Full Length Research Paper

Stable gene transformation in cowpea (*Vigna unguiculata* L. walp.) using particle gun method

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We investigated the possibility of transforming and obtaining transgenic cowpea (*Vigna unguiculata* L Walp) plants using the particle bombardment process. Meristematic explants that could give rise to whole fertile plants were used in transformation experiments with reporter and selectable marker genes driven by a 35S CaMV promoter. Conditions for optimal delivery of DNA to explants were established based on transient *gus* expression assays two days after bombardment. The size of microcarriers, microflight distance and helium pressure significantly affected transient expression of reporter genes. A total of 1692 explants were bombarded with DNA-coated particles and placed on 3 mg/l bialaphos selective medium. Only 12 regenerated shoots produced seeds eventually, and all were Gus negative even though 7 gave positive PCR signals with the *bar* primer. Eight out of 1400 seeds from T_o plants were GUS positive. DNA from eight of the GUS positive seedlings were amplified with both the *gus* and *bar* primers in PCR analysis but only two gave a positive Southern signal. Only two of the 3557 T_2 seedlings obtained were GUS positive. However, 3 seedlings survived Basta spray. The two GUS positive and 3 Basta surviving seedlings gave positive Southern hybridisation signals. Twelve T_3 seedlings from these were GUS positive and also gave positive Southern hybridisation signals. The positive reaction of T_1, T_2 and T_3 seedlings under Southern analysis confirms the stable integration of introduced genes and the transfer of such genes to progenies. However, the level of expression of introduced genes in cowpea cells is very low and this accounted for the high mortality rate of progenies under Basta spray.

Key words: Transformation, particle bombardment, gus assay, transient expression, reporter gene, basta, bar gene.

INTRODUCTION

Transformation of many plant species has become routine, permitting the introduction of characteristics that would not be possible by conventional breeding. Most legume transformation studies have used *Agrobacterium tumefaciens* to generate for example transgenic soya bean (Hinchee et al., 1994), chickpeas (Fontana et al., 1993), peas (Puouti-Kaerlas et al., 1990; Schroeder et al., 1993; Grant et al., 1995) and cowpea (Muthukumar et al., 1996; Kononowicz et al., 1993, 1997). Particle bombardment has also been used to produce transgenic soybean (McCabe et al., 1988), beans (Russell et al., 1993), peanut (Brar et al., 1994) and alfalfa (Pereira and Erickson, 1995). Stable integration and expression of transgenes in progeny of cowpeas using particle bombardment has not been reported. Various explants of *V. unguiculata* have been used in experiments with *Agrobacterium* or direct DNA transfer methods but the main obstacle has been difficulties in regeneration. Mature embryos (Penza et al., 1991; Akella and Lurquin 1993), leaf discs (Garcia et al., 1986, 1987) and cotyledons (Muthukumar et al., 1996; Kononowicz, et al., 1997) have been used. Neither Garcia and co-workers (1986, 1987) nor Penza and co-workers (1991) were able to recover plants from transformed tissues.

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Transformation of cotyledon explants of cowpea was performed using *Agrobacterium* (Muthukumar et al., 1996). Mature de-embryonated cotyledons with intact proximal ends were inoculated with *Agrobacterium tumefaciens* and cultured on a medium supplemented with 6-benzylaminopurine (BAP). Regenerated shoots selected on hygromycin were confirmed to be transgenic by Southern blot hybridisation. Chowrira et al., (1995) also reported transient expression, stable integration and expression of transgenes in the tissues and progeny of leguminous plants (including cowpea), after electroporation of DNA into intact nodal meristems *in planta*. Kononowicz et al., (1993, 1995, 1997), showed the presence of introduced genes in T_0 cowpea plants using particle bombardment. They were not able to obtain transgenic progeny. In this paper we describe a protocol for stable transformation of cowpea via particle bombardment, using the selectable marker *bar* (phosphinothricin acetyltransferase).

