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Agrobacterium-mediated transformation of eggplant (*Solanum melongena* L.) using cotyledon explants and coat protein gene of *Cucumber mosaic virus*

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Genetic transformation of eggplant (*Solanum melongena* L.) was attempted using cotyledon explants and *Agrobacterium tumefaciens* (strain LBA4404) carrying *Cucumber mosaic virus* (CMV) coat protein (CP) gene construct under the control of CaMV 35S promoter in p*ROK*2 binary vector. A total of 110 cotyledon explants were employed in 4 independent co-cultivation events, of which 66 putative transgenic plants were established in glasshouse. The integration of CMV-CP transgene in the genome of transgenic plants was confirmed by polymerase chain reaction (PCR) using CMV-CP specific primers. Southern hybridization tests with CMV-CP specific probe confirmed the insertion of single or double copy number of transgene in their genome, which successfully transcribed and translated 26 kDa protein. The regenerated transgenic plants were self-pollinated and grown under glasshouse conditions. T_0 generation transgenic plants showed a significant degree of resistance against CMV when screened by challenge inoculations.

Keywords: *Agrobacterium tumefaciens*, coat protein gene, cotyledon explants, *Cucumber mosaic virus*, eggplant, genetic transformation, *Solanum melongena*

Introduction

Eggplant (*Solanum melongena* L.), commonly known as brinjal in India, is a popular vegetable crop grown extensively in India along with other Asian, European and American countries[1]. Beside its high consumption as vegetable, eggplant is recommended for the ailment of diabetes, arthritis, asthma and bronchitis[2]. The production of eggplant is severely affected by several phytopathogens including viruses. The natural infections of various viruses on eggplant have been reported time to time from India and abroad. These include *Cucumber mosaic virus* (CMV)[3-7], *Potyvirus*[8-9]; *Eggplant mottled crinkle virus*[10-11], *Carlavirus*[12] and *Alfalfa mosaic virus*[13]. Among them, CMV has been recorded more frequently and sometimes it causes epidemic in eggplant and other solanaceous crops, which resulted in huge economic losses to the growers[6,14].

The use of resistant or tolerant cultivars as control measure of virus diseases has been suggested earlier[1]. However, neither the control of CMV nor the availability of any source of resistance gene has been reported in eggplant. For developing resistant varieties in eggplant, traditional breeding is limited by the sexual incompatibilities due to deleterious gene linkages, followed by difficulty in obtaining fertile progenies, besides the process takes a long time[15]. *Agrobacterium tumefaciens*-mediated transformation is an effective and widely used approach to introduce desirable genes into plants. There are reports on incorporation of marker and agronomical important genes in eggplant through *Agrobacterium*-mediated transformation[16-19]. However, no information exists in literature on transformation of eggplant utilizing viral gene/s. Therefore, *Agrobacterium*-mediated transformation of eggplant using coat protein (CP) gene of CMV and regeneration of transgenic plants have been attempted in the present study with the aim to develop resistance against CMV in eggplant.

Materials and Methods

Agrobacterium-mediated Transformation of Cotyledon Explants

For genetic transformation of cotyledon explants of eggplant cv. Pusa Purple Long, *A. tumefaciens* (strain LBA4404) harbouring p*ROK*2 binary vector carrying CMV-CP gene under the control of CaMV 35S constitutive promoter and *nos* terminator was used in this study[20]. Co-cultivation and transformation of

