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AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF *VIGNA UNGUICULATA* (L.) WALP.

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Cowpea (*Vigna unguiculata* sub sp. *unguiculata* (L.) Walp is an important annual fodder legume of the rainfed areas and can form a component crop in the multiple cropping systems. As a food, the grain is a rich source of dietary protein and staple in many countries. An efficient transformation protocol was developed for cowpea plants. Precultured distal half of cotyledons were co-cultured with *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector pBI121 for effective transformation. Histochemical assays showed extensive GUS positive activity zones in the transformed plantlets. The *uidA* gene was also detected by PCR analysis in the genomic DNA isolated from transformed plantlets. These results indicate stable transformation of cowpea using co-culture method.

Keywords: *Agrobacterium*; Cotyledon; Cowpea; Organogenesis; Transformation.

Introduction

In vitro regeneration of cowpea has been reported from shoot, root meristem and distal half of cotyledon explants[1-4]. Crop improvement by conventional breeding in this important legume crop is not as rapid as envisaged to meet the demands of increasing population, especially in seed quality improvement and developing virus- and insect-resistant varieties. There is an urgent need to improve several commercially grown varieties in India and elsewhere. Tools of genetic engineering can be exploited as an additional method for introduction of agronomically useful traits into established cultivars. For successful induction of desirable traits into the extensively cultivated varieties, efficient regeneration protocols as well as a gene delivery system need to be developed either by *Agrobacterium*-mediated gene transfer or the bombardment method. No report is available on shoot regeneration of cowpea from distal half of cotyledon explants that lack pre-existing meristems. In this paper, we report *de novo* shoot organogenesis from distal half of cotyledon explants in cowpea cv. DFH-1 and successful transformation of cowpea with *uidA* and *nptII* genes.

Materials and Methods

In vitro plant regeneration- Cowpea cv. Deenanath Fodder Horsegram (DFH-1) seeds were procured from the Regional Fodder Research Station of the University of Agricultural Sciences, Dharwad, Karnataka state, India were surface sterilized in 70% (v/v) ethanol for 3 min and 0.1% $HgCl_2$ for 1 min. Seeds were thoroughly rinsed four to five times with sterile double distilled water and then seeds were soaked in sterile water for 15 hours in darkness at 27±2° C. In all these experiments, mMS basal medium[5] (all the macro and micro elements except potassium nitrate were reduced to half strength with the addition of 1.0 g/l^{-1} L-glutamine, 0.5 g/l^{-1} Casein hydrosylate) with 3% Sucrose gelled with 0.8% agar-agar (Himedia) was used. The pH of the media was adjusted to 5.8 before sterilization. The medium was dispensed in 145 mm X 25 mm glass culture tubes containing approximately 15 ml of the medium and autoclaved at 1.04 Kg.cm^2 for 15 min.

The morphogenetic potential of cotyledonary segments (distal end) on mMS basal medium supplemented with 8.88 μM N^6- Benzylaminopurine and coconut water (15%) was tested. Cotyledons were split open from the presoaked seeds and the proximal meristematic ends were removed. Only the distal halves (3-3 mm^2) without any pre-existing axillary buds were cultured (one explant per tube) with adaxial surface touching the medium for a period of 4 weeks. 50 explants per treatment were used and the experiment was repeated three times. The cotyledonary explants (distal end) showing shoot primordia were subcultured on the same medium for nearly 2 weeks. The shoot primordia developed into shoots and showed further elongation when they were subcultured on mMS basal medium containing 2.22 μM BAP, Coconut water (CW) (15%) and Adenine sulfate AS (75 mg l^{-1}). The cultures were incubated at 26 ± 2°C on a 16 hour photoperiod under cool white fluorescent light (50 μmol m^{-2} Sec^{-1}) for 2 weeks. The regenerated shoots were transferred to half-strength mMS basal medium without growth regulators for rooting. The rooted plantlets were hardened and transferred to soil.