MATERIALS AND METHODS

Plant Material

IT83D-442, an elite cowpea cultivar selected at the International Institute of Tropical Agriculture (IITA) was used. Seeds were surface sterilised with 70% ethanol for 3 min, soaked in 20% sodium hypochlorite containing a few drops of Tween 20 for 30 min, and then rinsed three times with sterile distilled water. Seeds were left overnight in sterile distilled water and again surface disinfected with 20% sodium hypochlorite and Tween 20 for 30 min. After rinsing with sterile distilled water, the embryos were removed aseptically. Detached embryos were used as target tissues for bombardment. Isolated embryonic axes were cultured on Cp3 (Kononowicz et al., 1995), a shoot regeneration and elongation medium, for 24-48 hr at 26 °C in the dark before bombardment with DNA-coated gold particles. The pH of all media used in this study was adjusted to 5.8 prior to autoclaving.

Plasmid DNA

For microprojectile bombardment, the plasmid ptjk 142 was used. The ptjk 142 plasmid contains the *uid A* reporter gene (Jefferson et al., 1987) encoding β-glucuronidase (GUS) and a selectable marker gene *bar* (De Block et al., 1987) that encodes phosphinothricin acetyltransferase (PAT), both driven by the CaMV 35S promoter. The modifying enzyme phosphinothricin acetyltransferase confers resistance to bialaphos (Shinyo Sangyo, Tokyo, Japan), as well as to the related compounds phosphinothricin (PPT) and glufosinate ammonium. To optimise bombardment parameters, the plasmid pDPG 208 – carrying the *uid A* gene under control of the CaMV 35S promoter was also used.

Microcarrier coating with DNA

Plasmid DNA was adsorbed on gold microcarriers (1.0 μm in diameter, Bio-Rad) as described by Bio-Rad (Hertfordshire, UK). Thirty-five microlitre aliquots containing 2.0 mg of gold particles prewashed in ethanol were mixed with 25 μl of DNA (1 μg/μl), 250 μl CaCl$_2$ (2.5 M), 50 μl spermidine (0.1 M), and 220 μl de-ionized water in 1.5 ml microcentrifuge tube by vortexing. The resulting suspension was pulse centrifuged and the supernatant discarded. Particles were resuspended in 36 μl absolute ethanol by vortexing and briefly dipped in a water sonicator for 3 sec. 10 μl of the DNA-coated microcarrier suspension was used for each bombardment.

Particle bombardment of Primary Explants

Thirty embryonic axes maintained on Cp3 medium for 1 or 2 days after detachment were arranged in a circle of about 3 cm diameter in the centre of agar petri plates. Bombardment was carried out with a Biolistic PDS-100/He particle delivery system (Bio-Rad). All bombardments were performed at a pressure of 1100 psi, a 6 cm particle travel distance, an 11 mm gap distance (between the rupture disk and the macrocarrier), an 11 mm macrocarrier flying distance and a vacuum of 28 Hg inch.

Following bombardment, the explants were cultured for 2 weeks in the dark at 26°C on Cp3 medium for shoot proliferation and elongation. Explants were subsquently plated on rooting medium Cp4 (Knonowicz et al., 1995) in light (12 hr photoperiod, 20-25 μmolm^{-2}s^{-1} cool white fluorescent illumination). Developing shoots were recultured on fresh Cp4 medium at 2 weeks intervals. Shoot regeneration was conducted under continuous selection pressure (3 mg/L Bialaphos). Shoots that survived on selection medium for 3 weeks were transferred to soil after conditioning on peat-pellets in a temperature controlled environment. Plants were grown subsequently in the screen house until pod maturity.

Analysis of *gus* expression

Transient expression of the gus A gene was tested by histochemical staining of the tissues 48 hr after bombardment using X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase) as described by Jefferson et al (1987). Tissues were immersed in the gus A assay solution and incubated overnight at 37°C. Explants were decolourized with 2-3 rinses of absolute ethanol before counting blue spots under a low magnification laboratory microscope. The number of individual cells or cell aggregates that contained a blue precipitate was counted as expression units.

Herbicide screening and application

Seedlings were sprayed with 0.125-0.2% Basta (Hoechst, Brussels, Belgium) using a Hills Master Sprayer (Hills Industries, Caerphilly, UK) seven days after planting. In all cases, non-transformed controls died within 3-4 days after spraying. Survivors were scored after a week and subsequently resprayed. Only those that survived two sprays were grown to maturity in the screenhouse.