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cotyledon explants was carried out following slight modifications in protocols of Kumar and Rajam[18]. In this study, we used TDZ (0.1 mg/L) in combination with BAP (2.5 mg/L) and IAA (0.5 mg/L) in MS medium throughout the regeneration process. For selection of transformants, 50 mg/L kanamycin and 250 mg/L cefotaxim was used in the study instead of 30 mg/L kanamycin and 500 mg/L cefotaxim. *A. tumefaciens* carrying CMV-CP gene construct was grown for 48 h at 28°C with 200 rpm shaking in Luria Broth (LB) containing 50 mg/L kanamycin and 50 mg/L rifampicin. A pre-culture of the cotyledon explants was done for 2 d on MS medium. On the day of transformation, 48 h grown culture of chimeric *A. tumefaciens* was pelleted at a speed of 4500 rpm for 5 min at 4°C, then suspended and diluted to OD_{600nm} of 0.5 in liquid MS medium supplemented with 100 μ*M* acetosyringone. This bacterial suspension was used for infecting the explants for 30 min and then explants were blot dried onto a sterile filter paper and transferred onto Petridishes containing MS medium. Petridishes were sealed with parafilm and co-cultivated for 2 d at 25±2°C in the dark.

The co-cultivated explants were rinsed with sterile distilled water to remove bacterial contamination, blot dried and were transferred to selection medium (MS medium supplemented with 2.5 mg/L BAP, 0.5 mg/L IAA, 0.1 mg/L TDZ, 3% sucrose, 0.8% agar, 50 mg/L kanamycin and 250 mg/L cefotaxim) for shoot induction. Subculturing onto fresh medium was carried out every fortnight. Once the *Agrobacterium* contamination was brought under control, cefotaxim was eliminated from the media. Shoots were separated and transferred to rooting medium (half strength MS) containing 50 mg/L kanamycin. Rooted plantlets were transferred to pots containing sterile soil, sand and vermiculite (1:1:1, v/v/v) and maintained in glasshouse.

Transgene Integration and Copy Number of Putative Transgenic Plants

The presence of CP transgene in transformants was checked by PCR using CMV-CP gene specific primers. Total DNA from transgenic plants was isolated by the method described earlier[21]. PCR was performed in a 50 μL reaction containing ~20 ng of DNA template, 25 p*M* each of up-stream and down-stream primer, 0.2 *M* dNTPs each and 3 U *Taq* DNA polymerase (Genei Pvt. Ltd., India) in a thermal cycler (PTC200 MJ Research, USA). The PCR conditions were: denaturation at 94°C for 5 min, followed by 25 cycles of amplification with denaturation at 94°C for 1 min, annealing at 52^0C for 1 min and extension at 72°C for 1 min and a final extension of 7 min at 72°C. PCR products were electrophoresed in 1% agarose gel with a DNA marker: Lambda DNA digested with *Eco*R I/*Hind* III (Genei, Pvt. Ltd., India). To confirm the identity of PCR amplicons and integration of CP gene in the putative transgenic plants, DNA amplicons were blotted onto nylon membrane following the procedure[22]. The homologous probe was prepared by following the random primer labeling method[23] using CP gene of CMV strain[23] and allowed to hybridize with the amplicons.

To know the copy number of CP gene in the genome of transgenic plants, total DNA from the transgenic plants was digested by *Hind* III restriction enzyme. The plasmid DNA of disarmed p*ROK*2 (as positive control) and total DNA of un-transformed healthy eggplant (as negative control) was also cleaved by *Hind* III. The products were blotted onto nylon membrane following the protocol[22] and allowed to hybridize with CMV-CP specific probe.

Transgene Expression by Northern Hybridization and Western Blot Immune Assay

To detect the transcription of integrated CP gene, total RNA was extracted from 100 mg fresh leaf tissues of transgenic plants following the protocol[24] and RNA was suspended in 50 μL nuclease-free water. A total of 10 μg RNA was blotted onto nylon membrane following procedure[22] and allowed to hybridize with CMV-CP gene specific probe prepared as described earlier.

For translation analysis of CP, total protein was isolated from the leaf following the protocol[22]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by following the procedure described earlier[22]. The protein bands were transferred onto PVDF membrane and Western blot immunoassay was done using the antiserum of CMV (PVAS 242a, ATCC, Manassas, VA) as primary antibody, anti-rabbit IgG raised in goat conjugated with alkaline phosphatase enzyme (Sigma Aldrich, St. Louis, USA) as secondary antibody and p-Nitrophenyl phosphate (Sigma Aldrich, St. Louis, USA) as substrate. The translation of gene was judged by comparing with positive control.

