhou et al., 1983). The technique has been used to introduce total exogenous genomic DNA or plasmid DNA into other crops, including cotton (Ni et al., 1998; Zhou et al., 1983, 1988), rice (Duan and Chen, 1985; Luo and Wu, 1989), soybean (Lei et al., 1994, 1995; Liu et al., 1992, 1997; Zhao et al., 1995), wheat (Yu et al., 1999; Zhen et al., 1998), and watermelon (Chen et al., 1998). Most initial reports used total genomic DNA for transformation. In these studies, treated plants were monitored and compared with untreated controls for variations in plant morphology, fertility, pest resistance, seed composition, and peroxidase isozyme expression patterns (Lei et al., 1994, 1995; Liu et al., 1992, 1997; Zhao et al., 1995). One study showed RAPD analysis of treated soybean plants and suggested that the observed variation of treated plants was correlated with the introduction and integration of the donor DNA from wild soybean via the pollen-tube pathway transformation (Lei et al., 1994). Results from various molecular analyses, including PCR, slot-blot, PCR-Southern, and Southern blot, have been reported for the introduction of plasmid DNA carrying marker genes using the pollen-tube pathway technique (Chen et al., 1998; Luo and Wu, 1989; Ni et al., 1998; Yu et al., 1999; Zhen et al., 1998). Although some information was encouraging, the overall quality of the data was weak. Most results did not demonstrate integration of the transgene into the plant genome by using proper restriction enzyme digestions and Southern blot hybridization, which is considered a standard criterion for confirming the transgenic nature (Potrykus, 1990). In addition, no substantial progeny analysis data were presented to support claims of high efficiency.

Because of the lack of efficient regeneration and transformation procedures, soybean remains a recalcitrant crop for genetic transformation (Widholm, 1999). Therefore, the successful use of the pollen-tube pathway transformation technique in soybean should have many advantages. A number of studies have reported that exogenous DNA can be introduced into soybean via pollen-tube pathway transformation (Hu and Wang, 1999; Lei et al., 1994, 1995; Liu et al., 1992, 1997; Zhao et al., 1995). In addition, herbicide-tolerant and aphid-resistant soybean lines were obtained by introducing plasmid DNA carrying the *bar* and *gna* (*Galanthus nivalis* agglutinin) genes via this technique (D.P. Liu, personal communication, 2001). Over the past 3 y, we duplicated the pollen-tube pathway transformation technique using published protocols (Liu et al., 1992). Our objective was to evaluate the robustness of the technique in US soybean cultivars.

Materials and Methods

Plant materials

Pollen-tube pathway transformation experiments were conducted with the following soybean genotypes: cv. Minsoy (PI 27890), A99-22 (Genetic Type Collection number T219H, *Y₁₁Y₁₁*), and cv. Latham 640 (private variety) in 1999; A99-22, cv. Verde (PI 548624), and cv. Harosoy (PI 548573) in 2000; and Harosoy, Heilong 35, and cv. Williams 82 (PI 547890) in 2001. Heilong 35 is a Chinese cultivar,

kindly provided by Dr L.Z. Wang of the Chinese Academy of Agricultural Sciences.

Soybean seeds were planted in the field at the Bruner Farm near Ames, Iowa, which has a Clarion-Nicollet loam soil type (fine-loamy, mixed, superactive, mesic Typic Hapludoll and fine-loamy, mixed, superactive, mesic Aquic Hapludoll).

Plasmids

Plasmid DNA from pCAMBIA3301 (Figure 1C, CAMBIA, CSIRO, Canberra, Australia, Liz Deaves, personal communication, 1996), pTF102 (Figure 1A, Frame et al., 2002), pSHX004, or pSHX007 (Figures 1B and 1D) was used as a DNA donor. The constructs pCAMBIA3301, pTF102, and pSHX004 have been successfully transformed into soybean in our laboratory using the *Agrobacterium*-mediated cotyledonary node protocol (Zhang et al., 1999). All 4 constructs carry the *bar* gene, driven by a CaMV 35S promoter (Nagy et al., 1985), as a selectable marker conferring resistance to glufosinate. Constructs pCAMBIA3301, pTF102, and pSHX007 also carried the *gus* reporter gene driven by the CaMV 35S promoter. Construct pSHX007, with the same vector background as pCAMBIA3301, contained an additional 1.8-kb wheat 18S ribosomal RNA gene (Gerlach and Bedbrook, 1979). Construct pSHX004 had the same vector background as pTF102, but the *gus* gene was replaced by a 0.8-kb *Nicotiana* protein kinase gene (Kovtun et al., 2000).