Polymerase Chain Reaction (PCR)

Plants that regenerated following bombardment and selection were initially analysed by PCR. Two 25-mer primers which are homologous to the uid A gene (51-TTG CCC AGC TAT CTG TCA CTT CAC T-31 and 51-ATG TCA CAT CAA TCC ACT TGC TTT G-31) and two 24-mer primers homologous to the bar gene (51-GGG ACT TCA GCA GGT GGG TGT AGA-31 and 51-AAC CGC AGG AGT GGA CGG ACG ACC-31) were used. PCR amplifications of DNA were performed in 50μl volumes containing 100mM Tris-HCl (pH 9.0), 500mM KCl, 25mM MgCl2, 1% Triton X-100, 2.5 mM of each dNTP (Promega), 250ng of primers, 2U of DNA polymerase (Promega) and 500ng genomic DNA overlaid with 30μl mineral oil.

Amplifications were performed in a Perkin Elmer-Cetus DNA cycler 480. The amplification temperature cycle for the bar gene was as follows: Preheating at 94°C for 5 min followed by one cycle of 1 min each at 94°C, 60°C and 72°C and 34 cycles of 30 sec each at 94°C, 60°C and 72°C. For the *uid A* gene, there was a preheating at 94°C for 3 min and 1 cycle of 1 min each at 94°C, 55°C and 72°C and 34 cycles of 30 sec each at 94°C, 55°C and 72° C. The programmes ended at 4°C for cooling. Products were electrophoresed in 1.5% agarose gel and visualised under UV light by ethidium bromide staining.

Southern blot analysis

The modified CTAB DNA extraction procedure for *Musa* and *Ipomoea* (Gawel and Jarret, 1991) was adopted for genomic DNA isolation. Southern blotting and hybridization were performed as previously described (Southern, 1975; Sambrook et al., 1989). Genomic DNA (10 µg) was digested with *Hind* III, separated on 0.8% agarose gel, blotted onto nylon membrane (Hybond N, Amersham) and cross-linked to the filter with ultraviolet radiation. Non-radioactive labelling with digoxigenin-11-dUTP and hybridisation was carried out according to the manufacturer's manual (Boehringer Mannheim).
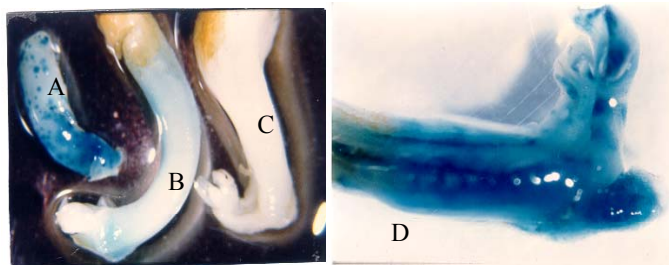

Figure 1. Positive gus reaction of bombarded mature embryos two days after bombardment. A, Bombarded with DNA coated particles; B, bombarded with particles not coated with DNA; C, Not bombarded; and D, Bombarded with DNA coated particles.

RESULTS AND DISCUSSION

Optimization of conditions for DNA delivery

To optimize DNA delivery, conditions for particle bombardment were established based on transient expression assay using histochemical techniques (Jefferson et al., 1987; Sanford et al., 1993). Reporter gene activity was evaluated by determining the expression of chimeric gene constructs (pDPG 208 and ptjk 142) in explants. These constructs consisted of the CaMV 35S promoter–*uid A* coding sequence. When subjected to GUS histochemical assay, cowpea explants exhibited characteristic blue cells typical of transient expression of the *uid A* gene two days after DNA delivery (Figure 1). The transformed cells appeared to be randomly distributed on the surface of explants. In some

