Transgenic Grain Legumes Obtained by *In Planta* Electroporation-Mediated Gene Transfer

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Abstract

Electroporation-mediated gene transfer into intact plant tissues was demonstrated in pea, cowpea, lentil, and soybean plants. Transient expression of a chimeric *gus* reporter gene was used to monitor the uptake and expression of the introduced DNA in electroporated nodal axillary buds in vivo. The branches that grew out of the nodal meristems were chimeric and expressed the introduced gene up to 20 d after electroporation. Transgenic R_1 pea, lentil, and cowpea plants were recovered from seeds originating on these chimeric branches as shown by Southern blot hybridization and GUS expression. Transgenic R_2 soybean and lentil plants were also obtained. Segregation ratios in these populations showed a strong bias against transgene presence or expression.

Index Entries: Electroporation; pea; lentil; cowpea; soybean; nodal meristems; transgenic plants.

1. Introduction

Electroporation-mediated direct gene transfer has been developed *(1)* and has been used in generating transgenic plants *(2)*. So far, mostly protoplasts have been the target of direct gene transfer methods, because the plant cell wall has been assumed to form an impenetrable barrier to DNA *(3)*. However, recent work has shown this assumption to be unfounded in several cases. Indeed, leaf sections, somatic and zygotic embryos, as well as suspension cells from several plant species have now been shown to be permeable to plasmid DNA and able to express various chimeric transgenes *(4–9)*. In one case, fertile transgenic plants were recovered from electroporated sugarcane cells *(10)*. Thus, this technique has excellent potential to generate transgenic plants, in particular in cases where technology has been limited by the lack of a reliable technique for regenerating complete plants from either protoplasts or tissue explants. In addition, electroporation does not necessitate expensive equipment, and optimization of parameters can be performed quickly. In the same vein, a recent review *(11)* stresses the severe limitations imposed by conventional plant transformation techniques and advocates the exploration of alternative DNA delivery techniques, such as electroporation of intact tissues, including meristems.

In this article, we used the meristematic tissues of axillary buds from pea, lentil, cowpea, and soybean plants as targets for DNA uptake. Since these tissues normally differentiate into shoots that bear flowers and eventually seeds, successful transformation of these meristems in vivo raises the possibility of obtaining transgenic seeds without the need for in vitro regeneration. Indeed, we also show that transgenic plants expressing GUS and harboring the *uid*A transgene are found among the R_1 and R_2 progeny of R_0 electroporated plants. This article thus provides molecular evidence to support our transformation protocol published earlier *(12)*.

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2. Materials and Methods

2.1. Plant Material

Pea (*Pisum sativum* L.) cv. Sparkle was grown in the greenhouse for 3 wk in potting soil (greenhouse soil mix with 60% peat, Washington State University Stores) placed in Deepot containers. Prior to electroporation, the apical portion of the plants was decapitated close to the node of a fully expanded leaf. The stipule and adjacent petiole were removed to expose the nodal bud. All other meristematic buds below that nodal bud were excised. The bud that was thus retained was contained in a V-shaped angle formed by the excised stem and the base of the petiole. After treatment (*see* Section 2.3.), the plants were placed back in the greenhouse and allowed to grow. R_1 progeny were raised from seeds collected from shoots that grew out from the intact buds subjected to electroporation in vivo. The seeds were surface-sterilized in 95% ethanol for 5 min, followed by agitation in 0.3% sodium hypochlorite for 15 min. Seeds were then washed in sterile distilled water three times and plated on MS salt medium (complete MS salt mixture, Gibco Laboratories, Grand Island, NY) supplemented with 0.2% gelrite and 3% sucrose. They were grown at room temperature in a 16-h light: 8-h dark cycle. Lentil (*Lens culinaris* Medik.) cv. Crimson, cowpea (*Vigna unguiculata* L. Walp) cv. Blackeye, and soybean (*Glycine max*) cv. Wye, were processed similarly, except that no axenic steps were used in any of the experiments.

2.2. Plasmid Construct and DNA Isolation

The *Eco*RI-*Hin*dIII fragment of p35SGUSINT *(13)* was cloned into pUC8, and the new vector is referred to hereafter as pGPT1.0. This fragment contains the *uid*A gene from *E. coli (13)* driven by the cauliflower mosaic virus 35S promoter and the *nos* terminator. In addition, this chimeric gene is interrupted by the second intron from the potato ST-LS1 gene. This construct has been shown to be expressed in cowpea *(14)*, pea, and lentil tissues (Z. Cai et al., unpublished).

Plasmid DNA was isolated according to the alkaline lysis method and purified by polyethylene glycol precipitation *(15)*. Plasmid preparations contained at least 80–90% supercoiled DNA as judged by agarose gel electrophoresis. DNA concentration was measured by the diphenylamine assay *(16)*.

2.3. Electroporation of Nodal Meristems

Pea nodal buds were injected with 2 µL of DNA solution (100–300 µg/mL pGPT1.0 DNA/spermine or Lipofectin™/MS salt solution) using a 10-µL Hamilton syringe. DNA was complexed with 2 m*M* spermine or 50 mg/mL Lipofectin™ as indicated in Table 1. The nodal buds were injected from the top through the apical dome to a depth of about 1 mm. Twenty minutes after injection, each plant was individually electroporated by dipping the exposed nodal bud into the DNA solution (100–300 µg/mL pGPT1.0 DNA/spermine or Lipofectin™/MS salt solution) contained in a circular electrode. Two square pulses of 99-ms duration each were delivered at 200 V, unless otherwise stated. The pulses were delivered 5 s apart using a Progenetor™ electroporation unit connected to a dc power supply. The gap between the electrodes was 0.6 cm. After electroporation, the plants were taken back and grown in the greenhouse. Control plants were either injected with plasmid DNA and not electroporated, or injected with pUC8 DNA or MS salts and electroporated.