Plasmid DNA was purified using the QIAGEN Plasmid Mixprep kit (QIAGEN Inc., Valencia, CA, USA). DNA solutions were adjusted to their final concentrations in double-distilled water before use.

Pollen-tube pathway transformation

Soybean pollen-tube pathway transformation was conducted according to the procedures described by Liu et al. (1992). All experiments were performed in the late afternoon on flowers that had been self-pollinated naturally that morning. Two wing petals and one keel petal were removed to expose the stigmas of soybean flowers. Stigmas were severed at the boundary between the ovary and stigma, and 10 μ L of the plasmid DNA (concentrations of 25, 80, 100, or 150 μ g/mL) was applied to the exposed stigma. The DNA solution was retained by the banner petal and calyx. Treated flowers were identified by tagging, and untreated flowers and buds at the same node were removed. The pods that developed from the treated flowers were harvested individually. Seeds from untreated plants were harvested as negative controls.

Progeny tests

Seeds harvested from treated flowers were germinated in soil (Universal Mix, Hummert, St. Louis, USA) in the greenhouse. Glufosinate-resistant transgenic soybean seeds that were derived from the cotyledonary transformation procedure (H. Shou, unpublished results, 2000) served as positive controls. Seeds from untreated flowers served as negative controls. Seedlings were sprayed with 100 mg/L glufosinate (Liberty®, Aventis, Strasbourg, France) 2 wk after

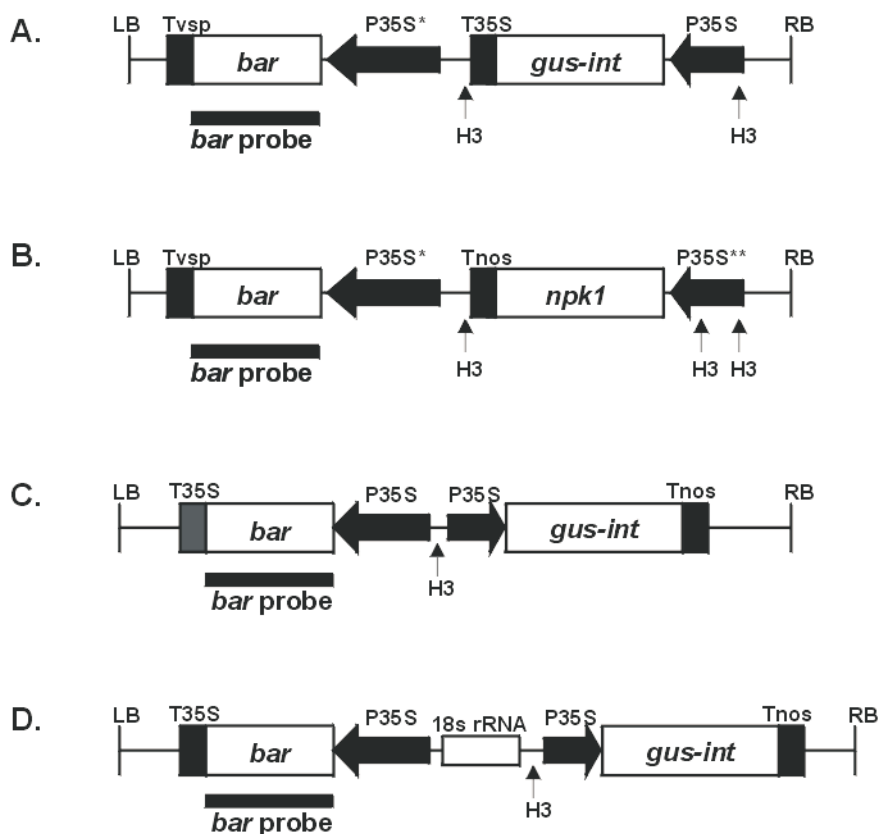


Figure 1. DNA constructs for soybean pollen-tube pathway transformation. (A) pTF102, (B) pSHX004, (C) pCAMBIA3301, (D) pSHX007. LB, left border; RB, right border; *bar*, phosphinothricin acetyltransferase gene; *gus-int*, β -glucuronidase gene containing an intron; *npk1*, *Nicotiana* protein kinase gene; P35S, CaMV 35S promoter; P35S*, enhanced 2X 35S promoter; P35S**, a modified 35S promoter (35SC4PPDK); T35S, CaMV 35S terminator; Tnos, nopaline synthase terminator; Tvsp, soybean vegetative storage protein terminator; H3, *Hind* III.

