Plant Cell Rep (2013) 32:1191–1198
DOI 10.1007/s00299-013-1415-4

ORIGINAL PAPER

Efficient genetic transformation of okra (*Abelmoschus esculentus* (L.) Moench) and generation of insect-resistant transgenic plants expressing the *cry1Ac* gene

M. Narendran · Satish G. Deole · Satish Harkude · Dattatray Shirale · Asaram Nanote · Pankaj Bihani · Srinivas Parimi · Bharat R. Char · Usha B. Zehr

Received: 1 February 2013 / Revised: 2 March 2013 / Accepted: 4 March 2013 / Published online: 17 March 2013

Abstract
Key message ***Agrobacterium*-mediated transformation system for okra using embryos was devised and the transgenic *Bt* plants showed resistance to the target pest, okra shoot, and fruit borer (*Earias vittella*).**
Abstract Okra is an important vegetable crop and progress in genetic improvement via genetic transformation has been impeded by its recalcitrant nature. In this paper, we describe a procedure using embryo explants for *Agrobacterium*-mediated transformation and tissue culture-based plant regeneration for efficient genetic transformation of okra. Twenty-one transgenic okra lines expressing the *Bacillus thuringiensis* gene *cry1Ac* were generated from five transformation experiments. Molecular analysis (PCR and Southern) confirmed the presence of the transgene and double-antibody sandwich ELISA analysis revealed Cry1Ac protein expression in the transgenic plants. All 21 transgenic plants were phenotypically normal and fertile. T_1 generation plants from these lines were used in segregation analysis of the transgene. Ten transgenic lines were selected randomly for Southern hybridization and the results confirmed the presence of transgene integration into the genome. Normal Mendelian inheritance (3:1) of *cry1Ac* gene was observed in 12 lines out of the 21 T_0 lines. We selected 11 transgenic lines segregating in a 3:1 ratio for the presence of one transgene for insect bioassays using larvae of fruit and shoot borer (*Earias vittella*). Fruit from seven transgenic lines caused 100 % larval mortality. We demonstrate an efficient transformation system for okra which will accelerate the development of transgenic okra with novel agronomically useful traits.

Keywords *Abelmoschus esculentus* · *Agrobacterium tumefaciens* · *Bt*—*Bacillus thuringiensis* · Shoot and fruit borer · Transgenic okra

Communicated by L. Peña.

M. Narendran · S. G. Deole · S. Harkude · D. Shirale · A. Nanote · P. Bihani · S. Parimi · B. R. Char (✉) · U. B. Zehr
Mahyco Research Centre, Maharashtra Hybrid Seeds Company Ltd., P.O. Box-76, Dawalwadi, Jalna-Aurangabad Road, Jalna 431203, Maharashtra, India
e-mail: bharat.char@mahyco.com

Introduction

Abelmoschus esculentus (L.) Moench., commonly known as okra or lady's finger is an important vegetable crop cultivated in many countries (Joshi and Hardas 1956). In India, okra is a major vegetable crop and 4.8 million tonnes of pods are produced from an area of 0.45 million hectares. Okra is a rich source of nutrients, and pods have been assessed to have protein levels of 4.41–4.55 % (Düzyaman and Vural 2003). As a member of the Malvaceae family, okra is susceptible to a number of pests and diseases (Hamon and van Sloten 1995). Yellow vein mosaic virus (YVMV) causes the most serious disease of this crop in the majority of okra growing regions (Ali et al. 2000), whereas the shoot and fruit borer- *Earias vittella* (Fabricius) is the most serious insect pest of this crop in Asia. Classical and mutation breeding programs aimed at resistance to diseases have rarely succeeded in okra (Rajamony et al. 2006; Joshi et al. 1960; Jambhale and Nerkar 1981). Genetic improvement by conventional plant breeding is protracted due to the lack of resistance sources to insect pests and diseases in okra germplasm. However, with the establishment of genetic engineering methods, a number of genes for resistance to insect pests, diseases, and nutritional enrichment can be incorporated into this crop.

