

Engineering Insect Resistance in Brinjal against Fruit and Shoot Borer

Leucinodes orbonalis

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ABSTRACT:

The Brinjal fruit and shoot borer(FSB) is the most destructive insect pest for Brinjal which causes yield losses of 60-70%.To reduce the effect of the pest in brinjal, Genetic transformation study was carried out using CO2 variety of brinjal explants with *Agrobacterium tumifaciens* to transfer the *cry2Ax1* synthetic gene to develop resistance against *Leucinodes orbonalis*. EHA 105 strain of *Agrobacterium* harbouring pCAMABIA 2301-*cry2Ax1* vector which contains *nptII* (neomycin phosphotransferase), 35S promoter and *nos* (nopaline synthase) terminator was used for the transformation. Thidiazuron (0.1 mg/l), used for induction of calli. Kanamycin (100 mg/l) used as a selectable marker, Cefatoxime (500 mg/l) controls the overgrowth of *Agrobacterium* in the selection medium. N6-benzylaminopurine (3.0 mg/l) and indole butyric acid (3 mg/l) used for the shoot and root induction, respectively. PCR analyses of transformed plant showed that the presence of transgene in the plant genome.

Key words: *Agrobacterium tumifaciens*,*Fruit and Shoot Borer, Brinjal, cry2Ax1, Cotyledons Explants, Genetic Transformation*

INTRODUCTION

Solanum molangiana (2n=24) is the most important vegetable crops in India which has high nutrient value. It is the best source for vitamin A, vitamin B and minerals like K. It contains

large amount of carbohydrate and lower amount of fat (Bajaj et al., 1979). The production of this important vegetable is severely limited by several soil-borne pathogenic viruses and the insect pest *Leucinodes orbonalis* which is commonly known as Fruit and Shoot Borer, belonging to the order Lepidoptera ([Collonnier et al., 2001](#)). It cause damages to the plant by fed into tender shoots, flower buds and fruit leads to the shedding of the plant and makes the fruits to unfit for human consumption and leads to the productivity loss. Usage of some chemical pesticide make the environment polluted as well it affect the human health (Choudhary and Gaur, 2009). Without these effects Plants can be raised against this pest by introducing cry genes into their genome by genetic engineering. Source of the cry genes are *Bacillus thuringiensis* a gram negative, soil borne pathogene. Insecticidal Crystalline Protein (ICP) product of cry genes, affect the midgut of the insect by binding the midgut receptor when they are injusted by them, which ultimately leads to the death of the insect. Agrobacterium –mediated transformation is one of the most widely used efficient methods for plant transformation. Guri and Sink (1988b) were the first to report *Agrobacterium*-mediated transformation of eggplant, quickly followed by others (Filippone and Lurquin, 1989; Rotino and Gleddie 1990; Fari et al. 1995; [Kumar et al. 1998](#); [Hanyu et al. 1999](#)). Although, the fact remains that the susceptibility of brinjal to infection by various *Agrobacterium tumefaciens* strains and it responds well to *Agrobacterium* mediated transformation with cointegrate and binary vectors ([Collonnier et al., 2001](#)).Therefore the transformation carried out using Agrobaterium in Brinjal plant with *cry* gene to develop resistant against Fruit and Shoot Borer.

Materials and Methods

Culture Media

The culture media was prepared with Murashige and Skoog's mineral salts supplemented with 0.8% agar and 3% sucrose was used as the basal medium. For aseptic germination of

seeds, 1/2- strength MS (Murashige and Skoog, 1962) basal medium was used. The induction of callus and differentiation of shoot buds was achieved on MS medium supplemented with 0.1 mg/l Thidiazuron (TDZ), kanamycin (50mg/l) as selective agent. The antibiotic cefotaxime (500 mg/l) was used to overcome *Agrobacterium* growth in the selection medium. For normal shoot development, shoot buds were cultured on MS basal medium containing 100 mg/l kanamycin and 500 mg l/1 cefotaxime for 15 days. Shoots were rooted directly in media containing IBA 250 mg/l, kanamycin50mg/l.

Bacterial Strain and Vector

Agrobacterium tumefaciens strain EHA 105 harbouring the vector pCAMBIA 2301-*cry2Ax1* was used for transformation. The vector pCAMBIA2301 harbours the neomycin phosphotransferase (*nptII*) gene driven by 35S promoter and nopaline synthase (*nos*) terminator, which confers resistance to the antibiotic kanamycin (plant selection marker). For inoculation, one single colony from a fresh bacterial culture plate was grown overnight in liquid Yeast Extract Peptone(YEP) medium with the appropriate antibiotics (50 mg/l) kanamycin and 20mg/l rifampicin at 28°C in a rotary shaker (440rpm).