Nodal meristems of cowpea, lentil, and soybean plants were injected similarly and electroporated at 100 V as described by Chowrira et al. *(12)*. In the latter three cases, Lipofectin™ only was used as a DNA protectant. Also, only covalently closed circular DNA was used for transformation. A full description of the entire experimental protocol was published earlier by Chowrira et al. *(12)*.

2.4. GUS Assay

Staining with X-glu was done as described in ref. *(17)* on pieces of leaf, petiole, tendril, stipule, sepal, petal, and anther, which developed on the shoots growing out of the electroporated buds and on pieces of leaf from R_1 and R_2 plants.

2.5. DNA Analysis

Plant genomic DNA was isolated according to the method of Doyle and Doyle *(18)*, and further

Table 1
GUS Staining in Floral Tissues
from R_0 Pea Shoots Under Different Electroporation Conditions

DNA concentration, µg/mL	DNA protectant	Volts	Pulse, ms	Fraction GUS^+ R_0
300	Spermine	0	0	0/40
100	Spermine	200	99	2/20
300	Spermine	200	50	1/23
300	Spermine	200	99	4/36
300	Lipofectin™	200	99	11/40

Floral tissues that stained included petals, stamens, anthers, and pollen, depending on individual plants.

purified by phenol-chloroform extraction subsequent to RNase treatment *(19)*.

The Genius™ kit (Boehringer Mannheim, Indianapolis, IN) was used for Southern analysis of genomic DNA isolated from R_1 plants. The GUSINT probe consisted of gel-isolated 1.8-kbp *Ssp*I-*Sst*I from pGPT1.0 (Fig. 1). This fragment contains a portion of intron II from the ST-LSI gene and all of the downstream *uid*A coding sequence *(16)*. DNA transfer, labeling of the probe, and detection were according to the manufacturer's protocol.

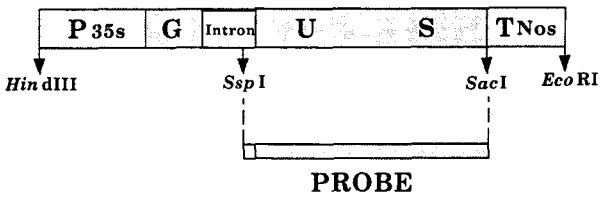


Fig. 1. Probe used in Southern blot experiments. The 1.8 kbp *Ssp*I/*Sst*I fragment from pGPT1.0 was gel isolated and labeled with DIG-11-dUTP. This fragment contains a portion of intron II from the ST-LSI gene and all the downstream *uid*A coding sequence.

3. Results and Discussion

Our initial experiments were actually aimed at reproducing the results of Dekeyser et al. *(5)*, which showed transient expression of transgenes in electroporated pieces of leaf from a variety of plants. Using the electroporation conditions described in Section 2., we observed transient expression of GUS in isolated pea nodal buds, up to 10 cell layers inside the tissues (not shown). This prompted us to adapt the electroporation technique to nodal buds *in planta* (Fig. 2), thereby avoiding all tissue-culture steps. Pilot experiments were performed with pea plants under a variety of electroporation and DNA protectant conditions. Only one set of experimental parameters was used for cowpea, lentil, and soybean.

3.1. Effects of Electroporation on the Survivability of the Nodal Bud

The single buds retained on the pea plants used for in vivo electroporation gave rise to branches bearing flowers and pods. Although 87% of the nonelectroporated plants gave rise to branches from these buds, the proportion of electroporated plants that successfully grew branches from these buds varied with voltage. At 100 V, viability was unchanged, whereas at 200 V, 84% of the buds survived and grew into a short branch bearing flowers and pods. Bud survival declined to 35% when the voltage was increased to 300 V.

3.2. GUS Assay on Shoots (R_0 Plants)

Twenty days after electroporation with a spermine–DNA complex, branches that grew from the electroporated pea buds were assayed for GUS activity in randomly harvested pieces of tissues. Approximately 5% (9/187) of these plants had pieces of shoots that stained positive with X-glu. Sectors of stained and unstained regions were found in all tissues that stained. Microscopic observations revealed blue crystals inside cells (Fig. 3A). When staining was observed, it was highly localized and never seen in the whole tissue being assayed. This pattern of staining was observed in leaves located as far as the fourth node on the