germination. Herbicide-susceptible seedlings were discarded 1 wk after spraying. Herbicide-resistant or partially resistant seedlings were kept for further histochemical GUS and Southern blot analyses.

Histochemical GUS assay and Southern blot analysis

The histochemical GUS assay (Jefferson et al., 1987) was used to screen for reporter gene expression in the herbicide-resistant or partially resistant seedlings derived from plants treated with constructs pCAMBIA3301, pTF102, and pSHX007. Total genomic DNA was extracted from fresh leaf material using the CTAB method (Reichardt and Rogers, 1994). Then, 10 μ g of DNA was digested with *Hind* III and separated by means of electrophoresis on a 0.8% agarose gel.

Southern blots and hybridizations (Southern, 1975) using the *bar* and *gus* gene cassettes as probes were conducted to verify the putative transformants.

Results

Podding rate of pollen-tube pathway transformation

From 1999-2001, we performed pollen-tube pathway transformations on 7 soybean genotypes using 4 plasmid DNAs with 4 different DNA concentrations (Table 1). A total of 5590 flowers were treated and 2400 pods were harvested with a success rate of 49.7%. The podding rate varied among years and genotypes and ranged from 21.1-77.3%. The choice of cultivars during the first 2 y was based on the availability of the plants in the field during the time of treatment. Researchers conducting the experiments were well trained in soybean hybridization; thus, flower abortion rates due to poor handling skills were minimized. Cultivar Verde showed a consistently low podding rate across 3 different DNA treatments in 2000. Cultivar Heilong 35 was included in the study in 2001 because it was previously shown to be successfully transformed by the pollen-tube pathway method (Lei et al., 1995).

Pollen tubes should arrive at the ovule approximately 6-8 h following self-pollination, thereby initiating fertilization (Carlson and Lersten, 1987). We chose flowers at this stage and applied the DNA solutions after the removal of the stigma. Approximately 70% of the treated pods lost the sharp apical part of the pod and became blunt compared to the untreated ones (Figure 2). The blunt tip pod is an important indicator that an appropriate amount of stigma was removed. Excessive stigma removal causes flower abortion, whereas insufficient stigma removal does not sufficiently expose the pollen-tube pathway for DNA delivery (DP Liu, personal communication, 2001).

Herbicide spraying results

All 4793 seeds harvested from the pollen tube pathway experiments were screened using herbicide spray. None of the seedlings showed complete resistance to the herbicide as did the positive control (Figure 3). Considering that the expression level of the *bar* gene may vary from event to event, we categorized the herbicide effects on seedlings into 2 groups: susceptible and partially resistant (Figure 3). Approximately 2.1%, a total of 101 seedlings, were partially resistant (Table 1) and further analyzed.

Histochemical GUS assay and Southern blot analysis results

All 101 seedlings that showed partial resistance to herbicide were further analyzed using both histochemical GUS assay and Southern blot analysis. As illustrated in Figure 4, the partially resistant plants (Panel B, PT1-PT6) obtained from the experiments did not have the integration of the *bar* gene, whereas the plants derived from the cotyledonary transformation procedure contained 2 or 3 copies of the *bar* transgene (Panel A, TCT1 to TCT3). The GUS assay also showed negative results, indicating that no transgene incorporation occurred in these plants.

Table 1. Summary of pollen-tube pathway transformation experiments in soybean.