Evaluation of Resistance in T_0 Generation of Transgenic Plants

In total 66 regenerated putative transgenic plants of four lines (A: 1-12, B: 1-18, C: 1-16, D: 1-20) were

challenged by CMV by mechanical inoculations using leaf crude sap (1:10 diluted) of *Nicotiana tabacum* cv. White Burley on which CMV-A[20] culture was maintained. A set of 20 seedlings of untransformed eggplant was also challenged as control. The challenged plants were observed for appearance of symptoms, if any, till 95 d of post inoculation.

Results

Genetic Transformation and Regeneration of Transgenic Plants

A high frequency shoot regeneration system employing cotyledon explants of *S. melongena* cv. Pusa Purple Long was obtained using MS medium supplemented with 2.5 mg/L BAP, 0.5 mg/L IAA and 0.1 mg/L TDZ. This optimized protocol was further used for the genetic transformation of eggplant (data not shown).

For genetic transformation, cotyledon explants were co-cultivated with *A. tumefaciens* containing expression cassette of CMV-CP gene in p*ROK*2 binary vector[20] following the protocol[18] with slight modifications: TDZ (0.1 mg/L) in addition of BAP (2.5 mg/L) and IAA (0.5 mg/L) supplemented with MS medium throughout the regeneration process. The selection of transformants was done by 50 mg/L kanamycin and 250 mg/L cefotaxim instead of 30 mg/L kanamycin and 500 mg/L cefotaxim as used earlier by Kumar and Rajam[18]. A total of 110 cotyledon explants were employed in 4 independent co-cultivation events, of which 85 explants survived upon two sub-culturing on selection medium containing 50 mg/L kanamycin. Explants which did not show any signs of regeneration or callus initiation, even after 2-3 successive transfers on selection medium were discarded.

Percentage survival of transformed explants shoots and plantlets of eggplant are detailed in Table 1. The 77.27% (85/110) explants survived on selection medium, from which 60% (51/85) showed shoot initiation. The 41.17% (21/51) explants were finally able to proliferate by direct shoot regeneration from the cut ends of explants after 15-20 d (Figs 1a & b). Such 21 explants from 4 different events (A: 5, B: 5, C: 6, D: 5) eventually produced 76 shoots (A: 15, B: 21, C: 18, D: 22) after 1 month (Figs 1c & d) with efficiency of 3.6 shoots per explant. These shoots excised at their 4-5 leaf stage from the base of mother explant, omitting callus, could be rooted successfully in rooting medium (Fig. 1e) with the rooting efficiency of 92.10% (70/76). Rooted shoot-plantlets were subsequently hardened in 1X Hoagland solution for 7 d in culture room conditions and then 67/70 plantlets were grown in vermiculite for another 10-12 d (Fig. 1f). The total of

Table 1—Percentage survival of transformed explants, shoots and plantlets of eggplant

Developmental stage	Transformed	
	Survived/total	Percentage
Explants survived after co-cultivation	85/110	77.27
Explants showed shoot initiation out of survived	51/85	60.00
Explants finally able to proliferate shoots	21/51	41.17
Total shoots proliferated from explants	76/21	361.90
Rooting of shoots	70/76	92.10
Hardening of rooted shoots/plantlets in 1X Hoagland solution	67/70	95.71
Number of established plants in vermiculite	66/67	98.50