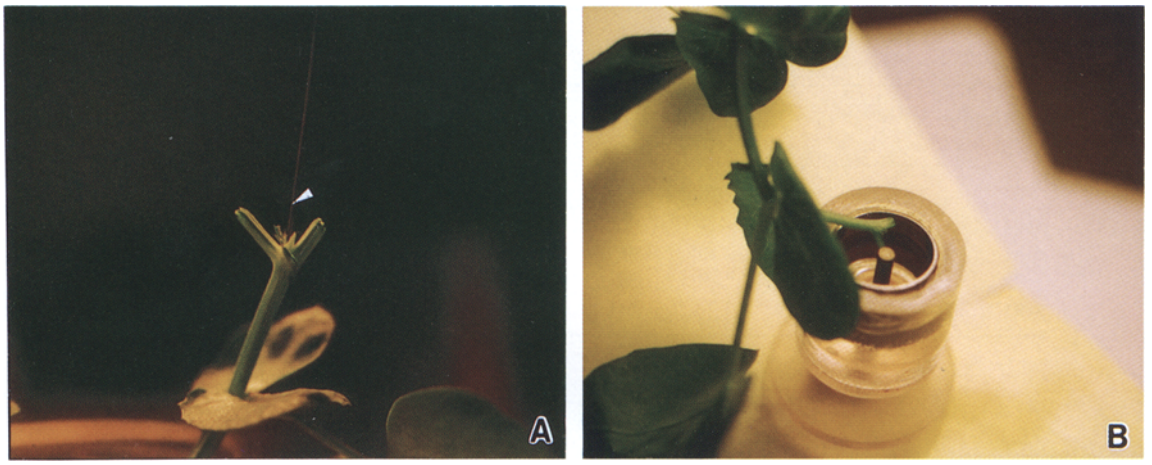
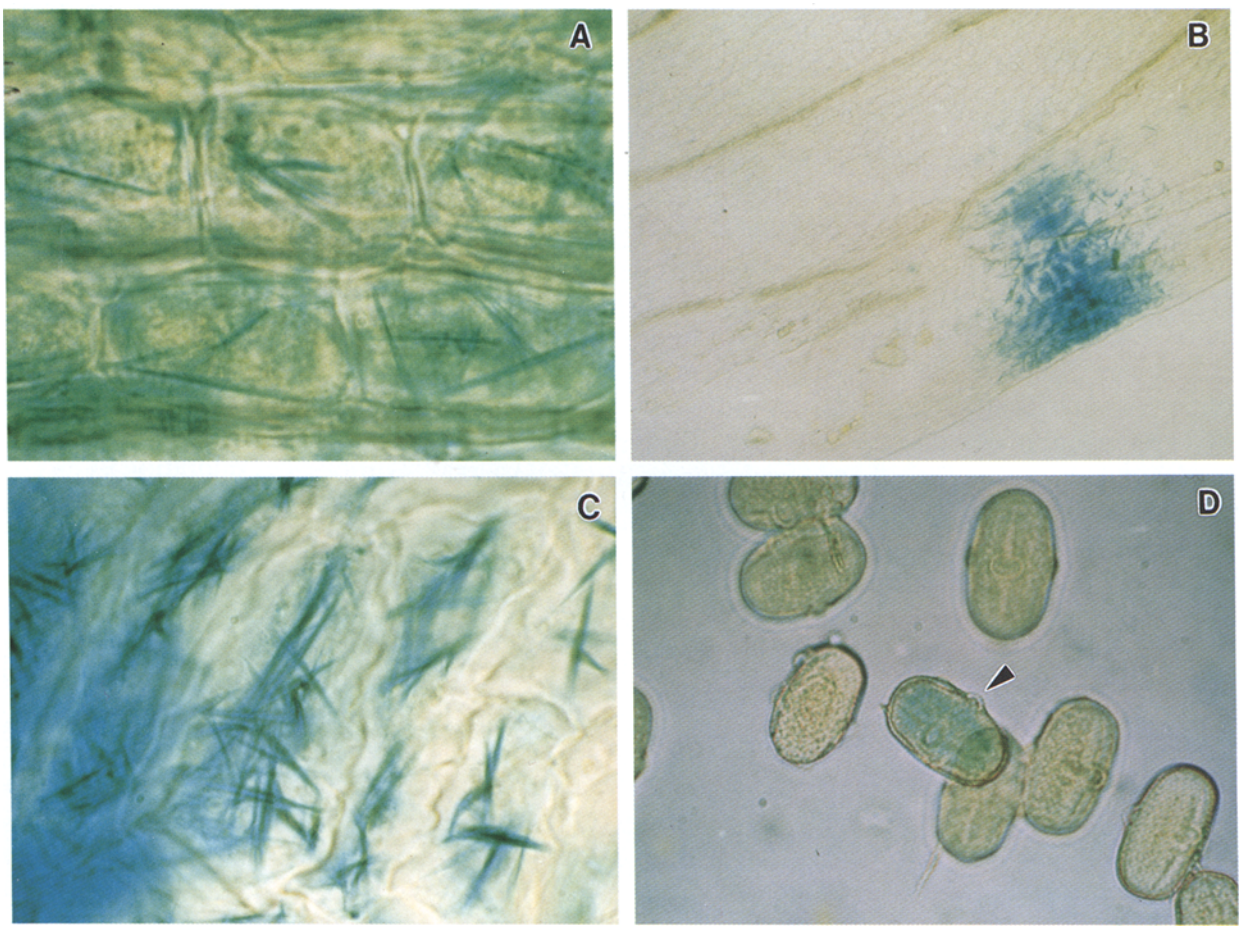


Fig. 2. Injection into nodal buds and electroporation. **(A)** Injection into nodal buds; the needle of the Hamilton syringe is indicated by an arrow. **(B)** After injection, the nodal bud is positioned between the outer ring (the cathode) and the center post (the anode), and subjected to electric pulses. For clarity, no electroporation medium was present in the chamber.



shoot that grew out of the electroporated bud. Also, we observed GUS-positive petals, anthers, and pollen on shoots bearing leaves that did not stain positive for GUS. These observations show that transgene expression can be detected up to 3 wk after electroporation into an intact bud and that patterns of expression vary considerably, possibly as the result of the timing of DNA-integrative events. Similar observations were made in cowpea, lentil, and soybean electroporated at 100 V, with Lipofectin™-DNA complex. In cowpea, 40 electroporated meristems gave rise to six GUS+ R_0 individuals; in lentil, 100 electroporated meristems generated 18 GUS+ R_0 individuals, whereas in soybean, three GUS+ Ro individuals were obtained from 40 electroporated plants. No staining was observed in samples from the 20 control plants that were maintained for each of the crops mentioned.

3.3. GUS Assay on Floral Parts of R_0 Pea Plants

In one series of experiments, 119 pea nodal buds were injected with plasmid DNA in the presence of protectants as indicated in Table 1, electroporated, and allowed to grow until the flowering stage. Staining for GUS activity was performed on floral tissues only. Eighteen of these plants were found to contain floral parts that stained positive for GUS. None of the 40 control plants (injected with complexed plasmid DNA, but not electroporated) showed any staining. As in the previous experiment, staining was sectorial as seen in the case of a fragment of petal (Fig. 3B and C). Stained anthers were found in flowers of three R_0 plants that contained a fraction of stained pollen (Fig. 3D). Such an observation is encouraging, since it suggests that a transgene may have been present in pollen mother cells at meiosis. Of course, at this stage, our results cannot exclude transfer of transcription or translation products from cell to cell during development.

