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In planta Agrobacterium-MEDIATED GENETIC TRANSFORMATION IN OKRA {*Abelmoschus esculentus* (L.) Moench}

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ABSTRACT

In Planta **methods have been developed in several plant species to overcome the problems associated with their tissue culture-based transformation. In present study, a method for genetic transformation in okra (*Abelmoschus esculentus*) was established using *in planta Agrobacterium*-mediated genetic transformation. The embryos of imbibed seeds pricked from meristematic region of plumule were used as explants for transformation. *Agrobacterium tumefaciens* strain EHA105 was carrying *cry1Ac* gene against fruit and shoot borer, CaMV 35S as promoter and *nptII* as plant selectable marker gene. The putative transgenic plants were confirmed by amplifying transgene through polymerase chain reaction.**

Key words: *Abelmoschus esculentus, Bacillus thuringiensis,* shoot and fruit borer, transgenic okra

INTRODUCTION

Okra {*Abelmoschus esculentus* (L.) Moench}, also called lady's fingers, gumbo or bhindi, is an important dietary vegetable crop grown in tropical and sub-tropical parts of the world. It is quite popular in India because of its easy cultivation and adaptability to varying moisture conditions. Worldwide, India ranked first in okra production with an area of 0.52 million ha and production of 6.1 million ton pods in 2016-17 (Anonymous, 2017). The major okra producing Indian states are Uttar Pradesh, Odisha, West Bengal, Andhra Pradesh, Karnataka and Assam. As a member of Malvaceae family, okra is an often-cross pollinated and allopolyploid plant with considerable variation in the chromosome numbers and ploidy levels. *Abelmoschus* genus has two cultivated species, *A. esculentus* and *A. caillei*. Of these, *A. esculentus* is most widely cultivated for its pods throughout Asia and Africa while *A. caillei* is cultivated for leaves and pods in West and Central Africa. Though *A. moschatus* occur as a wild species, it is also cultivated for aromatic seeds. Okra has medicinal and industrial value and is highly proteinaceous and good source of vitamins, calcium, potassium as well as iodine and other mineral matters (Duzyaman and Vural, 2003).

Okra is susceptible to a number of pests. Amongst these shoot and fruit borer - *Earias vittella* (Fabricius) is the most destructive insect pest causing considerable damage (Kashyap and Verma, 1983). Therefore, shoot and fruit borer is considered as the main bottleneck for okra cultivation, causing 10.1 to 50.0% losses (Brar *et al.*, 1994; Shukla *et al.*, 1997; Satpathy and Rai, 1998). The adult female lays eggs individually on leaves, floral buds and on tender fruits (Brar *et al.*, 1994). Larvae bore into the tender shoots, developing buds, flowers and fruits. The larvae feed inside the shoot before fruit formation and resulting in withering, wilting and drying of shoots. Plant gives a

bushy appearance as the side shoots arise. Later on, caterpillars bore into the fruits and feed on inner tissues as a result the infested plant bears smaller and deformed pods. Damaged plant tissues serve as entrance for disease causing microorganisms such as fungi. Many insecticides are used to control this pest leading to detrimental effect on environment and human health (Bansode *et al.*, 2015). On the other hand, genetic improvement by conventional plant breeding is limited due to the lack of resistance sources to insect pests in okra germplasm. Variation for resistance in okra germplasm is present but none of the varieties showed complete resistance or immune against shoot and fruit borer (Memon *et al.,* 2004). While, okra genetic transformation using crystal protein genes from *Bacillus thuringiensis*, specifically targeting lepidopteran insects provide enhanced insect resistance against fruit and shoot borer (Narendran *et al.,* 2013). However, reports on genetic transformation of okra for the production of transformed plants are very limited due to the absence of a reliable transformation method (Mallela *et al.,* 2009). Tissue culture is a major prerequisite for carrying out genetic transformation and there is need to develop an effective callusing and regeneration protocol in okra. Still after successful callusing and regeneration in a crop, many elite commercial cultivars remain recalcitrant to regeneration through tissue culture and are difficult to transform (Ganesan *et al.,* 2007). Therefore, in present study *in planta Agrobacterium*-mediated genetic transformation was attempted in popular cultivars of okra.

MATERIALS AND METHODS

Plant materials

Okra varieties 'Pusa Sawani', 'Punjab Padmani' and 'Prabhani Kranti' were used as experimental material. Healthy seeds of these varieties were procured from the Department of Vegetable Science, PAU, Ludhiana (India) in the year 2015-16.