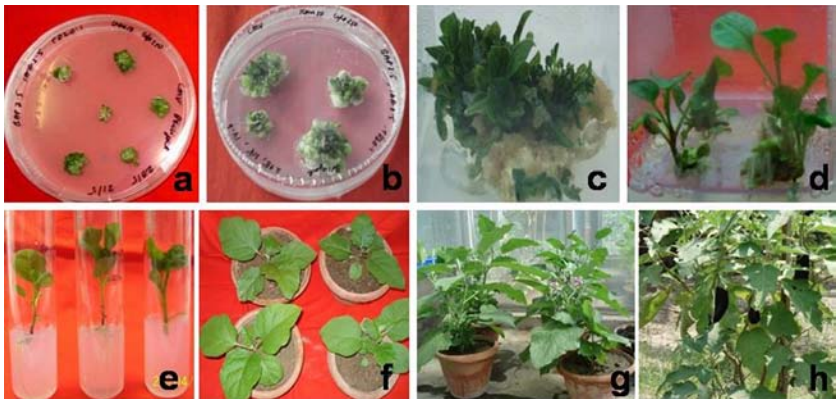

Fig. 1—Different stages of *in vitro* development of transgenic eggplant (*A. tumefacie*-mediated transformed cotyledon explants): Initiation of callusing at cut ends of cotyledon explants, followed by greenish compact callus and shoot proliferation after 15-20 d (a-b); vigorous shoot proliferation and shoot elongation after 1 month (c-d); 4-5 leaf stage shoots and their rooting (e); acclimatization of rooted shoots (f); & transformed plants showing flowering and fruiting (g-h).

66/67 regenerated putative transgenic plants of four lines (A: 1-12, B: 1-18, C: 1-16, D: 1-20) were finally established in glasshouse which showed similar morphology, viz., leaf size, shape, plant growth and flowering (Figs 1g & h) as compared to untransformed (healthy) plants.

Validation of Putative Transgenic Plants by PCR and Southern Hybridization

In PCR analysis, of 17 tested putative transgenic plants (at least one representative of each line), 11 showed the expected size (~650 bp) amplicons of CP gene in their genome, similar to the positive control. However, no such amplicon was obtained in negative control (untransformed eggplant) (Fig. 2a). The identity of amplicons was confirmed by Southern blot hybridization tests using CMV-CP probe, which showed strong signals of hybridization (Fig. 2b), confirming the integration of the CP gene into plant genome in the first step of transgene analysis.

To know the copy number of CP gene in the genome of transgenic plants, the total DNA of 5 selected transgenic plants (at least one plant from each line) along with one untransformed (negative control) and CMV infected tobacco (positive control) were digested by *Hind* III restriction enzyme and allowed to hybridize with CP specific probe. The DNA of transgenic lines hybridized well with the CP probe and resulted in strong signals indicating one or two copy of transgene in the genome of all five transgenic plants. Single copy number was found in B1 and D2 transgenic plants, while double copy number in A1, C1 and D1 plants (Fig. 3).

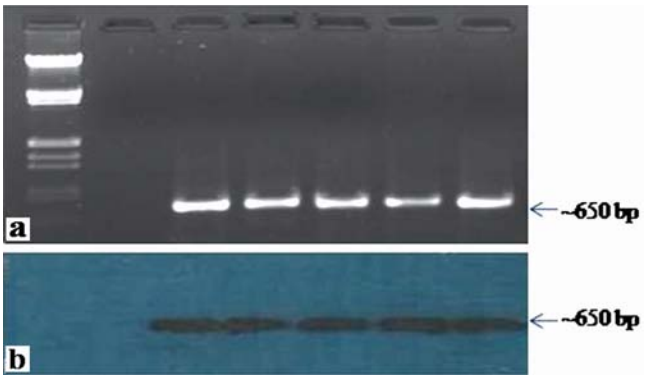

Fig. 2 (a & b)—a. PCR analysis of T_0 transgenic plants with CMV-CP specific primers showing positive amplification of expected size of ~650 bp; b. Southern blot hybridization of PCR products of transgenic plants with CMV-CP specific probe showing strong signal. (Lanes A1, B1, C1, D1 & D2: Putative transgenic plants; Lanes NC & M: Negative control and DNA marker (λ-DNA digested with *Eco*R I/*Hind* III), respectively).