Agrobacterium-mediated genetic transformation : The cotyledonary explants with distal halves (3-3 mm^2) and without any pre-existing axillary buds were separated and

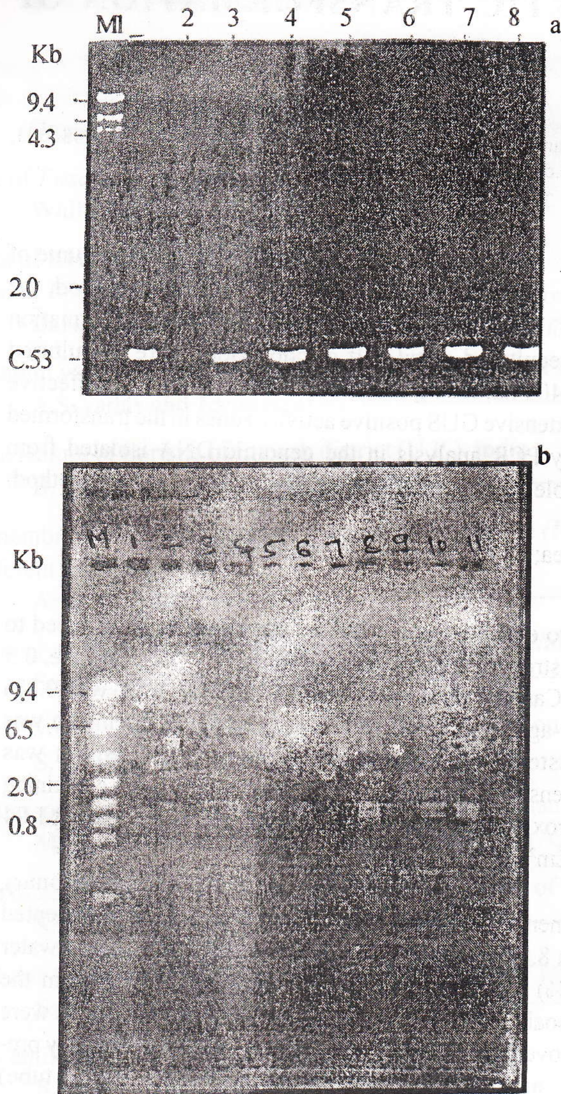

Fig. 1. PCR analysis of transgenic shoots by amplification of the *uidA* (GUS) and *nptII* genes from total plant DNA extracts. (a) Detection of the *uidA* (GUS) gene. Lane M-DNA molecular weight markers. Lane 1- plasmid (PBI121) positive control. Lane 2- untransformed plant (negative control). Lanes 3-8-transformed plants. (b) Detection of *nptII* gene. Lane M-DNA molecular weight markers. Lanes 3, 7, 9- untransformed plants (negative control). Lanes 1, 2- plasmid pBI121 (positive control). Lanes 4-6,8, 10, 11- transformed plants.

used as explants for transformation experiments. LBA 4404 strain of *Agrobacterium tumefaciens* harboring a binary plasmid pBI121 was used as the vector system for transformation experiments[6]. This plasmid carried the *nptII* selectable marker gene controlled by the nos promoter and the *uidA* reporter gene controlled by CaMV 35S promoter. Transformed *Agrobacterium* was grown at 28°C on YMA (yeast extract-5g l^{-1}, Casein hydrosylate-0.5g l^{-1}, mannitol-8g l^{-1}, ammonium sulphate-2g l^{-1}, NaCl-5g l^{-1}, agar-15g l^{-1}, pH- 7.0) medium supplemented with 50 µg/ml kanamycin and 100 µg/ml rifampicin for selection of the pBI121 vector. For inoculation, one single colony was grown overnight on liquid YMA at 28°C with appropriate antibiotic.

The explants were precultured for 2 days on mMS basal medium supplemented with 8.88 µM N^6-Benzylaminopurine and coconut water (15%) prior to co-cultivation with bacterial culture collected at late log phase (A_{600} 0.6). The cotyledons (100 explants) were gently shaken in the bacterial suspension for about 10 min and blotted dry on a sterile filter paper. Afterwards, they were transferred to the medium and co-cultivated under the same conditions of the preculture period (a 16 hour photoperiod under cool white fluorescent light (50 µmol m^{-2} Sec^{-1}) for 2 days). After co-culture, the explants were washed in the mMS liquid medium, blotted dry on a sterile filter paper and transferred to mMS basal medium containing 2.22 µM BAP, Coconut water (CW) (15%) and Adenine sulfate AS (75 mgl^{-1}) with antibiotics (100 µg/ml kanamycin and 300 µg/ml cefotaxime). Three subcultures are usually needed for the elimination of escapes. Latter, the concentration of kanamycin was reduced to 50 µg/ml and completely devoid of cefotaxime. The cotyledonary explants (distal end) showing shoot primordia were subcultured on the same medium for nearly 2 weeks. The regenerated shoots were transferred to half-strength mMS basal medium without growth regulators and kanamycin for rooting. All the experiments were repeated three times keeping all the parameters unchanged.

