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Full Length Research Paper

Transgene expression in cowpea *(Vigna unguiculata* (L.) Walp.) through *Agrobacterium* transformation of pollen in flower buds

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Pollen transformation shows potential as a fast and easy means of obtaining transformed plants carrying desirable transgenes. *Agrobacterium tumefaciens* has been suggested as the best natural plant genetic engineering system. Laboratory and screenhouse studies were undertaken to investigate the possibility of obtaining transformed progeny in cowpea by *Agrobacterium*-mediated transformation of pollen. Flower buds of selected cowpea accessions were inoculated the evening before opening with a late log phase culture of *A. tumefaciens* strain pGV 2260 carrying the transgene vector ptjk 142. The vector is a disarmed Ti plasmid carrying a chimeric fusion of the *UidA* and *Bar genes* for β-glucuronidase expression and bialaphos resistance respectively. Seedlings resulting from inoculated flowers were screened for expression of the genes by β-glucuronidase (GUS) assay and Basta spraying. GUS positive plants were analyzed by polymerase chain reaction and Southern hybridization. Up to 90% of inoculated flowers and developing pods aborted. Pod set from inoculated flower buds ranged between 8.92 and 10.51% with no significant difference in pod set among accessions. Nine seedlings showed positive GUS expression. None was tolerant to Basta. Seven seedlings showing positive GUS expression also showed positive signals under PCR and Southern analysis giving indicative evidence of transgene presence. Overall transformation frequency was 0.36%.

Key words: *Agrobacterium tumefaciens*, β-glucuronidase, bialaphos resistance, cowpea, pollen transformation.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important grain legume crop in the tropics and subtropics. It is a cheap source of protein in the diets of millions of relatively poor people in less developed countries of the tropics. Cowpea has relatively high lysine content and is thus an excellent improver of cereal grains (Bressani, 1985).

Cowpea has a very high yield potential (1.5 to 3.0 t/ha), but actual yields on small scale farms in much of the developing world average 0.2 to 0.4 t/ha (Murdock, 1992). This is largely due to a combination of biotic factors (insect pests, diseases, nematodes and parasitic weeds) and abiotic factors such as drought and heat, all of which contribute to large yield deficits. Remarkable progress has been made in breeding resistant lines to a good number of these constraints. However, numerous strains and variants of some pests and disease organisms coupled with difficulties in obtaining successful hybrids in crosses between cultivated cowpea and wild *Vigna* species, constrain breeding programmes that attempt to incorporate resistance genes.

The use of recombinant DNA technology shows great potential in circumventing these breeding constraints. Pollen transformation also holds potential as a rapid and efficient method of incorporating foreign genetic material

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into plants. Pollen grains, due to their abundance and ease with which they can be handled are ideal targets for introduction of foreign genes into the germ line (Eapen, 2011). Transformed pollen when used in pollination can carry foreign genes into plants through the normal fertilezation process. However, progress in the introduction of transgenes into pollen grains and their subsequent use in fertilization leading to development of transgenic plants are limited.

Agrobacterium tumefaciens-mediated transfer has been suggested as the best natural plant genetic engineering system (Lurquin, 1987). Pollen and the stigma have been observed to contain *vir* inducing compounds which stimulate *Agrobacterium* infection (Zerback et al., 1989). *Agrobacterium* has been observed to attach in a polar manner to growing pollen tubes. This can facilitate transfer of the T-DNA portion of the Ti plasmid into the pollen tube and eventual transformation of the sperm nucleus (Zerback et al., 1989). Hess et al. (1990) reported the transfer of the kanamycin-resistance gene into wheat through pollen by the *Agrobacterium* inoculation of the spikelets. Kumlehn et al. (2006) also reported the successful genetic transformation of barley (*Hordeum vulgare* L.), based on infection of androgenetic pollen cultures with *A. tumefaciens*. This study was therefore aimed at developing a transformation system for cowpea based on *Agrobacterium*-mediated transformation of mature pollen.

MATERIALS AND METHODS

Twelve (12) cowpea accessions comprising 7 elite IITA cultivars and 5 landraces were used in the study. The accessions were selected mainly on the basis of flower pigmentation. This is to determine, in addition to genotype effects, the effects of flower pigmentation (if any) on pollen transformation. All plants were raised in a containment screenhouse to reduce as much as possible the likelihood of foreign gene escapes due to human error as well as interference from biotic agents. Seeds of each accession were sown in 8-inch diameter pots. Ten pots were sown per accession at a seed rate of four seeds per pot. Seedlings were thinned to two per pot two weeks after planting.