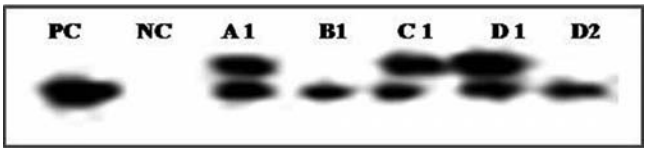

Fig. 3—Southern blot hybridization tests of total genomic DNA of transgenic plants digested with *Hind* III with CMV-CP specific probe showing single or double copy number of CP transgene in their genome. (Lanes A1, B1, C1, D1 & D2: Putative transgenic plants; Lanes NC & PC: Negative and positive controls, respectively)

Validation of Transcription and Translation of CP Gene

Total RNA of selected plants from each individual line (A1, B1, C1 and D1) was isolated and allowed to hybridize with probe specific to CP gene. Transgenic plants of all four transgenic lines showed hybridization signal, which confirmed the transcription of CP gene. Similar observation was made in case of positive control plant (CMV infected tobacco) (Fig. 4).

During Western blot immunoassay of transgenic plants, all the tested transgenic plants showed presence of a band of 26 kDa coat protein; similar as in case of positive control (Fig. 5). The level of CP was almost equal in all plants. However, no such response was observed in un-transformed healthy eggplant taken as negative control. A band of 52 kDa was also observed as a dimer of 26 kDa protein (Fig. 5).

Fig. 4—Northern blot hybridization of total RNA of transgenic plants with CMV-CP specific robe showing positive signals of transcription of CMV-CP gene. (Lanes A1, B1, C1 & D1: Putative transgenic plants; Lane PC is positive control)

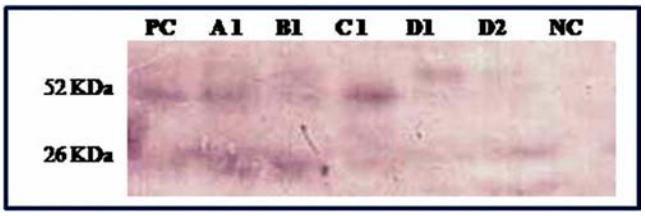

Fig. 5—Western blot immuno-assay of transgenic plants using antiserum of CMV-CP showing bands of 26 kDa protein representing the size of coat protein (CP) of CMV in all the transgenic plants. (Lanes A1, B1, C1, D1 & D2: Putative transgenic plants; Lanes NC & PC are negative and positive controls, respectively)

Evaluation of Resistance in Transgenic Plants in T_0 Generation

The data on resistance, tolerance or susceptibility to CMV were recorded based on the visual observation of symptoms on challenged transgenic plants. Symptoms appeared after 20 d post-inoculation were considered susceptible, while delayed symptoms till 60 d post-inoculation and appearance of no symptoms were considered tolerant and completely resistant, respectively. The data obtained on level of resistance/tolerance to challenge inoculations of CMV in T_0 generation transgenic plants are summarized in Table 2. The total resistance (sum of complete resistant and tolerant plants) against CMV achieved in the present study was as high as 70% in transgenic plants of line C, followed by 67, 65 and 61% in plants of lines B, D and A, respectively, as compared to the untransformed control plants (Table 2). The data on resistance of limited number of transgenic plants of four lines clearly indicated that significant degree of resistance/tolerance against CMV was achieved in transgenic eggplants in T_0 generation.