3.4. Nature of the Observed Staining

Experiments involving hundreds of plants, such as the ones described here, cannot be performed easily with axenically grown and manipulated material. In fact, many plants used in this study were grown in a greenhouse and surgically manipulated under nonsterile conditions. Therefore, it is possible that some of the observed staining may have been owing to bacterial contamination. Indeed, the *uid*A chimeric reporter gene used here is of prokaryotic origin and could be found in ordinary commensals of our plants, hence giving false-positive results. Two types of experiments were done to investigate the possibility that contaminants could account for the observed results. First, cultures of *Escherichia coli* DH5α (a strain commonly used in our laboratory) were shown as expected to strongly express GUS as judged by the standard X-glu assay. Such cultures were used to contaminate pieces of pea tissues deliberately for 24 h, which were then used in a GUS assay as conducted with our ordinary experimental material. Abundant formation of an amorphous blue precipitate was detected in the buffer, but no blue material was found intracellularly in the plant samples. These crystals could further be totally eliminated from the surface of the plant material with a single ethanol wash, a procedure routinely followed in this work. It should be noted from Fig. 3 that the blue crystals in our pea tissue samples are strictly confined by cell walls and are thus not bound to the cell surface. In addition, cell layers away from the surface were seen to contain similarly confined blue crystals simply by changing the focus of the microscope. Further, pieces of pea plants grown and manipulated as above were incubated overnight in LB medium or MS salts, both supplemented with 3% glucose at 25 and 37°C. Growth was observed in all cases, indicating the presence of microscopic contaminants, but none of the pelleted cultures

Fig. 3. *(opposite page)* GUS activity in different organs developing from buds injected and electroporated with pGPT1.0 DNA *in planta*. Staining was done 3 wk after treatment. **(A)** Blue crystals present in the cells of a stem originating from an electroporated bud. Magnification 100×. **(B)** Piece of petal staining positive for GUS. Magnification 10×. **(C)** Blue crystals in petal cells. Magnification 100×. **(D)** Pollen grain (arrow) staining positive for GUS. Magnification 600×.

stained positive for GUS. Therefore, it is unlikely that surface contaminants accounted for the GUS-positive sectors seen in our DNA-treated specimens. The possibility still exists, however, that highly localized endosymbionts could account for the observed effects. However, this possibility is remote, since 203 control pea plants, maintained in our pilot experiments, were never seen to stain for GUS. These controls were composed of 127 individuals not injected with DNA and not electroporated, 40 injected with pGPT1.0 DNA, but not electroporated, 21 injected with pUC8 DNA and electroporated, and 15 injected with MS salts and electroporated. Similarly, control cow pea, lentil and soybean shoots did not stain for GUS in the respective experiments.

Our results strongly suggest that chimeric shoots were obtained by in vivo injection and electroporation of intact nodal buds. That DNA can penetrate intact plant tissues and cells is in agreement with Lindsey and Jones *(20,21),* Dekeyser et al. *(5),* and Arencibia et al. *(10),* who showed that DNA can penetrate suspension cells of *Beta vulgaris,* small pieces of leaf tissues, and sugarcane suspension cells, respectively. Similarly, our results are in agreement with studies demonstrating DNA uptake and expression in intact mature and immature embryonic tissues *(4,6–9).*

3.5. GUS Expression in R_1 and R_2 Plants Obtained from Chimeric R_0 Individuals

3.5.1. Pea

In one series of experiments, seeds were obtained from 300 electroporated R_0 plants. One to five seeds were obtained from each treated plant, with an average of 2.8 seeds/plant. All R_1 progeny were raised axenically (thus minimizing the possibility that staining would be the result of contamination), and leaflets from each plant were stained with X-glu. Not all progeny from any given R_0 plant stained positive for GUS. Success in the stable transformation of an R_0 plant was counted when at least one positively staining R_1 plant was obtained from its seeds. Table 2 summarizes our results obtained with various treatments involving different combinations of electroporation conditions. The level of GUS expression in transgenic R_1 progeny was low compared to that in the R_0 generation, but intracellular crystals were clearly visible under the light microscope in all cells present in the examined samples. Also, R_1 progeny displayed a staining pattern different from that of R_0 plants. The latter showed blue foci (*see* Fig. 3B) with no evidence of intracellular blue crystals in the regions located outside the staining areas. Leaf tissue from R_1 plants, however, had blue crystals in all cells examined, but staining intensity varied macroscopically, indicating uneven expression of GUS in this tissue *(also see 12).* Similar observations were made by Jefferson et al. *(22)* and Stefanov et al. *(23).* The reasons for generally lower levels of GUS expression in transgenic R_1 plants are not clearly understood. Multiple integrations in the genome or sites of integration may play a role. GUS-positive R_0 plants showed relatively higher levels of GUS activity, although in small patches of cells, owing to their chimeric nature.

In another series of experiments, 120 pea plants were electroporated at 100 V with a DNA–Lipofectin™ complex and yielded a total of 17 chimeric shoots. The R_1 progeny of 14 of those plants were studied for GUS expression. Twenty GUS^+ individuals of 75 R_1 individuals were recovered DNA was extracted from 10 of those GUS^+ plants and analyzed by Southern hybridization. Owing to the small number of seeds obtained in experiments with peas, no attempts were made to study GUS expression in R_2.