Culture of *A. tumefaciens*

The *A. tumefaciens* strain pGV2260 which carries the plasmid vector ptjk142 was used. This plasmid (Figure 1) carries a chimeric fusion of the *UidA* reporter gene (Jefferson et al., 1987) which encodes the production of β-glucuronidase (GUS) and the *Bar* selectable marker gene (De Block et al., 1987) which encodes the production of phosphinothricin acetyltransferase (PAT). PAT confers resistance to the non-selective herbicide Bialaphos as well as to the related compounds phosphinothricin (PPT) and glufosinate ammonium. Both genes are under the control of the CaMV35S promoter. The *Agrobacterium* strain was grown in Terrific Broth (Sigma Chemical Co.) under antibiotic selection to maximize plasmid yield. Batches of sterile media (100 ml) containing 300 mg/L streptomycin and 100 mg/L spectinomycin were inoculated with the bacterial strain and cultured on a rotary shaker at room temperature until the bacterial growth was at the late log phase (Optical density at 600nm ca. 0.5). The bacterial culture was then stored at 4°C until use. Cultures were normally brought out and allowed to attain room temperature before use to ensure bacterial activity. Cultures for inoculation were normally not used beyond one week after preparation.

Transformation and screening procedures

Seeds of the selected cowpea accessions were grown in the screenhouse until flowering. *Agrobacterium* inoculation of flower buds commenced with the first flower buds that were initiated. Flower buds at the light green stage, that is, a day prior to opening were selected and 0.1 ml of the *Agrobacterium* suspension culture was injected into these buds using a Tuberculin syringe. This is to facilitate intimate contact of the *Agrobacterium* with the pollen grains as the pollen grains are released from dehiscing anthers, thereby resulting in the transfer of the transgenes into the pollen tube and the eventual transformation of the male or sperm nucleus. Inoculations were normally carried out between 6.00 and 7.00pm. Care was taken to avoid piercing the style, anthers and stigma with the needle during inoculation. Light pressure was applied on the flower buds to squeeze out as much of the excess bacterial suspension as possible from the inoculated flower buds. The excess bacterial suspension was soaked up from the flower buds with a paper towel. The flower buds were then tagged and the resulting pods were monitored to maturity. Pods were harvested immediately upon drying.

Seeds derived from the inoculated flower buds were planted out in sterilized soil in seed trays. A week after planting, the tips of the cotyledonary leaves of the seedlings were cut and assayed for GUS using 3mM 5-bromo-4-chloro-3-indoyl-β-glucuronic acid (XGlu) as described by Twell et al. 1989. Leaf samples were immersed in the GUS assay solution in cell wells and incubated overnight at 37°C. The plants whose leaf samples gave the characteristic blue coloration indicative of GUS activity (Figure 3) were selected from the trays into individual 8-inch diameter pots containing sterilized topsoil. The rest of the seedlings were sprayed with Basta; a non-selective herbicide containing Bialaphos as the active ingredient at a concentration of 0.075% to screen for Bialaphos resistance. The steps in *Agrobacterium* transformation of cowpea through pollen are as summarized in Figure 2.

Plant DNA extraction for molecular analysis

DNA was extracted from the putative transformants for molecular analysis using the Dellaporta et al. (1983) technique modified to accommodate more plant tissue for a higher DNA yield. Up to 3 g of young leaf samples of each putative transformant was extracted.

