Indian J. Genet., 71(1): 84-86 (2011)

Short Communication

Genetic transformation of *Vigna unguiculata* tissue by *Agrobacterium tumifaciens*

Jitendra Kumar Pal[1], Sanjeev Kumar and Major Singh*

Biotechnology Laboratory, Indian Institute of Vegetable Research, P.B. No. 01, P.O. Jakhini, Varanasi 221 005

(Received: July 2010; Revised: November 2010; Accepted: December 2010)

Vigna unguiculata L. (Walp.), commonly known as cowpea is a widely cultivated vegetable legume in tropical countries mainly for its green pods [1]. In India, cowpea pods are an integral part of the diet for a largely vegetarian population. The 100g of raw green seeds typically contain 338 calories, 22-25% protein [2]. Apart from low productivity, diseases are major problem in increasing the cowpea production. Out of many viral diseases golden mosaic disease is the most serious problem caused by cowpea golden mosaic virus (CGMV) incurring losses up to 100 % under epidemic conditions. The farmers have no option but to burn their crop to prevent further spread. To overcome this problem, development of transgenic virus resistant lines using viral gene construct is a vaible option [3] however, development of an efficient transformation and regeneration system for Indian varieties is still remains a major challenge in case of cowpea.

Agrobacterium tumefaciens-mediated transformation is an effective and widely used approach to introduce desirable genes into plants. In recent years many attempts have been made to genetically transform cowpea to incorporate genes of different traits [4-6]. Regeneration protocol in leguminous crops has been reported in green gram [7], mungbean, common bean [8], and soybean. The regeneration and transformation report in cowpea is very limited and no protocol is available for the Indian cowpea varieties. In present study, we have optimized a regeneration protocol in Indian cowpea variety Kashi Komal (IVRCP-4), and genetic transformation was achieved using reporter gene *nptII*.

Agrobacterium tumefaciens strain LBA4404 harbouring the binary vector pBinAR was used for transformation. Binary vector pBinAR harbors the neomycin phosphotransferase (*nptII*) gene, which confers resistance to the antibiotic kanamycin as a plant selection marker driven by the cauliflower mosaic virus *35S* promoter (CaMV *35S*) and nopaline synthase (*nos*) terminator. For inoculation, one single colony from a fresh bacterial culture plate was grown overnight in liquid Luria-Berteny (LB) medium with the appropriate antibiotics(50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin) at 28^{o}C in a rotary shaker (200 rpm).

Mature and healthy seeds of cow pea variety Kashi Komal (IVRCP-4) were handpicked and soaked in tap water for 15 min and surface disinfected with 1% (w/v) Cetrimide for 15 min. and finally surface sterilized using 0.1% (w/v) $HgCl_2$ for 2 min. inside a laminar air flow cabinet. For germination, one seed was inoculated in each culture tube containing 20 ml half-strength MS medium [9] solidified with 0.8% agar. Explants excised from 10-day-old seedlings were used for *Agrobacterium* co-cultivation. Multiple shoots were induced on MS medium containing 0.5 to 3.0 $mg1^{-1}$ BAP with or without 0.5 $mg1^{-1}$ IAA. Regenerated plants were rooted on MS basal medium, acclimatized and transferred to pot containing soil in glass house.

Eight to ten days old cotyledonary joints were

*Corresponding author's e-mail: singhvns@gmail.com
[1]Present Address: National Research Centre on Plant Biotechnology, IARI, New Delhi 110 012
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excised and used as explant for transformation. These explants were pre-incubated on hormone free MS medium for 2 days and used for co-cultivation. Pre-incubated explants were collected in a Petri plate and 10 ml of overnight grown *Agrobacterium* liquid culture (1 OD at 600 nm) were added and swirled gently for 10 minutes. After ensuring that all the cut edges of the explants were in contact with bacterial culture, explants were blot dried on a sterile filter paper and cultivated on hormone free MS medium for 2 days. These segments were transferred to selection medium (regeneration/ callusing medium containing 50 mg/l kanamycin) after two days of co-cultivation.

MS media supplemented with 0.8% agar and 3% sucrose was used as the basal medium. The induction of transgenic callus and differentiation of shoot buds was achieved on MS medium supplemented with BA (2 mg L^{-1}), with kanamycin (50 mg l^{-1}) as selective agent. The antibiotic cefotaxime (500 mg l^{-1}) was used to overcome *Agrobacterium* growth in the selection medium. For normal shoot development, shoot buds were cultured on MS basal medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime for 15 days. Regenerated shoots were rooted on MS basal medium with 50 mg l^{-1} kanamycin, acclimatized and plants were transferred in sterile soil and sand mixture (1:1) and irrigated with sterile water containing 50 mg l^{-1} kanamycin.

Seedlings and all *in vitro* plant materials were incubated at 25±2^{o}C under a 16/8-h (day/night) photoperiod. Light was provided by cool-white fluorescent lamps at photosynthetic photon flux of 60^{o}E m^{-2} s^{-1}.