3.5.2. Lentil

One hundred electroporated plants generated 18 GUS^+ chimeric R_0 shoots. The R_1 progeny of 10 of these plants were further analyzed for GUS expression. Results showed that 22 of 88 individuals were GUS-positive. One hundred and five R_2 individuals were obtained by selfing R_1 progeny of two of the R_0 lines (consisting of five GUS^+ plants from line #121 and four GUS^+ individuals from line 119, respectively). Three R_2 lines were analyzed for GUS expression and showed the presence of 4 GUS^+ individuals out of 34 plants (#121 L), 1 GUS^+/36 (#121 J), and 9 GUS^+/35 (#119 F). Fourteen GUS^+ R_2 plants were thus observed. This overall 14:91 ratio indicates a strong bias against

Table 2
Expression of GUS in the R_1 Generation of Electroporated Parents (Pea Plants)

DNA concentration, µg/mL	DNA protectant	Volts	Pulse, ms	Fraction of GUS^+ R_1
300 CCC	Spermine	None	None	0/80
300 CCC	Spermine	200	50	2/84
300 CCC	Spermine	200	99	13/52
300 CCC	Lipofectin™	200	99	29/81
100 CCC	Spermine	200	99	5/40
100 L	Spermine	200	99	6/43
Total				56/380

CCC: Supercoiled pGPT1.0 DNA.
L: pGPT1.0 DNA linearized with *Sac*I.
GUS expression was tested in leaf fragments.

either the presence or expression *uid*A in these R_2 plants. Several investigations of non-Mendelian inheritance of transgenic phenotype at the molecular level have indicated that loss of expression does not always correlate with the loss of the transgene, but rather with its inactivation *(24–28)*. We have indeed observed such an inactivation of integrated transgene in our experiments with pea transformation (*discussed* in Section 3.6.). Genomic DNA was extracted from two GUS-positive R_1 lentil plants (line 32C and line 119F) for Southern hybridization (*see* Section 3.6.).

3.5.3. Cowpea

Six GUS^+ chimeric shoots were observed on 40 electroporated individuals. R_1 progeny from three of these plants were analyzed for GUS expression with the following results: line C10 gave 2 GUS^+ progeny out of 27; line C15, 6 GUS^+ out of 23, and line C35, 1 GUS^+ out of 21 for a grand total, of 9 GUS^+ R_1 plants out of 52. GUS activity was not studied in R_2 plants. DNA was extracted from one GUS^+ individual from lines C10 and C15 each and used for Southern hybridization (*see* Section 3.6.).

3.5.4. Soybean

Forty R_0 soybean plants were electroporated, resulting in three individuals carrying chimeric GUS^+ shoots. R_1 progeny from those individuals were not analyzed, but were allowed to self-fertilize and produce an R_2 generation consisting of 56 individuals. Of those, 30 stained positive for GUS.

Table 3
Fraction of Individual Plants Found to Stain for GUS in Successive Generations[a]

	R_0	R_1	R_2
Pea	17/120	20/75	ND
Cowpea	6/40	9/52	ND
Lentil	18/100	22/88	14/91
Soybean	3/40	ND[b]	30/56

[a]All R_0 individuals were chimeric. All axillary buds were electroporated at 100 V with a covalently closed circular plasmid DNA-Lipofectin™ complex.
[b]ND: not done.

This 30:26 overall ratio is much higher than that observed in the case of lentil, but is still quite far from the 3:1 ratio expected if the R_1 plants are hemizygous for *uid*A. Here again, there is strong bias against the presence or expression of the transgene in the R_2 population. DNA was isolated for analysis from two R_2 individuals originating from line S_5B and four R_2 individuals originating from line S_2B. Line S_2B gave 11 GUS^+ plants out of 16. This particular line thus gave a 2.2:1 R_2 ratio.

Table 3 gives a grand summary of our numerical results obtained with plants electroporated at 100 V with a DNA–Lipofectin™ complex.

3.6. Presence of uidA in R_1 and R_2 Plants

The presence of the *uid*A sequence in R_1 pea, lentil, and cowpea plants and in R_2 soybean plants was investigated by Southern blot analysis. Figure 4A shows hybridization of the probe