Establishment of plant regeneration

The seeds were first washed under running tap water and surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 5 min. To remove the traces of sterilizing agents the seeds were washed twice with sterile distilled water. Leaf and hypocotyl from *in vitro* grown seedlings on MS medium were used as source of explant for callus induction. Three different concentrations of each growth regulator combinations *viz*., napthalene acetic acid (NAA) and benzyl amino purine (BAP); NAA and thidiazuron (TDZ); NAA and kinetin; 2,4 dichloro-phenoxy acetic acid (2,4-D) and BAP (Sigma, USA) were used for callusing. Once callus got induced, it was subcultured on same callusing media and after two subcultures the callus was cultured on regeneration medium having composition of Murashige & Skoog (MS) medium + BAP (2 mg L^{-1}) + NAA (0.25 mg L^{-1}).

Vector and Agrobacterium strain for transformation

Agrobacterium tumefaciens strain EHA105 harbouring a binary plasmid vector pBin19 carrying *cry1Ac* transgene (GenBank Accession No. KP195020; Sanyal *et al.,* 2011) was used for transformation of okra. The vector was procured from Dr. P. Ananda Kumar, National Research Centre on Plant Biotechnology, New Delhi, India. The *cry1Ac* gene and *nptII* gene were driven by cauliflower mosaic virus (CaMV) 35S and nopaline synthase (NOS) promoters, respectively. This construct contained *nptII* (kanamycin resistance gene) plant selectable marker gene which was used as a marker gene for the selection of transgenic plants during transformation. The integrity of *cry1Ac* transgene was verified by isolating the plasmid DNA followed by PCR amplification using transgene specific primers. The plasmid DNA was isolated by alkaline lysis method using PhoenIX™ Maxi plasmid DNA isolation kit (MP Biomedicals, USA) as per the Manufacturer's instructions. PCR was performed with initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 1 min for a total of 35 cycles in thermal cycler (Master cycler Gradient-Eppendorf™). Final extension was

carried out at 72°C for 7 min. Amplified DNA fragments were electrophoresed on 1.0% agarose gel and visualized under UV light and photographed using photo gel documentation system (Avegene, USA).

Single colony of *A. tumefaciens* EHA 105 strains hosting pBin 19 carrying *cry1Ac* transgene was cultured on yeast extract mannitol (YEM) medium [yeast extract 0.4 g, mannitol 10 g, NaCl 0.1 g, $MgSO_4.7H_2O$ 0.2 g and $K_2HPO_4.3H_2O$ 0.5 g L^{-1}; pH 7.0] supplemented with 50 mg L^{-1} kanamycin. The culture was incubated overnight at 28°C and kept under 180 rpm on an orbital shaker to obtain *Agrobacterium* suspension having optical density (OD) of 0.6. OD was measured at 600 nm on Nanodrop™ 1000 (Thermo Scientific, India).

In planta Agrobacterium transformation

The mature seeds of Pusa Sawani (450), 562 and 495 of Punjab Padmani (562) and Prabhani Kranti varieties of okra fruits thoroughly washed under running tap water and surface sterilized using 70% ethanol (v/v) followed by treatment with 0.1% (w/v) aqueous mercuric chloride solution for 5 min. After washing twice with sterile distilled water seeds were then soaked in water overnight and placed on an orbital shaker at 100 rpm for gentle shaking. Thereafter, the plumules of embryos were pricked gently with a sterile needle at the apical meristematic region. The tissue thus prepared was used for *in planta* transformation through *Agrobacterium*-mediated transformation. The pricked plumules were immersed in *Agrobacterium* suspension for 60 min at room temperature. Subsequently, these were washed with sterile water and sown in plug trays of 50 places containing potting mixture (soil and farm yard manure in 3:1 ratio). The trays were incubated in transgenic glasshouse at 25±2°C under a 14 h photoperiod. The germinating seedlings were moistened daily with water and leaves of 15 days old seedlings were used for PCR analysis.