Genomic DNA was isolated from the fresh leaves of putative transformed and normal (untransformed) plants using Plant DNA extraction kit (Qiagen, Germany) and subjected to PCR analysis using *nptII* gene specific primer (F; 5'-aagcgatagaaggcgatgcgctgc-3', R; 5'-caatcggctgctctgatgccgccg-3'). The DNA amplification was carried out in a 25µl reaction mixture consisting of 10 x reaction buffer, 200mM d NTPs, 25 mM $MgCl_2$, 10 picomoles of each Primer, 1µl plant genomic DNA (100-150ng) and 2 unit of *Taq* DNA Polymerase (Fermentas Life Sciences, UK). The PCR conditions were : initial denaturation 94^{0}C for 5 min, 94^{0}C for 1 min, annealing temp. 62^{0}C for 1 min, extension 72^{0}C for 2 min for 35 cycles and final extension 72^{0}C for 10 min. The amplification products were electrophorased in 0.8% agarose gel and exposed to UV light for visualization.

In the transformation study, callus induction was observed from the cut ends of hypocotyls and leaf segments after 21 days of incubation in selection medium (MS medium + 2.0 mgl^{-1} BA + 50 mg l^{-1} Kanamycin + 500 mg l^{-1} cefotaxime; Fig. 1a). In contrast, the untransformed explants showed necrosis and death within a week without any callusing on the selection medium. The frequency of surviving calli was 25% on selection medium. Earlier 15-19% of regeneration frequency have been reported on selection medium [1], however in our study, 13 out of 17 putative transformants died giving a survival rate of only 35%. When putative transformed calli were separated from the stock explants and subcultured on to fresh medium containing 50 mg l^{-1} kanamycin + 500 mg l^{-1} Cefotaxime, shoot buds initiation was observed from the callus (Fig. 1b). Later, the concentration of kanamycin was increased further to eliminate any escapes, and the putative transformants were still surviving even up to 500 mg l^{-1} kanamycin in the medium. At last 5 putative transformed plants were obtained.

Identification of transformed cowpea plants, was done by assaying the surviving plants for presence of *nptII* gene. All the putative transformants selected on the media were shown to be *npt II* positive. In contrast

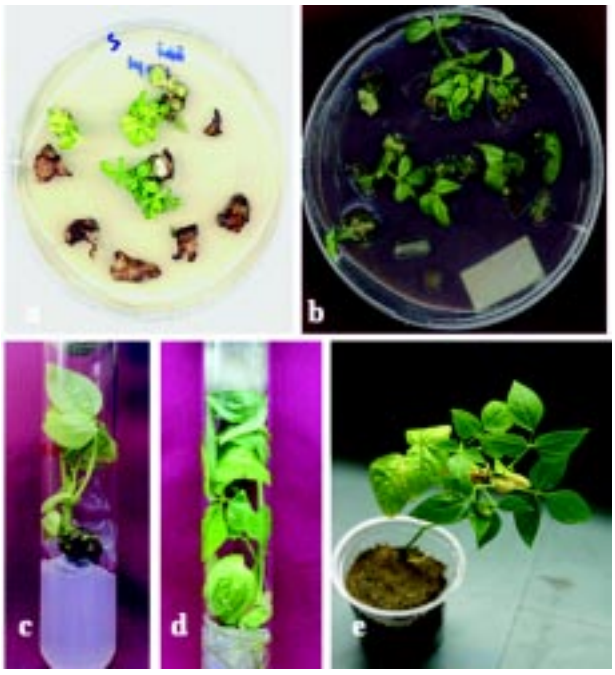

Fig. 1. Selection of cowpea transformants on selective medium (a) Multiple shoot initiation from putative transformants (b) elongation in the plantlet, (c) (d) rooted plantlet on MS medium, and (e) potted plant

the control and untransformed plants did not show the presence of *nptII* gene. Approximately 700 bp fragment specific to *nptII* was amplified with PCR (Fig. 2). This result is in contrast to earlier reports suggesting that *Agrobacterium* strain LBA4404 was least virulent on cowpea lines cultured *in vitro* [2]. However, it is very important to select the transgenic plants carrying a single copy of the foreign gene to avoid the possibility of subsequent gene silencing by homologous co-suppression [10].

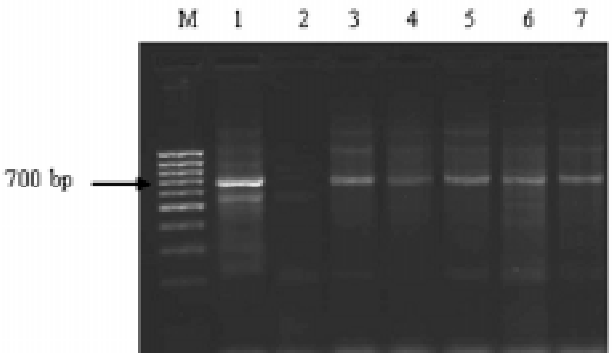

Fig. 2. PCR amplification of 700bp *nptII* gene specific fragment (markd with an arrow) using *nptII* gene specific primer, lane 1; purified plasmid DNA (positive control), 2; DNA from normal untransformed plant (negative control), 3-7; DNA samples extracted from putative transformants, M – marker 100 bp DNA ladder

In this study, transgenic cowpea was produced by *Agrobacterium*-mediated transformation through seedling derived explants. The ability to regenerate cowpea *in planta* [2] as well as the use of positive selection system may provide a means for recovery of stable transformants. The powerful combination of conventional and genetic engineering approaches has the potential of greatly enhancing the productivity of cowpea by increasing the resistance to pests and diseases. To be of value the transgenic plants must be efficiently express the transgene. Till now it was not achieved in cowpea. However, the present study showed that *Agrobacterium*-mediated transformation is a possible approach to develop transgenic plants in cowpea.

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