Polymerase chain reaction (PCR) analysis of putative transformants

Two 25-mer primers which are homologous to the *UidA* gene and two 24-mer primers homologous to the *Bar gene* were used in the PCR analysis. The base sequences of the primers are as follows:

GUS P1 5'-TTG CCC AGC TAT CTG TCA CTT CAC T-3'
GUS P2 5'-ATG TCA CAT CAA TCC ACT TGC TTT G-3'

Bar P1 5'-GGG ACT TCA GCA GGT GGG TGT AGA-3'
Bar P2 5'-AAC CGC AGG AGT GGA CGG ACG ACC-3'

A 25 µl amplification reaction mixture was prepared for each DNA

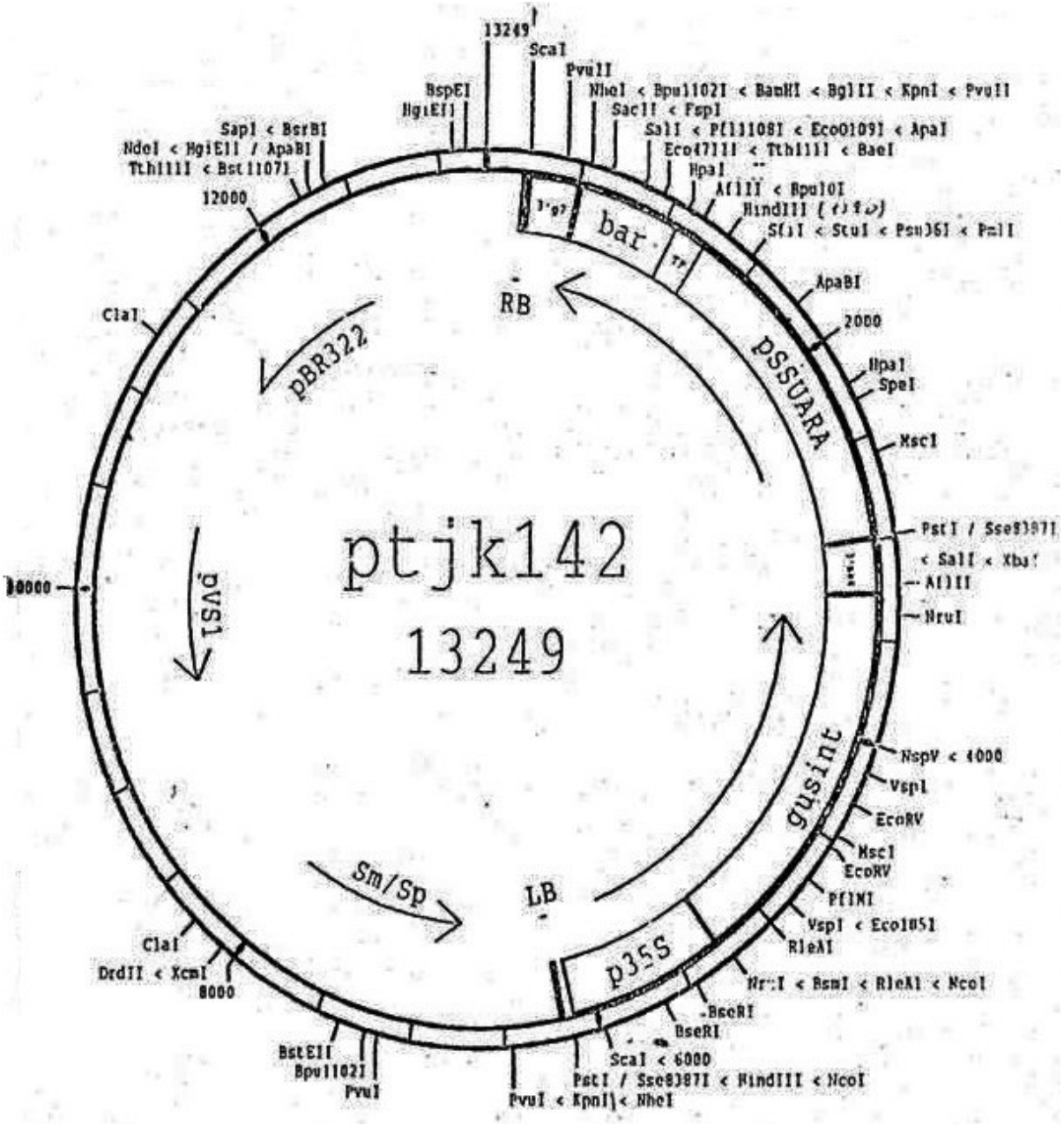

Figure 1. Restriction map of the plasmid ptjk142.