cases, the entire embryonic explants were totally blue, and this could be as a result of diffusion of GUS reaction product to adjacent cells. Control explants that were not bombarded or those that were bombarded with microcarriers not coated with DNA did not exhibit any *uid A* expressing cells. Similar *uid A* expression patterns were observed for cowpea and sweet potato previously (Kononowicz et al., 1995; Prakash and Varadarajan, 1992). Based on the transient *uid A* expression, particle size, target distance, helium pressure, number of bombardments and vacuum significantly affected transient expression of reporter genes. These findings are very similar to those reported for cowpea and barley (Knonowicz et al., 1997; King and Kasha, 1994). The interaction between bombardment pressure and target distance showed that the higher the pressure, the longer the distance. Best results were obtained at pressure of 1100 psi, target distance of 6cm and 28 Hg in vacuum. Bombarding the explants two times increased the number of cells giving transient gene expression and this could be due to the fact that multiple bombardments allow better coverage of the target areas and compensate for misfires from faulty and poorly set rupture discs as was the case in rice (Wang et al., 1988). By using the same range of sizes reported for maize (Klein et al. (1988), gene transfer in wheat was shown to be most efficient with microprojectiles 1.2 µm in diameter. The results obtained in optimizing different parameters are in line with those that have been obtained for other crops. Bombardment conditions chosen were those at which 70% of shoot embryos showed more than 20 blue spots per embryos hit.

Tissue Culture and Plant Regeneration

Two media, Cp3 (shoot elongation) and Cp4 (rooting) (Kononowicz et al., 1995, 1997) were used to regenerate plants. Embryonic axes used as primary target explants were obtained by excising embryos from mature seeds. Bombarded explants were cultured for two or three weeks in the dark on Cp3 medium. The culture conditions induced multiple shoots within a month from which plants ready to transfer to soil were obtained within two months when cultured in the absence of selectable agents. Similar results have been reported (Knonowicz et al, 1997). Bialaphos (3 mg/L) was used to select for transformed shoots after bombardment. Bombarded explants surviving for three weeks on bialaphos medium (Figure 2) as in *Phaseolus vulgaris* transformation (Russel et al., 1993) were grown up to adult plants. This concentration was very effective and eliminated the selection of many escapes. Previously for both legumes and cereals, concentrations ranging from 1-5 mg/L bialaplos have been used (Kononowicz et al., 1997; Spencer et al., 1990; Gordon-Kamm et al., 1990; Russel
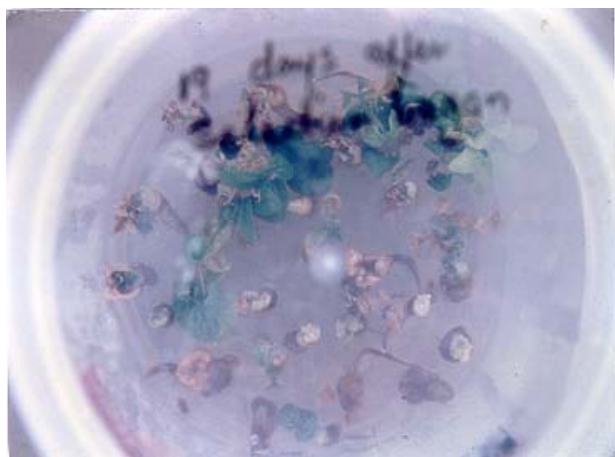
Figure 2. Surviving explants on bialaphos (3 mg/l) medium are greenish (19 days after selection was started). Control and untransformed shoots died within one week.

et al., 1993). Only 152 shoots survived out of a total 630 placed on the selection medium. The 152 shoots surviving selection accounts for 24% of those put on selection. Forty-eight shoots of those surviving selection (32%) were well rooted and conditioned for soil in jiffy peat pellets. At the end, twelve plants (8% of those selected) produced seeds. The rest were affected by fungal attacks. A similar case of low efficiency has been observed involving the kanamycin resistance gene in soybean (McCabe et al., 1988).