Experiment	Recipient parent	Donor DNA	DNA concentration (µg/ml)	No. treated flowers	No. pod set	No. seed set	% success rate of pod set	No. partial resistant seedlings
1999	A99-22	pCAMBIA3301	25	326	248	595	76.1	8
	A99-22	pTF102	150	144	84	149	58.3	3
	Latham 640	pCAMBIA3301	25	403	280	625	69.5	9
	Minsoy	pCAMBIA3301	25	93	51	106	54.8	2
2000	A99-22	pCAMBIA3301	80	154	82	121	53.2	3
	Harosoy	pCAMBIA3301	100	251	137	259	54.6	6
	Harosoy	pTF102	100	274	116	243	42.3	6
	Verde	pCAMBIA3301	80	681	165	228	24.3	5
2001	Verde	pSHX004	80	577	122	141	21.1	2
	Verde	pTF102	80	514	126	185	24.5	3
	Harosoy	pSHX004	100	177	67	150	37.9	4
	Harosoy	pSHX007	100	110	57	119	51.8	5
	Heilong 35	pCAMBIA3301	100	227	150	315	66.1	7
	Heilong 35	pSHX007	100	42	28	33	66.7	3
	Heilong 35	pTF102	100	44	34	75	77.3	3
	Williams 82	pCAMBIA3301	100	281	137	315	48.8	8
	Williams 82	pSHX004	100	359	120	254	33.4	6
	Williams 82	pSHX007	100	413	153	355	37.0	7
	Williams 82	pTF102	100	520	243	525	46.7	11
	Total Average				5590	2400	4793	49.7

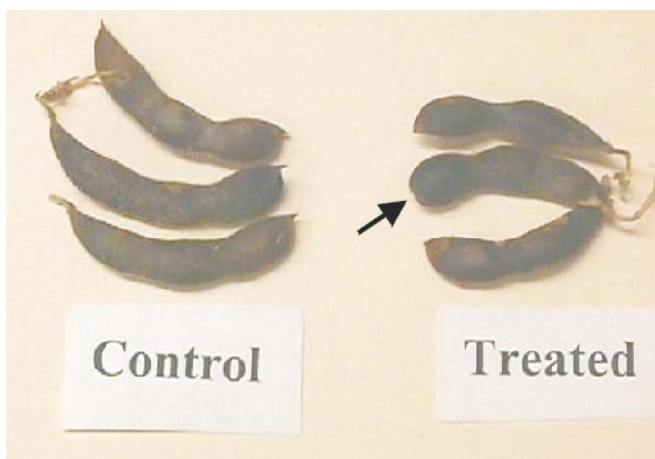


Figure 2. Appearance of the pods harvested after being treated for pollen-tube transformation. The arrow indicates the round apical part in pods derived from flowers with the stigma removed. Pods of the “control” were developed from nontreated flowers; pods of “treated” were obtained from the flowered that were operated.

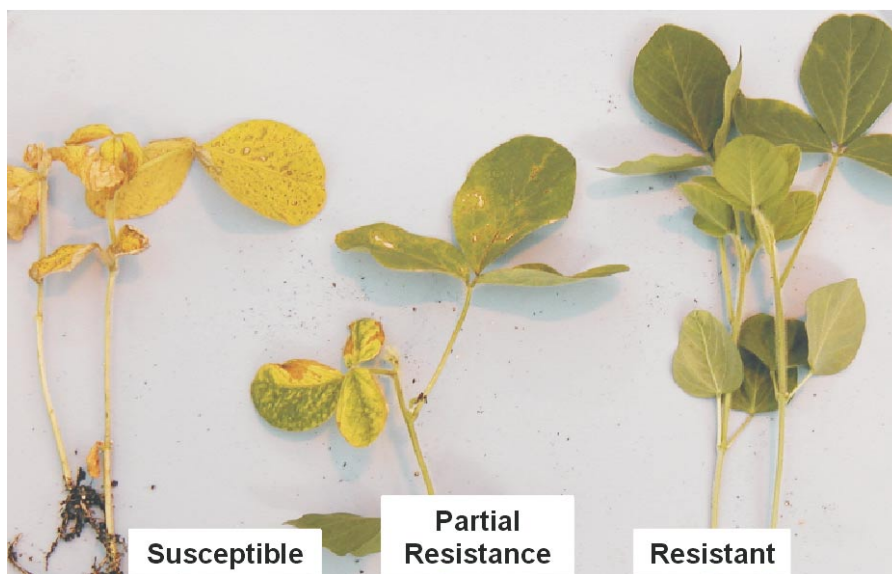


Figure 3. Appearance of herbicide-sprayed soybean progeny plants. Susceptibility of soybean progenies to 100 mg/L glufosinate sprayed on 2-week-old seedlings. The resistant seedlings were the transgenic plants obtained from *Agrobacterium*-mediated cotyledonary node protocol, serving as positive controls. The progenies derived from pollen-tube transformation were categorized into susceptible and partially resistant according to the symptoms of the herbicide effects.

