Soybean Transformation via the Pollen Tube Pathway

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ABSTRACT

The pollen tube pathway method of transformation has been reported to be successful in several crops including soybean. DNA can be transferred by cutting the stigma following pollination and applying the DNA solution on the severed style. DNA presumably reaches the ovary by flowing down the pollen tube and then integrates into the just fertilized but undivided zygotic cells. To provide the molecular evidence for this procedure, the plasmids pBI221 carrying a CaMV35S promoter-beta glucuronidase (*gus*) gene-nos terminator gene fusion construct and pBI0122 carrying a CaMV35S promoter-*bar*-g73' terminator gene fusion construct were used. Approximately 5000 seeds were produced from the flowers treated with DNA. None of the seeds from the plants treated with DNA containing the *bar* gene were found to be as resistant as the positive control. Morphological variation was observed in some plants, but this was not observed in the following generation. Approximately 2% of the seeds from plants treated with DNA containing the *gus* gene had a positive reaction in the GUS assay, however, no GUS activity was observed in the plant leaves from those seeds. Less than 3% of progeny seeds tested expressed a positive reaction and polymerase chain reaction (PCR) with seedling DNA did not detect the *gus* gene. Thus, we were unable to confirm the positive results of others when we used screenable marker genes.

INTRODUCTION

Soybean transformation technology has been the subject of intense efforts over the past few years. Genetic transformation of soybean provides a method to improve economically important traits such as resistance to diseases, and pests, and tolerance to herbicides, and value-added traits, which are conferred by a single gene. Horsch et al. (1985) reported transformation and plant regeneration in tobacco. Thereafter, soybean transformation was reported independently by Hinchee et al. (1988) and McCabe et al. (1988) using *Agrobacterium* mediated DNA transfer

and particle bombardment methods, respectively. Chee et al.(1989) and Al-Janabi (1992) have also reported successful *Agrobacterium*-mediated transformation. Finer and McMullen (1991) utilized particle bombardment with embryogenic soybean suspension culture to produce transformed plants and biotin-mediated delivery transformation was reported by Horn et al. (1990). Today, two methods are most commonly used for soybean transformation: *Agrobacterium*-mediated transformation of the cotyledonary node and gene gun particle bombardment of embryogenic cultures. These methods have been used to produce several successful examples of soybean transformation in recent years, including a glyphosate-tolerant soybean line, 40-3-2, by transferring two *CP4 EPSPS* genes with the particle acceleration method (Padgette et al., 1995), a soybean line with high resistance to corn earworm by introducing the *Bt* gene (Stewart et al., 1996), and a soybean line expressing the bovine ß-casein gene in the seeds (Maughan et al., 1999). Great challenges remain in soybean transformation since the methods are less efficient than those routinely used with other species (Finer et al., 1996). All the techniques described above involve complicated skills, and the rate of transformation is low. Development of a simple method with a high transformation rate would be very useful.

Introduction of exogenous DNA into a plant embryo through the pollen tube pathway after pollination was first reported by Zhou et al. (1983) in cotton. Hu and Wang (1999) reviewed the procedures used with this approach, the confirmation of the results, and the field performance of the transformed plants. The theory of this technique can be briefly described as follows: after pollination, the nucellar cells form a pathway to allow the pollen tube passage to the embryo sac; by removing the stigma and applying a DNA solution on the severed style after pollination, the exogenous DNA could presumably reach the ovary by flowing down the pollen tube and integrating into the just fertilized but undivided zygotic cells. The cells to be transformed at that time do not have normal cell walls. The genes of interest could then be integrated into the genome of the recipient cultivar. The transformed seeds could be obtained directly without protoplast preparation, cell culture, and plant regeneration. This method has been used in several crops, including cotton (Zhou et al., 1983, 1988), rice (Luo and Wu, 1988), and soybean (Liu et al., 1992; Lei et al., 1992) and variations were observed in the putatively transformed plants. Liu et al. (1992) reported that phenotypic variation was observed in approximately 3% of the progeny derived from soybean plants treated with the DNA from pea, and wild and semi-wild soybean. Variation occurred in maturity, growth habit, leaf and seed shape, and pubescence,