Polymerase chain reaction analysis on T_0 plants

Okra possess large amount of foliar mucilage that hinders DNA isolation, therefore modified cetyl trimethyl ammonium bromide (CTAB) method given by Ghosh *et al*. (2009) was used for DNA extraction from 15-20 days germinated okra. DNA was isolated from putative transgenic plants. To confirm the transfer of *cry1Ac* transgene PCR analysis was done (Narendran *et al.,* 2013). The transgene specific PCR primers 5'-TGGAGAACGCATTGAAACCG-3' and 5'-TGTTGCTGAAT CCGGAACGC-3', were designed to amplify an expected amplicon of 927 bp. PCR amplification was carried out with 20 µL PCR reaction mixture containing template DNA (100 ng), primers (10 µM), buffers (5X), dNTPs (10 mM), $MgCl_2$ (25 mM), GoTaq® DNA polymerase (5 units µL) of Promega, USA and distilled deionized water in a thermal cycler (Eppendorf Master Cycler, Germany). The PCR profile consisted of initial denaturation at 94°C for 5 min; followed by 35 cycles each with denaturation at 94°C for 1min., primer annealing at 54°C for 1min., and primer extension at 72°C for 1 min in a thermal cycler (Master cycler Gradient-eppendorf™). The final extension step was carried out at 72°C for 7 min. Amplified products were analyzed by gel electrophoresis on 1% agarose gel along with 1 kb DNA ladder (Promega). The PCR products were resolved by running gel at 5 V/cm-for 1-1.5 h.

RESULTS AND DISCUSSION

Establishment of plant regeneration

A reproducible protocol for *in vitro* regeneration is a prerequisite for varietal improvement through genetic engineering. Callus obtained from explants is an ideal material for genetic transformations (Finer and McMullen, 1990). In present study, using different media compositions in all the genotypes, hypocotyl was more callogenic (83.3) as compared to leaf (Fig. 1 & 2); therefore, hypocotyl was found better source of explant for callogenesis (Anisauzzaman *et al.,* 2008). Variation in callus forming ability of different explants types has been reported in many other plants (Ishii *et al.*

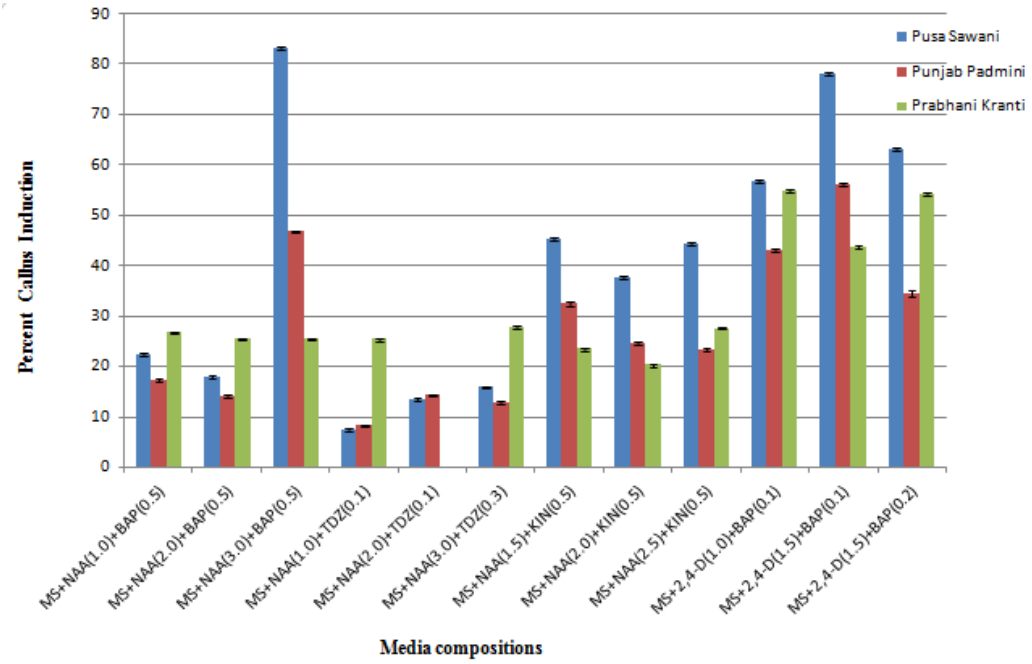

Fig. 1: Comparative effect of various media compositions on callus induction from hypocotyl in different okra varieties