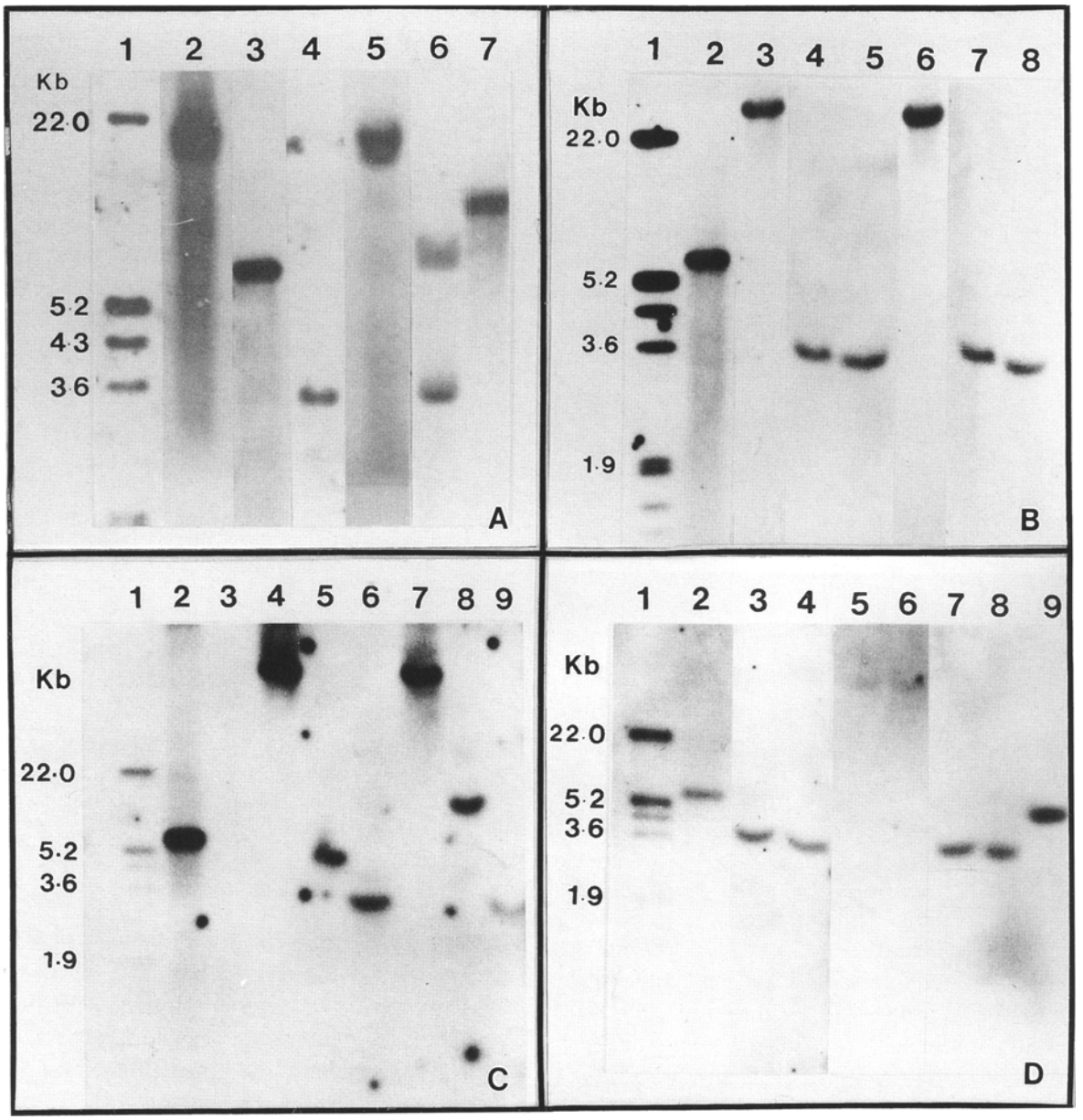


Fig. 4. Southern blot analysis of introduced chimeric gene in R_1 GUS-positive progeny of pea, lentil, cowpea, and R_2 GUS-positive progeny of soybean, transformed by *in planta* electroporation of nodal meristem. Genomic DNA was isolated from frozen leaf tissue, and 15 µg of restriction enzyme digested or undigested DNA were blotted onto positively charged Zeta-probe membrane. Southern hybridization was done with DIG-labeled *Ssp*I/*Sst*I fragment of pGPT1.0 (Fig. 1) as GUSINT probe. **(A)** Hybridization pattern in two independent transgenic pea R_1 plants (14C and 23H): lane 1, mol-wt marker; lanes 2 and 5, undigested genomic DNA; lanes 3 and 7, DNA digested with *Eco*RI (a single cut giving rise to junction fragments); lanes 4 and 6, DNA double digested with *Eco*RI/*Hin*dIII (releasing the intact 3.5-kbp chimeric transgene). Digested and undigested genomic DNA from untransformed control plants did not show any hybridization signal with GUSINT probe (data not shown). **(B)** Southern hybridization pattern in, lentil R_1 plants (two independent transgenic lines, 32C and 119F): lane 1, mol-wt marker; lane 2, linearized pGPT1.0 DNA (30 pg), used as a positive control; lanes 3 and 6, undigested DNA; lanes 4 and 7, genomic DNA digested with *Eco*RI (giving rise to a junction fragment of 3.6 kbp in both the transgenic lines); lanes 5 and 8, double-digested genomic

to unrestricted DNA isolated from leaves of two GUS^+ R_1 pea plants (lanes 2 and 5). This figure further indicates that genomic DNA from these plants when digested with *Eco*RI (which created a single cut in the plasmid pGPT1.0) showed a single band of 6.1 kbp (lane 3) or 8.0 kbp (lane 7). Double digestion of the genomic DNA with *Eco*RI and *Hin*dIII (lanes 4 and 6) yielded in both cases a fragment of 3.5 kbp, consistent with the cassette including the chimeric *uid*A gene as present in the vector. The 6.5-kbp band seen in lane 6 was probably the result of partial digestion of the genomic DNA. Together, these results demonstrate integration and expression of the reporter gene in these plants. Interestingly, as mentioned before, one GUS-negative R_1 pea plant was shown to contain an intact *uid*A cassette (data not shown), indicating the presence of a silenced transgene.

Similarly, R_1 lentil and cowpea DNA revealed hybridization signals for the high-mol-wt, hybrid fragments, and intact *uid*A gene, when digested with the appropriate restriction enzymes and probed. In lentils, the two independent transgenic lines (32C and 119F) tested exhibited very similar restriction enzyme digestion patterns (Fig. 4B). The size of the hybrid fragments (hybrid between the lentil genomic DNA and the chimeric *uid*A gene), when cut with *Eco*RI was 3.6 kbp in both the transgenic lines (lanes 4 and 7). Double digestion with *Eco*RI and *Hin*dIII released the intact *uid*A gene in both lines (lanes 5 and 8). Integration into lentil genome was further demonstrated when the probe hybridized to the undigested genomic DNA at high molecular weight (lanes 3 and 6). In the case of cowpea (Fig. 4C), unrestricted genomic DNA from two independent transgenic lines showed hybridization signal at high molecular weight (lanes 4 and 7). A single enzyme digestion with *Eco*RI yielded hybrid fragments of sizes 5.1 kbp (lane 5) and 6.3 kbp (lane 8). The intact *uid*A gene was released when double digested with *Eco*RI and *Hin*dIII (lanes 6 and 9).

Genomic Southern analysis in the case of soybean was performed on DNA samples from R_2 progeny. Transgene integration in the soybean genome is shown in Fig. 4D. Interestingly, the restriction pattern of GUS^+ soybean genomic DNA when digested with *Eco*RI and probed was very similar to that seen in lentil R_1 genomic DNA. Both the independent transgenic lines in soybean (lanes 3 and 7) and in lentils (Fig. 4B, lanes 4 and 7) yielded a hybrid fragment of size 3.6 kbp. When digested with *Hin*dIII (which also created a single cut in the plasmid pGPT1.0), soybean genomic DNA yielded hybrid fragments of different sizes (5.0 kbp in case of S_2B_2, lane 9 in Fig 4A and 16 kbp in case of S_5B_3, data not shown). The complete *uid*A gene (3.5 kbp) was released when double-digested with *Eco*RI and *Hin*dIII (lanes 4 and 8). Hybridization signal at high-mol wt in unrestricted genomic DNA is shown in lanes 5 and 6. The reason why independent transgenic lines in lentil and soybean produced very similar hybrid fragments when digested with