sample. The amplification mixtures contained 5 µl of 500 ng/µl genomic DNA template. The amplifications were carried out using a Perkin Elmer GeneAmp PCR System 9600. The amplification temperature cycle for the *UidA* gene was as follows: preheating at 94°C for 3 min, 1 cycle of 1 min each at 94, 55 and 72°C, respectively and 34 cycles of 30 s each at 94, 55 and 72°C, respectively. The reaction was ended by cooling to 4°C. For the *Bar gene*, there was preheating at 94°C for 5 min followed by one cycle of 1 min each at 94, 60 and 72°C and 34 cycles of 30 seconds each at 94, 60 and 72°C, respectively. The reaction was ended by cooling to 4°C. After the amplification reaction, the products were separated by electrophoresis in a 1.4% agarose gel and visualized under ultraviolet light by ethidium bromide staining.

Southern analysis of putative transformants

Genomic DNA (10 µg) was digested with the enzyme *Pst I*, separated on 0.8% agarose gel, blotted onto nylon membrane (Hybond N+, Amersham) using the procedure of Sambrook et al. (1989) and cross-linked to the membrane with ultraviolet radiation. The DNA membrane blots were stored at 4°C until use. The plasmid was also restricted with *Pst I* to free the regions containing both the *UidA* and *Bar* coding sequences. To carry out plasmid restriction, 10 µl of the plasmid DNA solution (30 µg/ml) was placed in a sterile microfuge tube and mixed with sufficient sterile distilled water to make up to a final volume of 30 µl. 20 units (2 µl of the restriction enzyme at 10 units/µl) was added to the DNA