Plant Material:

The CO₂ brinjal seeds were sterilized with sterile water, alcohol and 4% sodium hypochloride followed by rinse with sterilized water thrice and dried with Whatmann no. 1 filter paper for 20 minutes. Seeds were inoculated in bottles with half – strength medium and kept in tissue culture room. After 12days of germination, the seedlings were used for preculturing.

Genetic Transformation

Pre-culturing

The hypocotyls and cotyledon of 7-day old *in vitro* grown seedlings were excised and kept in pre-culture medium containing MS salts and vitamins containing TDZ (0.044 mg/litre). The plates were cultured for 2 days.

Transformation

Agrobacterium EHA 105 (pCAMBIA 2301-*cry2Ax1*) was streaked on YEP medium with rifampicin 10 mg/litre and kanamycin 50 mg/litre and grown overnight at 28°C. The plate was stored at 4°C. A single colony from mother plate culture was inoculated into 3 ml of YEP broth with kan 50 mg/ml, rif 10 mg/ml and incubated at 220 rpm in a shaker overnight. Next day, 30 ml culture of *Agrobacterium* was prepared by adding 0.5 ml of 3 ml inoculum to 30 ml of YEP broth (app) with kan 50 mg/ml, rif 10 mg/ml. It was incubated for minimum 8-9 hours at 220 rpm to get curd like growth of *Agrobacterium* culture. The *Agrobacterium* cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the pellets dissolved in 1 ml of AAM broth. About 30 ml of AAM broth was mixed with 30 µl of acetosyringone (0.1M) in a centrifuge tube. One µl of this mixture was poured on co-cultivation media (CCM, MS salts and vitamins, 0.044 mg/l TDZ, 1 ml/litre of 0.1 M acetosyringone) overlaid with whatman no. 1 filter paper. The AAM+ACS mixture was added to the *Agrobacterium* culture (1 ml). Then this suspension was used for infection of explants from pre culture medium for 20-30 min. The explants were blot dried on whatman No.1 filter paper and transferred onto CCM plates. The plates were incubated in the dark.

Regeneration

After 2 days of co-cultivation the explants were washed with 50 ml of sterile distilled water containing 300 mg/l cefotaxime for 5-10 min. Then the explants were washed with sterile distilled water twice and blot dried with Whatman no.1 filter paper and transferred onto regeneration medium (MS salts and vitamins, 0.044 mg/l TDZ, 500 mg/l cefotaxime) and incubated in light for two days. After two days, the co-cultivated explants were transferred

onto selection media (MS salts and vitamins, 0.044 mg/l TDZ, 500 ml/l cefotaxime).and incubated in growth chamber for 15 days. Every 15 days, the explants were transferred to a fresh selection media. Three selections were done for each batch of co-cultivated explants. The tissues survived were transferred into shooting media (MS salts and vitamins, 0.044 mg/l TDZ, 500 ml/l cefotaxime) in bottle

Rooting

The shoots were transferred onto rooting media (MS salts and vitamins, 0.044 mg/l TDZ, 500 ml/l cefotaxime, 3 mg/l IBA) and grown in a bottle.

Hardening

After the development of roots in the shoots they were transferred into pot containing sterilized red soil sand, vermicompost. Then the plants were grown in green house.

Molecular analysis of the Plants

The regenerated plants were screened for the integration of the gene through PCR analysis. The presence of the *cry2Ax1* and *nptII* genes was confirmed by the polymerase chain reaction (PCR) using gene-specific primer sequences. Plant genomic DNA was isolated from fresh leaves of putatively transformed and non-transformed (control) plants of the different varieties using the CTAB (cetyl trimethyl ammonium bromide) method described by [Doyle and Doyle \(1990\)](#). The *nptII* primer sequences (5'-3') were GAG GCTATT CGG CTA TGA CTG and ATC GGG AGG GGC GAT ACC .