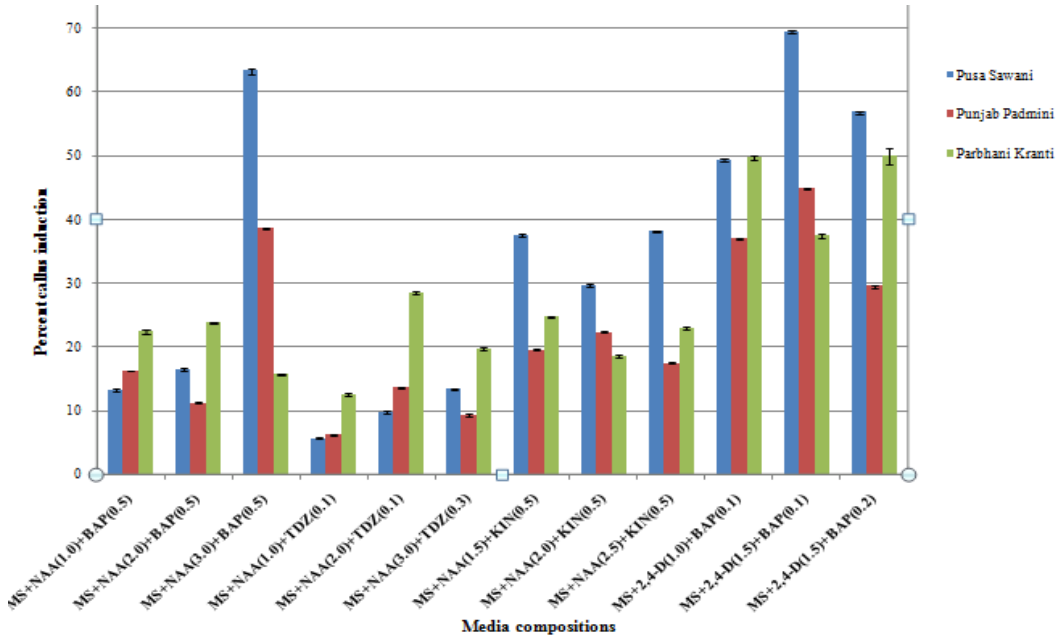

Fig. 2: Comparative effect of various media compositions on callus induction from leaf in different okra genotypes

2004). Among the genotypes, the per cent callus formation was maximum (83.3) in Pusa Sawani followed by Punjab Padmini (56.2) and Parbhani Kranti (54.8). Among the different growth hormones combinations used MS medium containing NAA (3.0 mg L^{-1}) + BAP (0.5 mg L^{-1}) was the best medium. This indicated that for tissue culture-based transformation protocol selection of genotype is a crucial step for successful transformation of okra.

Calli obtained from leaf and hypocotyls of all the genotypes were cultured regeneration medium to check their regeneration ability. Although plant regeneration from calli obtained from cotyledonary axil (Roy and Mangat, 1989); hypocotyl (Haider *et al.*, 1993; Ganesan *et al.*, 2007;

Anisauzzaman *et al.*, 2008); leaf and cotyledon (Mallela *et al.*, 2009) of different okra genotypes were reported but none of the medium composition and explants worked equally well in the genotypes under investigation. Species of Malvaceae family are well known for their recalcitrant nature as these do not show plant regeneration from callus (Sunilkumar and Rathore, 2001; Kumar *et al.*, 2004). It has been observed that initially within 15-20 days of culturing on regeneration medium the callus showed change in colour from white to green, showing some signs of regeneration, but later the calli turned black without induction of any shoot bud.

In planta Agrobacterium-mediated genetic transformation

To overcome the effect of genotypes, the tissue culture-free *in planta Agrobacterium*-mediated genetic transformation method was attempted in okra. Embryos with pricked plumules (Fig. 3) of 430, 555 and 483 seeds of three okra varieties *viz*., Pusa Sawani, Punjab Padmani and Prabhani Kranti, respectively, were inoculated with *Agrobacterium* suspension (Table 1). The inoculated seeds were germinated in soil in trays which were kept in glasshouse (Fig. 4). Out of these 284, 401 and 351 seeds of Pusa Sawani, Punjab Padmani and Prabhani Kranti, respectively, were germinated. Negative controls were maintained in each experiment. The transgenic plants were phenotypically normal and fertile (Fig. 5).

Fig. 3: Needle pricking of seeds at plumule end for genetic transformation

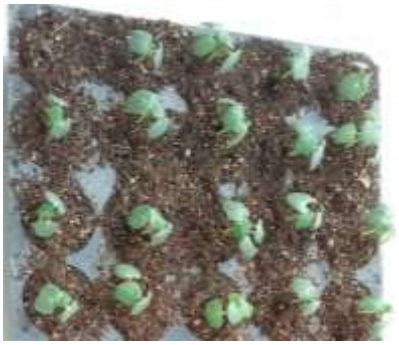

Fig. 4: *In planta Agrobacterium* inoculated seeds germinated in trays in glass house

Fig. 5: PCR positive plants a) Pusa sawani b) Punjab padmani

PCR analysis

After 15-20 days of germination, the DNA was isolated from putative transgenic plants using CTAB method. Okra possess large amount of foliar mucilage that hinders DNA isolation. Mucilage often binds to other secondary metabolites such as phenolics, tannins and alkaloids and co-precipitates with DNA during isolation. Contamination of mucilage and other secondary metabolites not only makes the DNA unmanageable during pipetting but also hinders further downstream applications such as PCR or other enzymatic reactions since they inhibit Taq polymerase activity (Fang *et al.*, 1992) and interfere directly or indirectly with the enzymatic reactions (Weishing *et al.*, 1995). Therefore, modified CTAB method given by Ghosh *et al.* (2009) was used for DNA extraction.