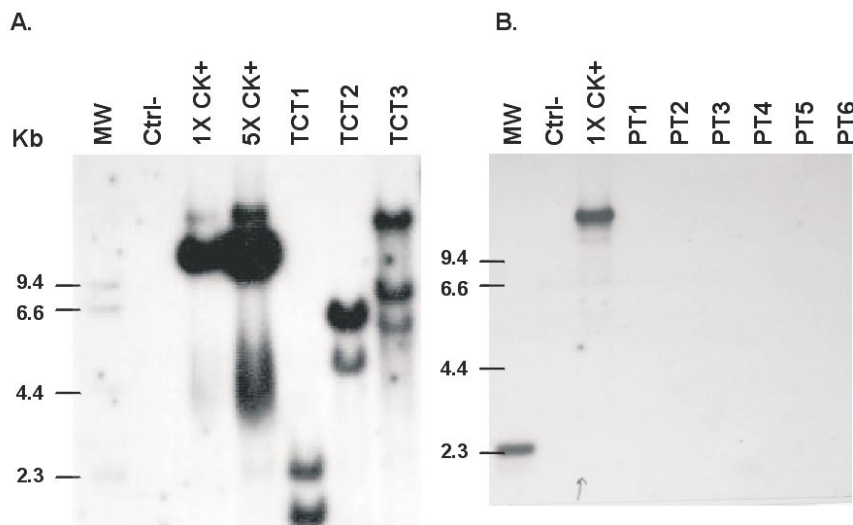


Figure 4. Southern blot hybridization of soybean genomic DNA. Soybean plants obtained from *Agrobacterium*-mediated cotyledonary transformation (A) and pollen-tube transformation procedure (B). TCT1, TCT2, and TCT3 are DNA of independent transformants obtained from *Agrobacterium*-mediated cotyledonary transformation with binary vector of pCAMBIA3301, pTF102, and pSHX004, respectively. PT1, PT2, PT3, PT4, PT5, and PT6 are DNA of the herbicide partially resistant soybean plants from pollen-tube transformation procedure. MW, λ DNA / *Hind* III molecular marker; Ctrl-, negative check (genomic DNA from nontransformed soybean plants); 1X CK+ and 5X CK+, nontransgenic soybean genomic DNA spiked with 63 pg and 315 pg digested pTF102 plasmid DNA severed as positive checks of 1 and 5 copies of transgene, respectively.

Discussion

We were unable to reproduce the pollen-tube pathway transformation technique for delivering plasmid DNA into soybean plants using the published protocol (Liu et al., 1992). Moore et al. (1996) also reported failure to achieve transgenic soybean and cotton plants using a similar approach. In their experiment, all 50 soybean seeds and 226 cottonseeds harvested from treated flowers tested negative after herbicide screening.

To date, the pollen-tube pathway method has been reported to successfully deliver both total genomic and plasmid DNA into plants (Luo and Wu, 1989; Ni et al., 1998; Yu et al., 1999; Zhen et al., 1998). The delivery of exogenous genomic DNA via this technique in soybean has also been reported (Hu and Wang, 1999; Lei et al., 1994, 1995; Liu et al., 1992, 1997; Zhao et al., 1995), although molecular evidence to confirm transformation has been limited. Transformation efficiency reported in these laboratories was estimated at 0.5%-3% with either genomic or plasmid DNA (DP Liu, personal communication, 2001). During the second and third years of our experiments, we were able to obtain technical advice and assistance from Dr D.P. Liu, who developed the technique for soybean and reported success in delivering genomic DNA into soybean (Liu et al., 1992,

1997). In addition, in our third year of field experiments, we used Heilong 35, a cultivar reported to be a successful recipient parent (Lei et al., 1995). Nevertheless, we did not obtain any transgenic soybean plants in any cultivar tested. We conclude that the pollen-tube pathway transformation technique is not reproducible in soybean.

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