RESULTS

Two hundred seeds were inoculated for transformation of brinjal plants. Explants were obtained from 10-12days seedlings ([Fig 1a](#)). Totally 755 explants (hypocotyls (350) & cotyledon (405) were cultured in preculture medium containing TDZ 0.044mg/l ([Fig 1b](#)). After two days of preculturing, they were infected with *Agrobacterium* along with ACS. Then the explants were placed in co-cultivation medium for the transfer of gene. After two

days, the explants were transferred to pre-selection medium containing cefotaxime. Then they were transferred onto selection medium containing kanamycin and cefotaxime. After 3 weeks of incubation on selection medium with growth regulators, callus grew profusely along the surface of the infected explants (Fig 1c). In contrast, the non-transformed turned necrotic. Transgenic calli from the former could be selected efficiently in the presence of 100 mg/l kanamycin, as has been reported earlier for leaf explants (Rotino and Gleddie, 1990). After three rounds of selection, 55 explants showed proliferation and they were transferred into regeneration medium. After the shoots are developed (Fig 1d), they were transferred onto the rooting medium supplemented with IBA which facilitated the development of the roots (Fig 1e). Plants with well developed roots were transferred greenhouse for hardening (Fig. 1f). A total ten plants were recovered and maintained in the transgenic greenhouse.

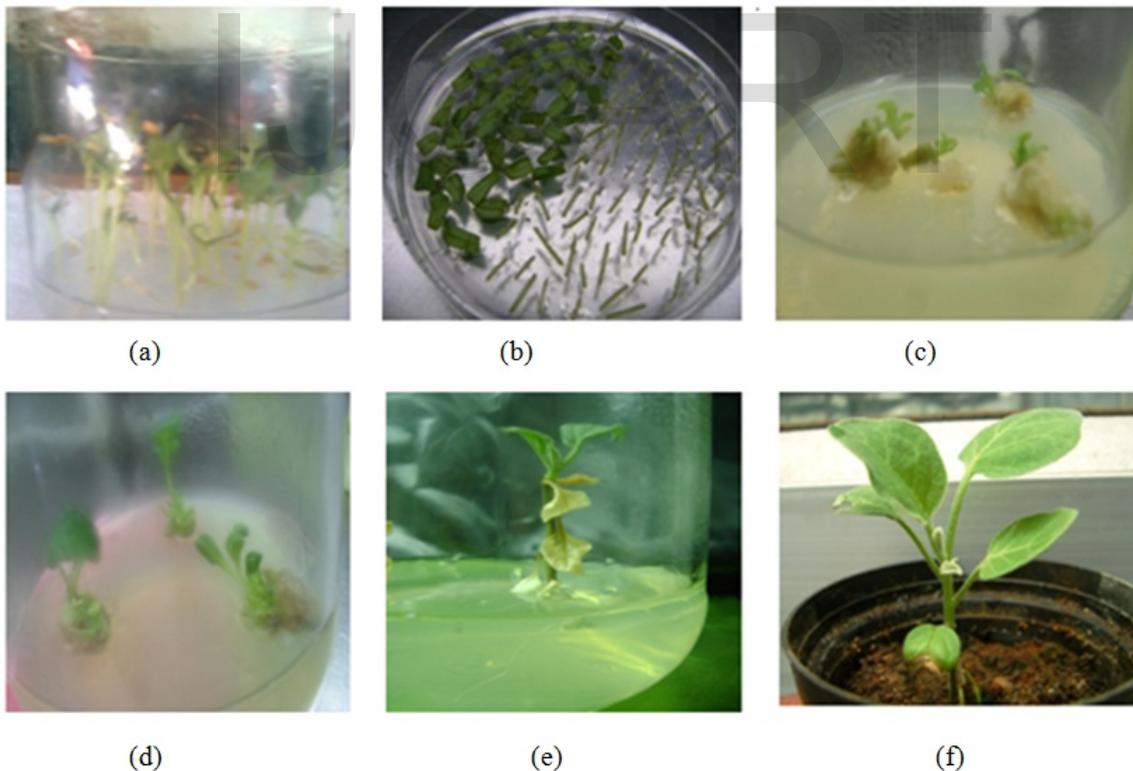


Fig. 1: Genetic Transformation of brinjal explants with *cry2Ax1* using *Agrobacterium*; a) seedling on 1/2 MS medium; b) Pre-culture; c) Co-cultivated explants on selection media d) Regenerated shoots on shooting medium; e) Regenerated plants on rooting medium and f) Regenerated plants

Molecular analyses of transgenic plants

The presence of transgenes, *cry2Ax1* and *nptII* in two putative transgenic plants was confirmed through PCR analysis using gene specific markers (Fig 2a&2b). The presence of *cry2Ax1* gene was confirmed by the amplification of 600bp internal fragment in the PCR experiment. The PCR analysis indicated that selection of calli at 100 mg /l kanamycin was sufficient to eliminate escapes.