flower, and hilum color. A similar approach was taken by Lei et al. (1992) when they used DNA from semi-wild soybean and cultivated soybean with high protein concentration and early maturity. Progenies with higher protein concentration and earlier maturity were observed and peroxidase isozyme analyses indicated that the progenies with altered traits had band patterns different from the recipient cultivars. By introducing ^{3}H-labeled DNA from bluish dogbane (*Apocynum venetum*) into cotton, Zhang et al. (1992) indicated that the route of the exogenous DNA into the embryo sac was through the pollen tube and the exogenous DNA was randomly taken up by the eggs, zygotes, synergid and polar nucleus. However, those results did not provide clear evidence indicating that the DNA introduced was successfully integrated into the genome and expressed in the progeny of plants. Most of the variations have not been verified at the genomic level, and we do not know if the variations in progeny plants were caused by the damage resulting from the procedure or by DNA integration into the chromosomes. Using the pollen tube pathway method, Luo and Wu (1988) observed that transformation had occurred in rice by using a cloned gene for neomycin phosphotransferase, *nptII,* as a reporter. In their study, Southern blot analysis revealed that up to 20 % of the rice seeds from treated florets were transformed and the transformed rice plants contained copies of the *nptII* gene, varying from 1 to 300 copies. Some of transformed plants could produce neomycin phosphotransferase.

The objective of this research was to determine the efficacy of the pollen tube pathway method of transformation in soybean by using a selectable marker and a reporter gene that can be verified phenotypically and finally at the molecular level in the subsequent generations.

MATERIALS AND METHODS

Plasmid constructs used

The two plasmids used in this study were pBI221, a pUC19 cloning vector encoding ampicillin resistance and carrying a CaMV35S promoter-beta glucuronidase gene-nos terminator gene fusion construct; and pBI0122, a plasmid similar to the ampicillin resistant vector carrying a CaMV35S promoter-*bar*-g73' terminator gene fusion construct. The *bar* gene encodes resistance to the herbicide Liberty (phosphinothricin). Plasmid DNA was diluted to 400 ng/µl in TE buffer and stored at 4^{0} C for use.

Materials

Soybean cultivars Williams 82, Macon, Clark 63 and Iroquois were used as recipients of DNA in the field at the Crop Sciences Research and Education Center, University of Illinois at Urbana-Champaign. The cultivars were planted in the summer of 1997 in the field with three different planting dates. Plots were three rows wide and 5 m long with 1.5 m between rows in the field and at the seedling stage the plants were thinned to approximately 15 cm apart.

DNA application

The DNA application procedure of Zhou et al. (1988) and Lei et al. (1992) was used with some modifications. At 8:00 in the morning, flowers judged to be pollinating later that day were marked with tags below the node with the flowers. The other flowers were removed, and only the flowers that would open that day were kept. Between 1:00 and 4:00 pm on the same day when the flowers opened, the petals were separated and one third of the stigma was severed with a small pair of scissors. Approximately 5-8 µl of exogenous DNA were placed on the severed surface of the style with a microsyringe. After 30 min, the flowers were checked. If the DNA was gone, more DNA was applied on that flower. Five, ten, and twenty days after treatment, the nodes were checked to remove new flowers. At harvest, pods were separated based on types of DNA, and date and time of treatment.

Greenhouse evaluation with herbicide

A3322, susceptible to phosphinothricin, and its isoline, designated as A3322B (transformed with the *bar* gene conferring resistance to phosphinothricin) used in this study were provided by Asgrow Seed Company and the herbicide, Liberty, was provided by Dr. Loyd Wax, USDA-ARS, at the University of Illinois. A3322, A3322B, Williams 82, Macon, Clark 63 and Iroquois were used for the test of optimal herbicide concentration for screening putatively transformed plants. One of two unifoliolate leaves on each plant was dipped into the herbicide solution approximately 10 days after planting in sand benches in the greenhouse. Each plot included 15 plants, and 13 aqueous solutions of the herbicide Liberty, 0.01%, 0.05%, 0.10%, 0.15%, 0.20%, 0.25%, 0.30%, 0.35%, 0.40%, 0.45%, 0.50%, 0.55%, and 0.60%, were used. The treated leaves were observed for necrosis five and ten days after treatment. The optimal concentration of herbicide was determined based on the concentration that caused leaf necrosis of susceptible plants and had no effect on the leaves of resistant plants (A3322B).