Molecular evaluation of T_0 plants

Even though the T_0 plants were all GUS negative, DNA from all 12 plants were amplified with both the *bar* and *uid A* primers (Table 1). Southern hybridisation of some of the PCR products with *bar* gene probe confirmed the identity of the amplified products (gel results not shown for T_0 plants). Similar confirmatory assays have been done for cowpea, banana and plantain (Kononowicz et al., 1997; Sagi et al., 1995). The amplification of DNA samples from GUS negative plants and the subsequent confirmation by Southern analysis proves that the plants were transformed with the exogenous DNA integrated into the genome. There are reports of *uid A* genes fused with selectable marker genes (*npt-II* or *bar*) not being expressed even though the selectable markers were expressed (Pereira and Erickson, 1995; Russel et al, 1993; Fitch et al., 1992; McCown et al., 1991).

Molecular evaluation of T_1 plants

Basta spraying, GUS assay, PCR and Southern analysis were used to evaluate all T_1 seedlings (Table 1). One
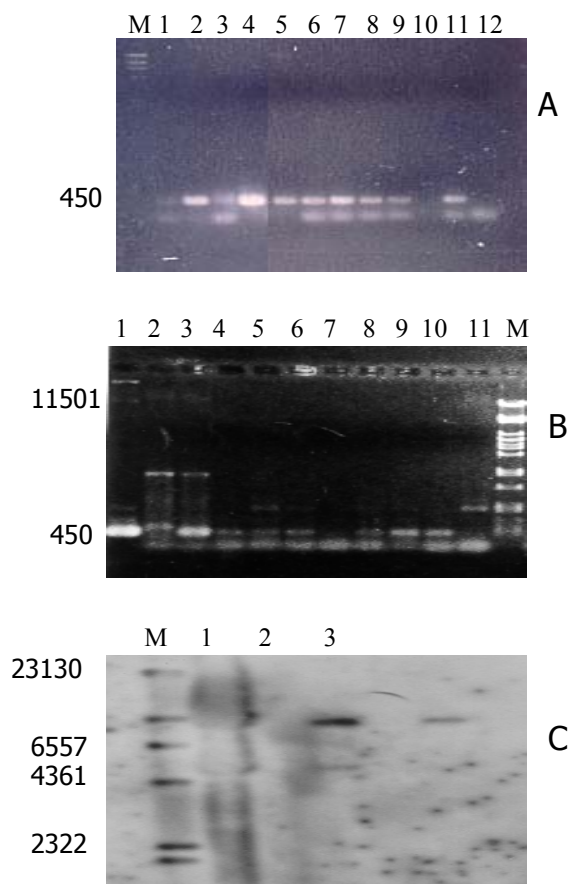

Figure 3. Molecular analysis of T1 plants. **A.** Lanes 5-12 show 450bp pcr products with the *bar* primer. Lanes 2 and 4 are ptjk142 plasmid and tobacco transformed with the bar gene respectively, while lanes 1 and 3 are negative controls pcr cocktail without DNA template and untransformed tobacco respectively. **B.** Lanes 4-11 show 450bp pcr products with the *gus* primer. Lanes 3 and 1 are ptjk142 plasmid and tobacco transformed with the *gus* gene respectively, while lane 2 is negative control pcr cocktail without DNA template. **C.** Southern analysis of T1 Plants. Lane 1 is ptjk142 plasmid and lanes 2 and 3 are transgenic plants. Genomic DNA from plant and plasmid were digested with Hind III.

thousand four hundred T_1 seedlings were raised from the twelve T_0 plants. Two hundred and thirty-nine of these were subjected to gus assay and only eight showed positive gus reaction. The GUS reactions were very strong with the midribs showing blueness. Using T_0 plants, the potential for germ-line transformation can be evaluated based on the observations of Christou and McCabe (1992). They observed that whenever any part of the vascular system of a soybean leaf produced from meristem bombardment expressed the reporter gene, it was possible to pass that gene to its progeny at very high frequency. Thus these plants have a very high chance of germline transformation. GUS positive plants were spared from Basta spray and only one seedling survived

Table 1. Molecular evaluation of T_0 plants and their T_1, T_2 and T_3 progenies.