The *cry1Ac* gene and *nptII* gene were driven by cauliflower mosaic virus (CaMV) 35S and nopaline synthase (NOS) promoters, respectively (Fig. 6). The isolated DNA was verified for the presence of *CRY1Ac* transgene through PCR using transgene specific primers. Plasmid DNA harbouring C*ry1Ac* gene sequence was used as positive control and seedlings from non-inoculated embryos were used as negative control. The results revealed amplification of a 927 bp fragment corresponding to the size of transgene (Fig. 7). Plasmid DNA harbouring C*ry1Ac* gene sequence was used as positive control and seedlings from non-inoculated embryos were used as negative control. Out of 1036 plants verified for presence of *CRY1Ac* gene, two plants i.e. one plant of Pusa Sawani and one plant of Punjab Padmini were confirmed to be PCR positive (Table 1) for presence of *CRY1Ac* gene (Fig. 6)

Table 1: Genetic transformation (%) in Pusa Sawani, Punjab Padmini and Parbhani Kranti varieties of okra

Varieties	No of seeds inoculated with *Agrobacterium*	No of seeds responded in glasshouse	No of PCR positive plants	Genetic transformation (%)
Pusa Sawani	430	284	1	0.42
Punjab Padmini	555	401	1	0.33
Parbhani Kranti	483	351	0	0
Total	1468	1036	2	0.13

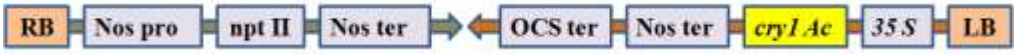

Fig. 6: Schematic map of transgene construct showing *cry1Ac* under the control of CaMV 35S promoter and Nos terminator. *RB* right border, *LB* left border

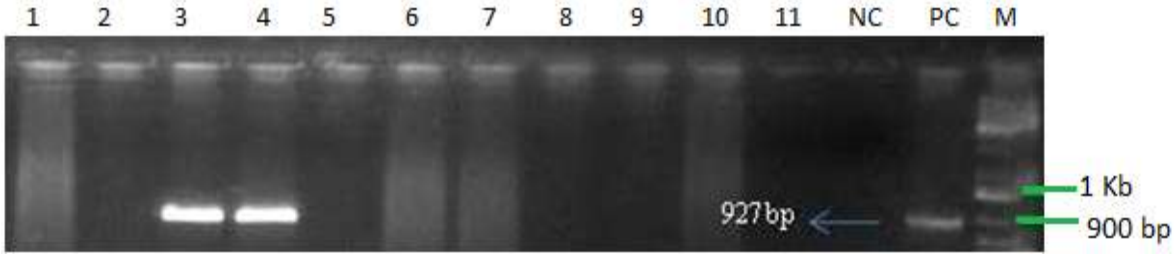

Fig. 7: PCR confirmation of putative transgenic plants M = 1 kb plus DNA ladder, PC = positive control, NC = Negative control, W = Water, 3 = PCR positive plant of Pusa sawani, 4 = PCR positive plant of Punjab Padmini

indicating successful T-DNA uptake following simplified *Agrobacterium* transformation. The transformation frequency was 0.42 and 0.33 in Pusa Sawani and Punjab Padmini, respectively. Narendran *et al.* (2013) reported transformation efficiency of 1.88% using *Agrobacterium*-mediated genetic transformation of embryos in okra. These transgenic plants were then transferred to the pots and maintained in transgenic glass house. The transgenic plants were phenotypically normal and fertile. The present study demonstrated that *in planta Agrobacterium*-mediated genetic transformation takes less time as compared to the tissue culture-based genetic transformation protocol. In tissue culture regeneration of transgenic plants takes longer time and also there is problem of somaclonal variation in *in vitro* cultures of somatic embryogenesis maintained for longer periods. Thus, an efficient genetic transformation protocol will accelerate the ability to genetically transform okra with a number of other economically important traits.

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