After the determination of the optimal herbicide concentration, the putatively transformed seeds along with their corresponding negative control seeds and A3322B and A3322 were planted in sand benches in the greenhouse to be screened for plants carrying the *bar* gene. If the plants appeared to be resistant to the herbicide, they were transplanted to large pots (30 cm diameter) from the sand beach and grown to maturity.

GUS and PCR assays

A small piece of cotyledon opposite the hilum was removed from each seed and placed into 96-well plates for GUS assay. The seed remnants were saved. With each set of 240 putatively transformed seed chips assayed, 20 negative controls and two positive controls (soybean transformed with the *gus* gene provided by Dr. Wayne Parrott, University of Georgia) were stained by the histochemical assay in an X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution. The stock solution for the histochemical assay of GUS activity included 0.1 M NaPO4 (pH 7.0), 0.1 M K3Fe(CN)6, 0.1 M K4Fe(CN)6, and 0.2 M NaEDTA (pH 7.0). Immediately before use, 25 ml of buffer solution was prepared with 11 ml sterile water, 12.5 ml 0.1 M NaPO4 (pH 7.0), 125 µl 0.1 M K3Fe(CN)6, 125 µl 0.1 M K4Fe(CN)6, and 1.25 ml 0.2 M NaEDTA (pH 7.0). After 16.7 mg of X-Gluc was dissolved in 166.7 µl of N,N-dimethyl formamide, 25 ml buffer was added, and then 20 µl of trition X-100 was added to the X-Gluc solution. After loading 100 µl of X-Gluc solution into each well, the plate was covered with a lid and incubated at 37^0 C in the dark for 48 hr. The color of the seed chips in each well was observed, compared with the controls, and recorded.

After the GUS assay, the chipped seeds that gave a positive response were sterilized in 5% Clorox solution for 10 min and germinated on the FRSG (Finer embryo germination medium with sucrose and gelrite, Finer and McMullen [1991]) medium or planted directly in pots filled with sand. After the roots of seedlings formed in the FRSG test tubes, the plants were transferred into small peat pots, and then transplanted into large pots for growth to maturity. The plant leaves and progeny seeds were assayed with X-Gluc solution. Positive progeny seeds were grown again. Leaf samples were taken from both the first and second-generation plants for DNA isolation.

The CTAB method from Keim et al. (1988) with some modifications was used for leaf DNA isolation. Two types of primers specific for *gus* gene amplification were used in the PCR reactions. The sequences for the first pair of primers were 5`-GUSXB- GGG GGA CTC TAG

AGG ATC CCC GGG TGG TCA GTC CCT TAT G and 3`-NOSKE-CGA ATT GGG TAC CGA ATT CGA TCT AGT AAC ATA GAT GAC. The sequences for the second pair of primers were 5`-GUS1-TTA CGT CCT GTA GAA ACC, and 3`-GUS2R-GTC CCT CCG TTT GTT ACT (Cho et al., 1995). The amplification program consisted of 2 min at 94^0 C followed by 45 cycles of 1 min at 94^0 C, 1 min at 45^0 C and 1 min at 72^0 C. A final cycle of 72^0 C for 7 min was completed before the reaction mixtures were held at 4^0 C. Plasmid, pBI221, and DNA from *Astragalus sinicus* transformed with the *gus* gene (Cho et al., 1998) were used as positive controls and DNA from recipient cultivars were used as negative controls.

RESULTS

Flowers treated and seeds harvested

In the field during the summer of 1997, approximately 3,670 flowers were treated with plasmid DNA (2,375 with the *gus* gene and 1,295 with the *bar* gene). A total of 5,109 seeds were harvested from the 1,965 pods that formed, 1,942 seeds from flowers treated with the *bar* gene (Table 1), and 3,167 seeds from flowers treated with the *gus* gene (Table 2). Some of the seeds were smaller and more shrunken than normal seeds.

Herbicide resistance screening

When A3322, A3322B, and the recipient cultivars, Williams 82, Clark 63, Macon, and Iroquois were used to determine the optimal herbicide concentration for screening, 0.05% concentration of Liberty was considered to be optimal based on leaf necrosis.