The *β*-glucuronidase (GUS) histochemical assay was used as a rapid way to detect the presence of the *uidA* gene (GUS) in the putative transformants as described by Jefferson *et al.*[6] using leaf segments in regenerated shoots from explants. For GUS histochemical assay, 1 mM X-Gluc solution (5-bromo-4chloro-3indolyl-glucuronide) was used as a substrate. Leaf tissues were collected and washed with a solution of 400 mg l^{-1} carbenicillin before the assay. The dishes containing leaf tissues with the X-Gluc solution were incubated in the dark at 37°C overnight and recorded the GUS expression. Presence of GUS and *nptII* was confirmed by PCR amplification of the *uidA* and *nptII* using two specific primer sequences. Plant DNA for PCR analysis was isolated as described by Hills and van Staden[7]. Specific primers for GUS (*uidA*) primer sequences (5'-3') were TTC GCG TCG GCA TCC GCT CAG TGG CA and GCG GAC GGG TAT CCG GTT CGT TGG CA. The *nptII* primer sequences (5'-3') were GAG GCT ATT CGG CTA TGA CTG and ATC GGG AGG GGC GAT ACC GTA. The PCR reaction contained 1 µl (100-150 ng) of genomic DNA, 50 pmoles of each primer, 0.25 mM of each dNTPs, 2.5 units Taq DNA

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polymerase, 3mM $MgCl_2$. Reactions were carried in a thermal reactor first at 94°C for 5 min followed by 30 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min. PCR reactions were analyzed by electrophoresis on 1.2% agarose gels and detected by ethidium bromide staining, photographed under ultraviolet light.

Results and Discussion

Totipotent cells are apparently available and are distributed all over the surface of the explants, as shown by the production of buds all along the explants. The availability of a large number of totipotent cells on the surface of a single cotyledonary segment (explants) enhances the possibility of genetic transformation by microprojectile bombardment. Bud formation is also associated with a wounding site, a prerequisite for *Agrobacterium*-mediated transformation[8-10]. In the present study, an important factor which enhanced the transformation efficiency of cowpea was the 2-day preculture of the cotyledonary explants, which probably served to reduce wound stress and increased the number of competent cells at the wound site. These results suggest that under the experimental conditions used in the present study cells of cotyledons may have competence for shoot induction signals and that the competence may vary in different cowpea explants[11-12]. Leaf segments of regenerated shoots were subjected to *in situ* GUS assay. The expression of *uidA* gene was verified by histochemical staining of the leaf of the transgenic plants. The *nptII* positive regenerants showed the typical indigo blue coloration of X-Gluc treatment, while the negative ones did not. Also, more than 60% of the regenerants were GUS positive. This seems to be a typical expression pattern of the CAMV 35S promoter regulated *uidA* gene in young tissues. Presence of the *uidA* and *nptII* genes in the plant genome was confirmed by PCR analysis revealed that 0.53 kb *uidA* and 0.8 kb *nptII* DNA fragments amplified from genomic DNA of all putative transgenic plants. However, no *uidA* and *nptII* PCR products were seen for untransformed control plants. Figure 1a shows that all the samples of transgenic plants (lanes 3-8) gave the predicted size of DNA fragment of 0.53 kb of *uidA* gene. No band was detected in the DNA sample from an untransformed control plant (lane 2). The 0.53 kb DNA fragment was also amplified from the pBI121 plasmid as positive control (lane 1). DNA products with the expected size of 0.8 kb were amplified from total genomic DNAs of the putative transgenic plants (Figure 1b, lanes 4-6, 8, 10 and 11). These DNA fragments were not detected in the untransformed control plants (Figure 1b, lanes 3, 7, 9). As a positive control, the *nptII* gene products were also amplified from the pBI121 plasmid (Figure 1b lane 1, 2). Based on the resistance to kanamycin and PCR detection, presence of both *uidA* and *nptII* genes were confirmed in the transgenic cowpea plants. The protocol can be used efficiently for the introduction of more desirable genes and is currently being used for producing transgenic plants with desirable genes and hence useful for improving the crop through genetic manipulations[13].

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