Discussion

CMV is a major constraint for cultivation of solanaceous crops including eggplant which resulted in huge economic losses[6,8]. Therefore, the genetic transformation of *S. meolngena* cv. Pusa Purple Long was attempted using cotyledon explants with the aim to develop resistance against CMV. *A. tumefaciens* (strain LBA4404) harboring p*ROK*2 binary vector as expression cassette carrying the CMV-CP gene under the control of CaMV 35S constitutive promoter and *nos* terminator was used in this study, as Srivastava and Raj[20] demonstrated successful resistance in *Nicotiana benthamiana* model plants by using the same construct. These transgenic *N. benthamiana* plants exhibited complete resistance, remained symptomless throughout life and showed reduced or no virus accumulation after CMV challenge. Keeping in mind the successful resistance against CMV in tobacco model plant, the same strategy has been attempted for development of resistance in commercially important eggplant

Table 2—Screening of resistance/tolerance in T_0 transgenic lines of eggplant against challenged inoculations by CMV-A[20]

Line	Total no. of plants taken	Susceptible plants*	Tolerant plants*	Complete resistant plants*	Total resistance (%)
A	12	4 (33.3)	7 (58.3)	1 (8.3)	61.6
B	18	5 (27.7)	10 (55.5)	3 (16.6)	67.1
C	16	4 (25.0)	10 (62.5)	2 (12.5)	70.0
D	20	6 (30.0)	11 (55.0)	3 (15.0)	65.0
Control	20	19 (95.0)	1 (05.0)	- (00)	00.0

Susceptible plants = Symptoms appeared after 20 d post inoculation, Tolerant plants = Delayed symptoms till 60 d post inoculation, Complete resistant plants = No symptoms appeared till complete life, Control = Untransformed challenged eggplants plants.
*Figure in paranthesis show per cent value

After the successful optimization of regeneration protocol from cotyledon explants of eggplant cv. Pusa Purple Long, the transformation methods were optimized, which is an important prerequisite step for the development of transgenic plants. Co-cultivation and transformation of cotyledon explants of eggplant was carried out following the slight modifications in protocols of Kumar and Rajam[18]. However, we used TDZ (0.1 mg/L) in addition to BAP (2.5 mg/L) and IAA (0.5 mg/L) in MS medium for shoot initiation as well as shoot elongation. For selection of transformants, 50 mg/L kanamycin was able to check the regeneration of untranformants instead of 30.0 mg/L kanamycin previously used by Kumar and Rajam[18]. Cefotaxim at a concentration of 250 mg/L was used to control the growth of *Agrobacterium* during subcultures of transformants instead of 500 mg/L used earlier.

During transformation from 4 different events, 77.27% cotyledon explants of eggplant survived on selection medium and after two subcultures only 60% of them showed shoot initiation, while rest of them did not show any sign of regeneration and were discarded. Later, only 41.17% explants were proliferated into shoots with an efficiency of 3.6 shoots per explant. In total of 66/67 regenerated putative transgenic plants of four lines (A: 1-12, B: 1-18, C: 1-16, D: 1-20) were finally established in the glasshouse.

Screening of the putative transgenic plants by PCR and Southern blot hybridization tests confirmed the integration of CMV-CP transgene in their genome in single or double copy numbers. The Northern and Western blot immuno-assay analyses of these transgenic plants also confirmed the expression of transgene leading to translation of 26 kDa protein. The T_0 generation transgenic plants when screened for resistance by challenge inoculations of CMV, using a

high inoculum (1:10 dilution of virus culture), showed significant level of resistance/tolerance against CMV, the homologous virus, under glass house conditions.

Attempts to develop resistance against CMV using CP gene was first initiated in 1988 by Cuozzo and co-workers[25] and generated transgenic tobacco lines, which demonstrated high degree of resistance against CMV. After that several methods employing the use of genetic engineering have been successfully demonstrated based on the hypothesis of pathogen derived resistance[26]. Recently, Srivastava and Raj[20] demonstrated protein mediated resistance in transgenic *N. benthamiana*, a model plant, by using CP gene of CMV. The present protocol developed for the eggplant transformation could have a wide scope in future in the generation of transgenic plants using other gene of interest.

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