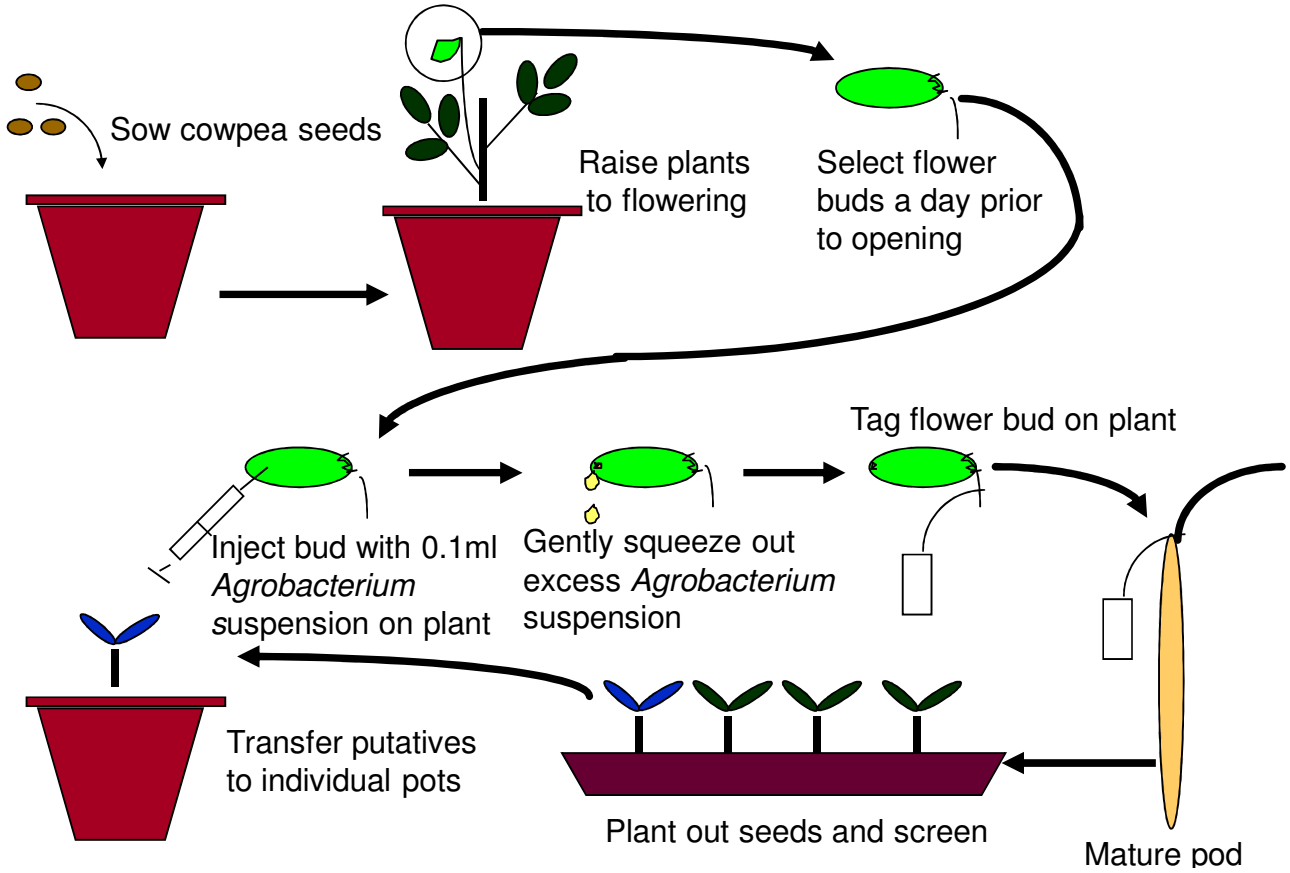

Figure 2. Steps in *Agrobacterium* transformation of cowpea through flower bud inoculation.

solution, mixed by gentle vortexing for about 2 s and then incubated at 37°C overnight. The reaction was stopped by adding 0.5M Ethylene diamine tetra acetate (EDTA) (pH 8.0) to a final concentration of 10mM.

The DNA fragments containing the genes were separated by agarose gel electrophoresis. The bands corresponding to each fragment was cut out of the agarose gel and each was purified using the Qiagen® DNA purification kit according to the manufacturer's procedures. Each gene-containing DNA fragment was then labeled using the non-radioactive Digoxigenin DNA labeling kit from Boehringer Mannheim according to the manufacturers' instructions manual. The labeled DNA was stored at -20°C until used. Hybridization of DNA blots with the labeled DNA probe was also done according to the instructions from Boehringer Mannheim. After hybridization, the DNA bands were detected colorimetrically on the blots by the addition of the color substrate solution (45 µl NBT and 35 µl X-phosphate). The membranes were incubated at room temperature in the color substrate solution long enough for the DNA bands to show.

RESULTS

Initially, an average of up to 95% of inoculated flowers aborted within 48 h after opening. When the excess *Agrobacterium* suspension was squeezed out from the flowers, the average rate of flower abortion dropped to about 90%. At $P \leq 0.05$, no significant differences were observed among the cowpea accessions in the percentage of pods set after *Agrobacterium* inoculation of the flower buds (Table 1). The percentage pod set ranged between 8.92 and 10.51%.

A total of 364 pods developed to maturity from which 1936 viable seeds were obtained. 30 seedlings per accession were first screened by spraying with Basta at 0.125%. None of the seedlings however, showed tolerance to Basta. All died within a week of spraying. Another screening was carried out with the remaining 1576 seeds. In this set of seeds, however, selection for putative transformants was first based on the expression of the screenable marker (the *UidA* gene for GUS) rather than on Bialaphos tolerance. The tips of the cotyledonary leaves of the seedlings were first cut and assayed for GUS. The seedlings that showed positive GUS expression (Figure 3) were then selected from the trays into individual 8-inch diameter pots before the remaining seedlings were sprayed with Basta at a concentration of 0.075%. Nine seedlings were recovered indicating positive GUS expression. None of the remaining seedlings showed tolerance to Basta when sprayed. When the GUS-positive seedlings were subjected to PCR

Table 1. Means of percentage pod set among the cowpea accessions after *Agrobacterium* inoculation.

Accession	Number of Inoculations	Pod set (%)*
IT 90K 284-2	390	10.51[a]
IT 82E-16	331	10.27[ab]
IT 86D-719	359	10.03[abc]
IT89KD 374-57	372	9.95[abc]
TVu 10241	272	9.93[abc]
IT 82D-716	303	9.90[abc]
TVx 3236	328	9.76[abc]
TVu 10050	319	9.72[abc]
IT84S 2246-4	342	9.65[abc]
TVu 1390	227	9.25[ab]
TVu 8340	253	9.09[c]
TVu 1362	213	8.92[c]

*Mean separation by the t-test at P ≤ 0.05. Values followed by the same letter are not significantly different from each other.