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DNA with *Eco*RI/*Hin*dIII (giving rise to intact 3.5-kbp *uid*A chimeric gene). GUSINT probe did not hybridize to DNA from untransformed control plants (data not shown). **(C)** Hybridization pattern in two cowpea transgenic R_1 lines, C10-2 and C15-21: lane 1, mol-wt marker; lane 2, linearized pGPT1.0 DNA (30 pg), used as a positive control; lane 3, genomic DNA from untransformed control plant digested with *Eco*RI; lanes 4 and 7, undigested genomic DNA from 10-2 and C15-21, respectively; lanes 5 and 8, genomic DNA digested with *Eco*RI (resulting in junction fragments of different sizes); lanes 6 and 9, genomic DNA double digested with *Eco*RI/*Hin*dIII (releasing the intact 3.5 kbp chimeric *uid*A gene). **(D)** Hybridization signal in two independent transgenic R_2 soybean lines S_2B_2 and S_5B_3: lane 1, mol-wt marker; lane 2, linearized pGPT1.0 DNA (10 pg), used as a positive control; lanes 3 and 7, genomic DNA digested with *Eco*RI (junction fragment of 3.6 kbp hybridized to the probe in both the transgenic lines); lanes 4 and 8, genomic DNA double digested with *Eco*RI/*Hin*dIII (the probe hybridized to the intact *uid*A chimeric gene of 3.5 kbp); lanes 5 and 6, undigested genomic DNA (hybridization signal seen at high-mol wt); lane 9, genomic DNA from line S_2B_2 digested with *Hin*dIII (probe hybridized to a junction fragment of 5.0 kbp). Digestion of S_5B_3 DNA with *Hin*dIII resulted in a junction fragment of 16 kbp (data not shown). Untransformed control DNA did not show any hybridization signal (not shown).

*Eco*RI is unknown. However, the same observation was made in two independent transgenic barley lines obtained by macroinjecting an *npt*II-carrying vector into the inflorescences. Two kanamycin-resistant F2 plants originating from independent primary transformants did display the same restriction pattern in Southern blots when the probe was an internal fragment of the *npt*II gene *(29)*.

Estimation of transgene copy number was done by Southern hybridization with *Eco*RI-*Hin*dIII-digested DNA from transgenic lentil 119F. DNA from untransformed lentil was supplemented with the equivalent of 1, 2, 3, and 4 copies of the *uid*A gene (10, 20, 30, and 40 pg/10 µg lentil DNA, respectively). Band intensities indicated the presence of 2 copies of the *uid*A cassette/genome (not shown). Copy number experiments were not done with the other crops, but Southern blots in Fig.4 suggest integration of 1–3 copies for the *uid*A transgene.

4. Conclusions

The technique described in this article shows that cells present in the apical dome of grain legumes can take up and express a *uid*A reporter gene in growing tissues after macroinjection and electroporation *in planta*. Furthermore, the presence of GUS-expressing individuals containing an integrated reporter gene among the R_1 and R_2 offspring shows that expression is not necessarily of the transient type. Thus, our procedure constitutes an attractive alternative to *Agrobacterium*-mediated gene transfer in legumes and requires no tissue-culture step whatsoever, often a major stumbling block in these plants.

The transformation frequencies based on R_1 and R_2 GUS-expressing plants, even though quite variable from experiment to experiment, were high enough to allow detection of transgenics without the need for a selectable marker. Based on this, we have applied our protocol toward the production of pea plants tolerant to pea enation mosaic virus through coat protein-mediated tolerance or resistance. Such plants were obtained and were shown to contain the pea enation mosaic virus coat protein gene by Southern hybridization, PCR, and Southern-PCR. Furthermore, coat protein gene expression was demonstrated by Western blot experiments, whereas ELISA tests on challenged plants showed much reduced virus titers in tolerant transgenic plants (Chowrira let al., in preparation).

Further work will be necessary to investigate the nature of the non-Mendelian R_2 segregation ratios we observed. Unusual ratios have been documented in a large number of cases involving many different plant species *(30–32)*. This could be accounted for by the loss or the inactivation of the transgene, and this topic has been extensively reviewed by Finnegan and McElroy *(33)*. However, we have not yet determined whether R_2 plants not expressing *uid*A still harbor it. Also, it will be important to determine whether or not transgenic plants obtained through our protocol continue to segregate the transgene in subsequent generations.

In conclusion, our findings further demonstrate the usefulness of tissue electroporation in the production of transgenic plants. To our knowledge, this is the first article in which axillary buds were used as targets for DNA delivery, although transgenic rice *(34)* and maize *(35)* plants were previously obtained after electroporation of embryos.

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References

1. Fromm, M. E., Taylor, L. P., and Walbot, V. (1986) Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proc. Natl. Acad. Sci. USA* **82,** 5824–5828.
2. Fraley, R. T. (1989) Genetic engineering for crop improvement, in *Plant Biotechnology* (Kung, S. D. and Arntzen, C. J., eds.), Butterworth, pp. 395–407.
3. Potrykus, I. (1991) Gene transfer to plants: assessment of published approaches and results. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42,** 205–225.