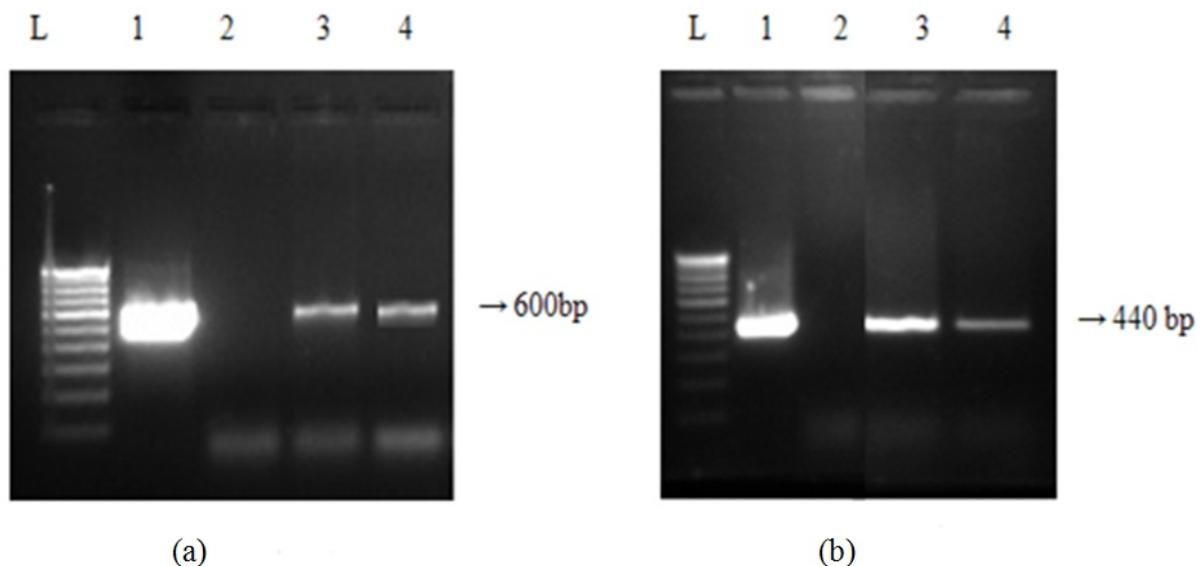


Fig. 2: Molecular analyses of transgenic plants a) PCR amplification of *cry2Ax1* and b) PCR amplification of *nptII*(L - 100bp ladder ;1 - Positive control; 2 - Negative control; 3 - Sample 1; 4 - Sample 2)

DISCUSSION

In the present study, results of our experiments confirmed earlier observations that lowering bacterial density (Srivastava et al., 1988; Henzi et al., 2000) and preculture of explant on callus inducing medium (Sangwan et al., 1992; Ovesna et al., 1993) help to improve transformation frequency. The phenolic compound acetosyringone has been known to induce vir gene ([Shimoda et al., 1990](#)) and increase transformation frequency. Although, addition of acetosyringone to bacterial culture medium was not beneficial, its presence during co-culture stage was found to improve transformation efficiency (Henzi et al., 2000). Co-cultivation temperature and duration are also in agreement with the earlier report of Metz et al. (1995). This study implies that, both callus induction and shoot regeneration were achieved at same kanamycin level in the transformed. This is in contrast to the observations of [Curtis et al. \(1999\)](#) who found that organogenic calli initiation required a reduction in kanamycin concentration from 200 mg /l to 100 mg /l. When the transgenic calli were separated from the parent explant and sub cultured on fresh media, several shoot buds were visible on the surface of callus. These subsequently elongated at a low rate. In contrast, transgenic buds elongated quickly (within 15 days) into shoots when cultured on basal media. PCR with *nptII* primers provided a rapid means of screening regenerants. Fragments of the predicted size were amplified, thereby it was confirmed that the presence of gene in the plant genome ([Fig 2a&2b](#)).

CONCLUSION

Agrobacterium tumefaciens-mediated transformation is an effective and widely used approach to introduce desirable genes into plants. The brinjal fruit and shoot borer (FSB) is the most destructive insect pest causing extensive damage to brinjal yield. To control this

insect pest, farmers all over the world use large quantities of chemical insecticides singly or in combination. The extensive use of chemical pesticides causes environmental threats. The objective of the present study was to develop brinjal resistant to shoot and fruit borer.

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