	T_0 Plants				T_1 Plants				T_2 Plants				T_3 Plants			
	Total	Fraction assayed	+ve	% +ve	Total	Fraction assayed	+ve	% +ve	Total	Fraction assayed	+ve	% +ve	Total	Fraction assayed	+ve	% +ve
Gus Assay	12	12	None	0	1400	239	8[a]	4	3557	401	2[a]	0.5	598	598	12[a]	5
Basta spray (0.2%)	ND				1400	1392	1	0.07	3557	3555	3	0.08	598	586	0	0
PCR Bar Primer	12	12	12	100	9	8	8	100	ND				ND			
PCR-gus Primer	12	12	12	100	9	8	8	100	ND				ND			
Pcr/ Southern	12	7	7	100	1	1	1	100	ND				ND			
Southern	ND				2	2	2	100	5	5	5	100	12	12	12	100

a = Gus positive seedlings were not sprayed with basta
nd=not done

Figure 4. Three T_2 Plants surviving basta spray (A), and unsprayed control plants (B) and Sprayed control plants (C).

The spray out of a total of 1392 assayed. The high mortality rate of T_1, T_2 and T_3 plants under Basta spray does not necessarily indicate the non-integration of the bar gene or high application levels of the Basta. Plants that did not express the phosphinothricin-resistance gene in the leaf tissue but were shown to have the gene by Southern analysis have been reported for peas (Grant et al., 1995). The 0.2% of Basta applied is within the ranges reported for other crops even though different crop plants may have different responses to the herbicide (Christou et al., 1991; Gordon-Kamm et al., 1990). The most logical conclusion when plants which definitely carried the bar gene (clear PCRs and Southerns) react negatively on the leaf-painting assay or spraying with Basta will be that the level of expression is not enough to induce resistance against Basta.

DNA samples from eight GUS positive T_1 plants subjected to PCR analysis were all amplified with both the *bar* and *gus* primers. Southern analysis of genomic DNA samples from two of the plants confirmed the stable integration of the bar gene (Figure 3). In any method to produce transgenic seed plants, the critical aspect is whether or not the plants can pass the introduced traits to the progeny. The chimeric T_0 plants obtained were fertile and passed the traits to their progeny even though the level of expression of the bar gene seems to be very low.

Molecular evaluation of T_2 plants

Four hundred and one (401) T_2 seedlings out of a total of 3557 were subjected to GUS assay. Only two gave good positive reaction (Table 1). Seedlings that reacted negatively and all other T_2 seedlings totalling 3555 were sprayed with Basta (0.2%). Three seedlings survived the herbicide spray (Figure. 4). The five T_2 plants that either were *gus* positive or survived herbicide treatment were subjected to Southern analysis. Genomic DNA from all five hybridized with *bar* gene probe (data not shown).

Molecular Evaluation of T_3 plants

Five hundred and ninety-eight T_3 plants were evaluated by Gus assay and Basta spraying. Twelve of these were positive and were therefore not subjected to Basta spraying. All the negative ones were sprayed with Basta (0.2%) but none survived (Table 1). All twelve positive plants were subjected to Southern analysis. The bar gene fragment was detected in the genomic DNA extracted from all twelve plants (data not shown).

Conclusion

The positive reactions under PCR and Southern analyses of genomic DNA samples from T_1, T_2 and T_3 plants confirm the transfer of the *bar* gene upto the third generation. The *bar* gene was detected in 5 T_2 and 12 T_3 plants. Despite the low level expression of the *bar* gene under Basta spray, three T_2 plants were able to survive almost unaffected by Basta. Two T_2 and twelve T_3 plants were also gus positive. All T_2 and T_3 plants that were gus positive and resistant to Basta (0.2%) were also positive in Southern analysis using the *bar* probe. To obtain transformant T_1 progeny, the L_2 layer of the meristem is normally the target for the DNA coated particles (Christou, 1992; Christou et al., 1993), since this layer gives rise to the germline cells that produce sperm and egg (Sussex, 1989; Irish, 1991; Szymkowiak and Sussex, 1992). An earlier report on developing a transformation system for cowpea (Knonowicz et al., 1997), only confirmed the integration of the bar gene into the genome of T_0 plants. No transformed T_1 progeny was obtained from transgenic chimeras. Thus this work is the first report of generation of transgenic cowpea by particle bombardment with molecular evidence of stable integration of introduced gene in T_1, T_2 and T_3 progenies.

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