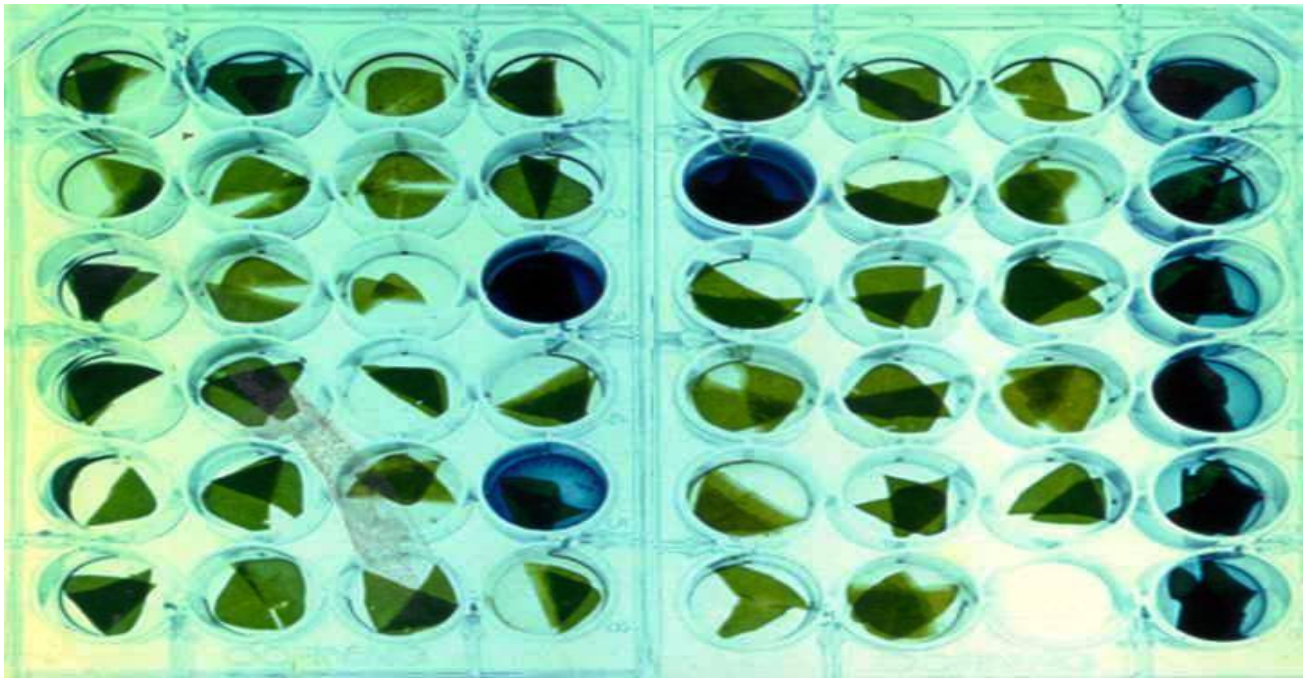

Figure 3. Cell wells with leaf samples from putative transformants showing positive GUS expression. The wells on the far right column contain samples from positive transgenic tobacco control.

analysis using the primers for the *UidA* gene, 7 of them gave positive signals indicating the presence of the *UidA* gene in their genome (Figure 4). None gave positive signals when subjected to PCR analysis using the *Bar* primers. Southern analysis with labeled DNA for the *UidA* gene showed positive Southern signals (Figure 5) indicating the presence of the *UidA* gene in the genome. The cowpea accessions and the number of transformants derived from them are shown in Table 2.

DISCUSSION

Pollen grains carry the male genome during the process of fertilization and are thus the ideal vectors for direct gene transfer into plants (Dupuis and Pace, 1993). The high rate of flower abortion in cowpea after *Agrobacterium* inoculation however, remains a constraint to a high turnover of transformants. It has been observed (Fawole, University of Ibadan, personal communication)