Each screening for Liberty resistance included the putatively transformed seeds and one row each of the recipient cultivars, and A3322, and A3322B as the negative and positive controls, respectively. Among the 1,942 seeds screened from four cultivars, no seedling was found to be as resistant as the positive control A3322B; however, 14 plants with intermediate resistance to Liberty were found. These plants were grown to maturity in 30 cm diameter pots. When 10 progeny seedlings from each plant were tested again with the same herbicide concentration, all were susceptible to the herbicide.

From the 1,942 seeds obtained from the *bar* gene treated plants, nine phenotypic variant plants were observed (Table 1). Among the variants, five were from Clark 63; three were from Williams 82; and one was from Iroquois. The abnormal phenotypes included one or three

unifoliolate leaves or abnormal shaped unifoliolate or trifoliolate leaflets. However, progeny grown from the seeds produced by these variants had normal morphology.

GUS assay and PCR detection

The 3,167 seeds produced from plants treated in the field with pBI221 carrying the *gus* gene were screened using the GUS histochemical assay. Blue coloration, similar but less intense than that of the positive controls, was found for 70 seeds. Among them, 49 seeds were from Clark 63, three from Williams 82, 16 from Iroquois, and two from Macon. However, when leaves from plants grown from these seeds were tested for GUS expression with X-Gluc, all tests were negative. Twelve plants were grown to maturity and among the 390 seeds produced, 2.6% were GUS positive. PCR reactions were conducted with DNA from leaves of these plants as well as 20 first generation plants and nothing was amplified with two different pairs of *gus* gene primers. The plasmid DNA and DNA from *Astragalus sinicus* transformed with the *gus* gene produced the correct size fragments. This indicates that the *gus* gene was most likely not present in these plants.

DISCUSSION AND CONCLUSION

Most pollen tube pathway transformation results were reported in the late 80's and early 90's (Luo and Wu, 1988; Lei et al., 1992; Liu et al., 1992) with little progress being reported recently. Hu and Wang (1999) reviewed the procedure and results of pollen tube pathway transformation in soybean and concluded that the pollen tube pathway and ovarian injection procedures can be used in transgenic plant production. Based on the results from this experiment, although partial resistance, abnormal phenotypes, and positive GUS activity were found among those seeds produced by flowers treated with exogenous DNA, no evidence indicated successful transformation. The partial resistance to Liberty herbicide identified in the previous generation may have been the result of inconsistency of application of the herbicide solution or the resistant gene was lost in the next generation. The abnormal phenotypes observed in previous generation might have been caused by the action of the procedure and did not result from heritable genetic changes since all plants were normal in the next generation. In this research, the GUS activity expressed in some of seeds may have been endogenous or transient expression in which the DNA was inside the cells but not integrated into a

chromosome. The seed GUS assay procedure might not be a definitive test to detect the presence of an incorporated *gus* gene, but it still can be useful as a rapid, initial evaluation. Most of the previous reported successes did not include molecular analyses to actually confirm integration of the transformed sequence. It is possible that the reported results are in error or the exact requirements for consistent results may not yet be known. Moore et al. (1996) also reported no success using the *bar* gene with soybean in a smaller scale experiment. Following the DNA movement through the pollen tube pathway after pollination with radioisotope- or fluorescence-labeled DNA would be one experiment that could provide additional information about the potential for this procedure.

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Table1. Summary of flowers treated with the plasmid carrying the *bar* gene and initial evaluation of harvested seeds.

Cultivars	Flowers treated	Seeds harvested	Partial resistant plants in first generation	Abnormal phenotypes
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Williams 82	350	620	5	3
Clark 63	300	495	2	5
Macon	195	306	3	0
Iroquois	450	521	5	1
Total	1295	1942	14	9

Table 2. Summary of flowers treated with the plasmid carrying the gus gene and initial evaluation of harvested seeds.

Cultivars	Flowers treated	Seeds harvested	Seeds with a positive GUS assay
	----------	-----no.-----	----------
Williams 82	200	240	3
Clark 63	1010	1583	49
Macon	190	264	2
Iroquois	975	1284	16
Total	2375	3167	70