4. Akella, V. and Lurquin, P. F. (1992) Expression in cowpea seedlings of chimeric transgenes after electroporation into seed-derived embryos. *Plant Cell Rep.* **12,** 110–117.
5. Dekeyser, R. A., Claes, B., De Rycke, R. M. U., Habets, M. E., Van Montagu, M. C., and Caplan, A. B. (1990) Transient gene expression in intact and organized rice tissues. *The Plant Cell* **2,** 591–602.
6. Klöti, A., Iglesias, V. A., Wünn, J., Burkhardt, P. K., Datta, S. K., and Potrykus, I. (1993) Gene transfer by electroporation into intact scutellum cells of wheat embryos. *Plant Cell Rep.* **12,** 671–675.
7. Senaratna, T., McKersie, B. D., Kasha, K. J., and Procunier, J. D. (1991) Direct DNA uptake during imbibition of dry cells. *Plant Sci.* **79,** 223–228.
8. Songstad D. D., Halaka, F. G., DeBoer, D. L., Armstrong, C. L., Hinchee, M. A. W., Ford-Santino, C. G., Brown, S. M., Fromm, M. E., and Horsch, R. B. (1993) Transient expression of GUS and anthocyanin constructs in intact maize immature embryos following electroporation. *Plant Cell, Tissue and Organ Culture* **38,** 195–201.
9. Chaudhury, A., Maheshwari, S. C., and Tyagi, A. K. (1995) Transient expression of gus gene in intact seed embryos of Indica rice after electroporation-mediated gene delivery. *Plant Cell Rep.* **14,** 215–220.
10. Arencibia, A., Molina, P. R., de la Riva, G., and Selman-Housein, G. (1995) Production of transgenic sugarcane (*Saccharum officinarum* L.) plants by intact cell electroporation. *Plant Cell Rep.* **14,** 305–309.
11. Songstad, D. D., Somers, D. A., and Griesbach, R. J. (1995) Advances in alternative DNA delivery techniques. *Plant Cell, Tissue and Organ Culture* **40,** 1–15.
12. Chowrira, G. M., Akella, V., and Lurquin, P. F. (1995) Electroporation-mediated gene transfer into intact nodal meristems in planta: generating transgenic plants without in vitro tissue culture. *Mol. Biotechnol.* **3,** 17–23.
13. Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L., and Rocha-Soza, M. (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated transformation. *Mol. Gen. Genet.* **220,** 245–250.
14. Penza, R., Lurquin, P. F., and Filippone, E. (1991) Gene transfer by cocultivation of mature embryos with *Agrobacterium tumefaciens*: application to cow pea (*Vigna unguiculata* Walp.). *J. Plant Physiol.* **138,** 39–43.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
16. Gendimenico, G. J., Bouquin, P. L., and Tramposch, K. M. (1988) Diphenylamine-colorimetric method for DNA assay: a shortened procedure by incubating samples at 50°C. *Anal. Biochem.* **173,** 45–48.
17. Jefferson, R. A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5,** 387–405.
18. Doyle, J. J. and Doyle, J. L. (1990) Isolation of plant DNA from fresh tissue. *Focus* **12(1),** 13–15.
19. Maniatis, T., Fritsch, E. F., and Sambrok, J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20. Lindsey, K. and Jones, M. G. K. (1987) The permeability of electroporated cells and protoplasts of sugar beet. *Planta* **172,** 346–355.
21. Lindsey, K. and Jones, M. G. K. (1987) Transient gene expression in electroporated protoplasts and intact cells of sugar beet. *Plant Mol. Biol.* **10,** 43–52.
22. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO J.* **6(13),** 3901–3907.
23. Stefanov, I., Fakote, S., Bogre, L., Pauk, J., Feher, A., and Dudits, D. (1994) Differential activity of the mannopine synthase and the CaMV 35S promoters during development of transgenic rapeseed plants. *Plant Sci.* **95,** 175–186.
24. Scheid, O. M., Paszkowski, J., and Potrykus, I. (1991) Reversible inactivation of a transgene in *Arabidopsis thaliana. Mol. Gen. Genet.* **228,** 104–112.
25. Matzke, M. A., Primig, M., Trnovsky, J., and Matzke, A. J. M. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *The EMBO J.* **8,** 643–649.
26. Meyer, P., Linn, F., Heidmann, I., Meyer, H., Niedenhof, I., and Saedler, H. (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize AI gene construct in transgenic petunia and its colour phenotype. *Mol. Gen. Genet.* **231,** 345–352.
27. Meyer, P. and Heidmann, I. (1994) Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. *Mol. Gen. Genet.* **243,** 390–399.
28. Renckens, S., de Freve, H., van Montague, M., and Hernalsteens, J.-P. (1992) Petunia plants escape from negative selection against a transgene by silencing foreign DNA via methylation. *Mol. Gen. Genet.* **233,** 53–64.
29. Mendel, R. R., Clauss, E., Hellmund, R., Schulze, J., Steinbib, H. H., and Tewes, A. (1990) Gene transfer to barley, in *Progress in Plant Cellular and Molecular Biology.* Proceedings of the VIIth International congress on Plant Tissue and Cell Culture, Amsterdam, The Netherlands (Nijkamp, H. J. J., Van Der Plas, L. H. W., and Van Aartrijk, J., eds.), Kluwer Academic Publishers, Dordrecht, p. 73.
30. Peng, J., Wen, F., Lister, R. L., and Hodges, T. K. (1995) Inheritance of *gus*A and *neo* genes in transgenic rice. *Plant Mol. Biol.* **27,** 91–104.

31. Goto, F., Toki, S., and Uchimiya, H. (1993) Inheritance of a co-transferred foreign gene in the progenies of transgenic rice plants. *Transgenic Res.* **2,** 302–305.
32. Deroles, S. C. and Gardner, R. C. (1988a) Expression and inheritance of kanamycin resistance in a large number of transgenic petunias generated *by Agrobacterium*-mediated transformation. *Plant Mol. Biol.* **11,** 355–364.
33. Finnegan, J. and McElroy, D. (1994) Transgene inactivation: plants fight back! *Bio/Tech.* **12,** 883–888.
34. Li, B. J., Xu, X. P., Shi, H. P., and Ki, X. Y. (1991) Introduction of foreign genes into the seed embryo cells of rice by electro-injection and the regeneration of transgenic rice plants. *Sci. China* **34,** 923–930.
35. D'Halluin, K., Borne, E., Bossut, M., De Beuckeleer, M., and Leemans, J. (1992) Transgenic maize plants by tissue electroporation. *Plant Cell* **4,** 1595–1505.

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