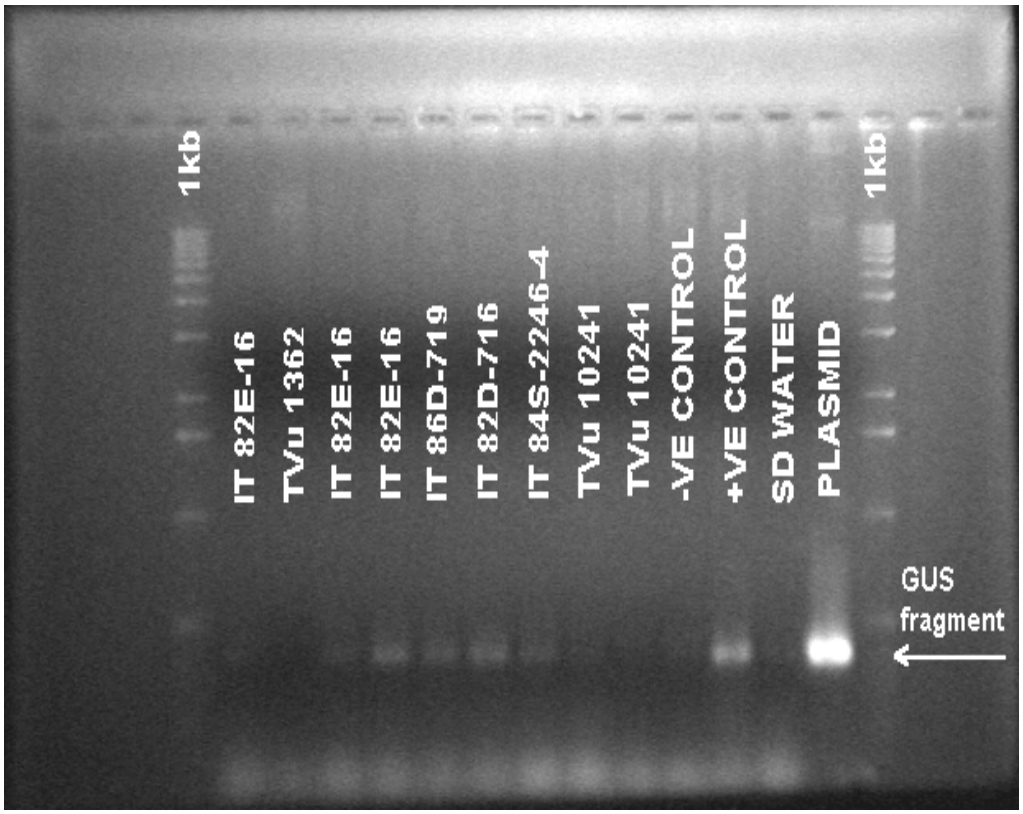

Figure 4. GUS PCR signals from transgenic cowpea derived from transformation by *Agrobacterium* inoculation of flower buds showing amplification for the *UidA* gene.

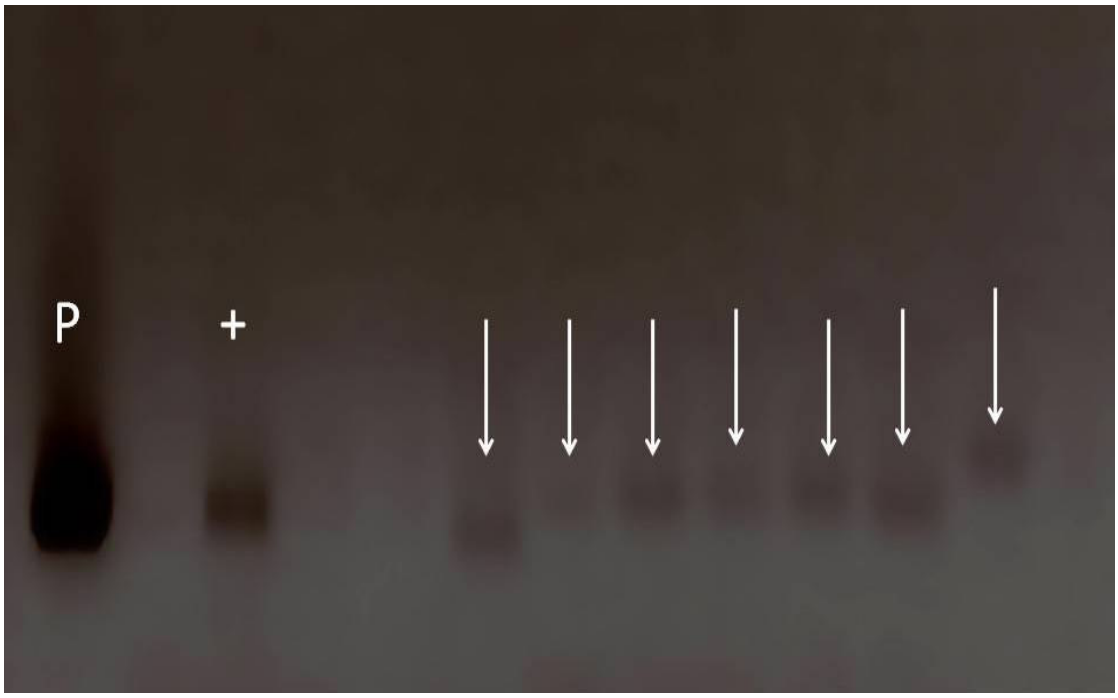

Figure 5. Southern signals from transgenic cowpea derived from transformation by *Agrobacterium* inoculation of flower buds showing hybridization for the *UidA* gene (arrows). P = Plasmid control: + = Positive tobacco control.

Table 2. The cowpea accessions and the number of transformants derived from them.

Cowpea accession	Seedlings screened	Number of GUS transformed	GUS transformed (%)
IT 86D-719	173	1	0.58
TVx 3236	154	0	0
IT 82D-716	180	1	0.56
TVu 8340	138	0	0
IT 84S-2246-4	198	1	0.51
TVu 1390	150	0	0
IT 89KD-374-57	152	0	0
TVu 10050	108	0	0
TVu 10241	178	1	0.56
IT 82E-16	204	3	1.47
IT 90K-284-2	144	0	0
TVu 1362	157	0	0
Totals	1936	7	0.36

GUS, β-glucuronidase.

that cowpea flowers harvested immediately after a rain rarely result in seed set when used for artificial pollination. The high abortion rate observed in *Agrobacterium-inoculated* flowers could be a consequence of the liquid medium in which the *Agrobacterium* was suspended thereby suggesting that cowpea pollen is hydrophobic.

When compared with up to 5% transformation reported by De Kathen and Jacobsen (1990) for the *Agrobacterium* transformation of epicotyl and nodal explants of pea, the observed transformation rates may be considered rather low. It is possible that the low transformation rates observed might be due to the level of virulence of the *Agrobacterium* strain used (Fillipone and Lurquin, 1989). The low transformation rates may also be due to the effect of temperature. Dillen et al. (1997) in their studies with *Agrobacterium* strains carrying the *UidA* gene reported a notable decrease in transient GUS expression at temperatures above 22°C. At 27°C, expression was low and was undetectable at 29°C. In the screenhouse where the transformation studies were carried out, ambient temperatures at the time of *Agrobacterium* inoculation ranged from 23 to 32°C. This temperature range may have significantly affected the transformation efficiency of the *Agrobacterium* strain used.

The non-recovery of bialaphos resistant or tolerant plants could be due to non-incorporation of the *Bar gene* into the cowpea genome. It is also possible that the *Bar gene* may have been inserted into a reading frame not recognized by the transcription mechanism of the cowpea genome. There is also the possibility that gene inactivation or gene silencing may have occurred on the transgenes resulting in the low transformation rates recorded. Inactivation of the transgenes may have been by the process of methylation (Finnegan and McElroy, 1994). Ulian et al. (1996) also observed gene inactivation in *Petunia hybrida* plants transformed with the *nptII* gene for kanamycin resistance. The cowpea accession IT 82E-16 had the highest transformation percentage suggesting a genotype-influenced transformation rate. This however, needs to be further ascertained. Flower pigmentation does not appear to influence transformation frequency. The difference between the number of GUS-positive plants obtained in the GUS assay and the number obtained in the PCR analysis may be due to the presence of false positives. Hybridization patterns obtained in the southern analysis indicate that only one copy of the GUS transgene was inserted in each transformant.

From the study, *Agrobacterium-mediated* transformation of pollen was observed to have potential as a rapid and inexpensive technique for gene transfer to plants. Its major appeal is in the circumvention of tedious regeneration and tissue culture processes. The approach could be adapted to all seed setting species that are amenable to *Agrobacterium* transformation. Further work is needed to understand the hydrophobic nature of cowpea pollen with a view to reducing flower abortion after *A. tumefaciens* inoculation in order to increase transformation efficiency.

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