Okra is severely affected by the shoot and fruit borer, and the larvae bore into shoots or fruits (pods) and consume the internal contents, causing the withering of the plant and reduction in marketable value of the pods (Brar et al. 1994). The extent of crop loss in okra due to infestation of *Earias* spp. was estimated in the range of 12–54 % in different parts of India (Brar et al. 1994; Shukla et al. 1997; Krishnakumar and Srinivasan 1987; Verma et al. 1984; Satpathy and Rai 1998). The severity of damage varies from place to place during different seasons. Farmers often apply pesticides on the crop on alternate days in some localities, where pest pressure is high. In India, more than 400 genotypes have been evaluated for resistance against shoot and fruit borer, and none of these lines could be rated as highly resistant or immune (Dhankhar et al. 2009). Even though relative intervarietal variation in resistance has been observed in okra against shoot and fruit borer, none of the varieties showed complete resistance in these studies (Memon et al. 2004). While genes from *Bacillus thuringiensis* have been successfully engineered into crop plants to get resistance to specific insect pests in a number of species (Fischhoff et al. 1987; Metz et al. 1995; Kumar and Sharma 1994), this has not been realized in okra due to the absence of a reliable transformation method.

The development of a method for the genetic transformation of okra is of importance because it can be used for the transfer of beneficial genes into this crop. The okra genome is quite unique with a large number of chromosomes $2n = 130$ (Nwangburuka et al. 2011; Joshi and Hardas 1956) and precocious chromosome movement observed during metaphase in wide hybridization (Fatokun 1987). The absence of a transformation system has hampered the progress in okra genetic engineering and genomic research. Availability of a genetic transformation system will facilitate research aimed at exploring the okra genome.

The prerequisite of genetic transformation is an efficient tissue culture system that allows selection and regeneration of transgenic plants. Tissue culture-based direct shoot regeneration from cotyledon and cotyledonary node explants were reported by Mangat and Roy (1986), and also reported regeneration of okra plants from callus tissue derived from cotyledonary axil (Roy and Mangat 1989). Although we regenerated plants from cotyledon explants using this tissue culture protocol, we were unable to utilize these protocols for establishing an efficient genetic transformation system for okra. Ganesan et al. (2007) reported somatic embryogenesis and plant regeneration of okra through suspension culture. We standardized a new tissue culture protocol for okra using zygotic embryo explants. This is based on direct multiple shoot bud induction from plumule of embryo and integration of this system with *Agrobacterium*-mediated transformation.

Here, we report the establishment of a reliable genetic transformation system for okra and development of insect-resistant transgenic *Bt* okra lines.

Materials and methods

Plant material

Okra (*Abelmoschus esculentus*) genotype used in this research was a proprietary in-bred line of Maharashtra Hybrid Seeds Company Ltd., Jalna, India. The seeds were surface sterilized in 0.1 % (weight/volume) $HgCl_2$ in distilled water for 30 min. These seeds were washed thrice in sterile distilled water and imbibed overnight in sterile water. The embryos were isolated from these seeds in aseptic conditions and rinsed thrice in sterile distilled water, blotted dry on filter paper, and placed on sterile filter paper soaked in sterile water.

Agrobacterium–mediated transformations

Transformation using cry1Ac and nptII genes

The plasmid CAMBIA 2300 carried the c*ry1Ac* gene driven by an enhanced CaMV 35S promoter (Kay et al. 1987; Perlak et al. 2001) and the *nptII* gene as a plant selectable marker in the T-DNA (Fig. 1). This plasmid was introduced into the *Agrobacterium tumefaciens* strain EHA105 and used for the transformations. *Agrobacterium* was inoculated into 25 ml 2YT medium (Sambrook et al. 1989) containing 50 mg/l kanamycin and 10 mg/l chloramphenicol and kept on a rotary shaker at 28 °C until an optical density (600 nm) of 1.8 was reached, and this suspension in 2YT medium was used in inoculating the explants. A total of five experiments were performed using this construct, and the numbers of explants used in each of these experiments are shown in Table 1.

Inoculation of explants with Agrobacterium and shoot bud induction

Isolated embryos were wounded by stabbing 2–3 times at the plumule with a syringe needle (23G, 1″, 0.6 × 25 mm) and inoculated in *Agrobacterium* suspension (EHA105 carrying the *cry1Ac* and *nptII* genes) for 15 min, blotted dry on sterile filter paper, and transferred at the rate of 20 embryos per petridish on MSB3 medium (MS salts—Murashige and Skoog 1962, B5 vitamins—Gamborg et al. 1968, Zeatin 2 mg/l, agar 0.8 %, sucrose 3 %, pH of all the media adjusted to 5.8).

These petri dishes were incubated at 26 ± 2 °C for 2 days co-cultivation. After 2 days of co-cultivation, the

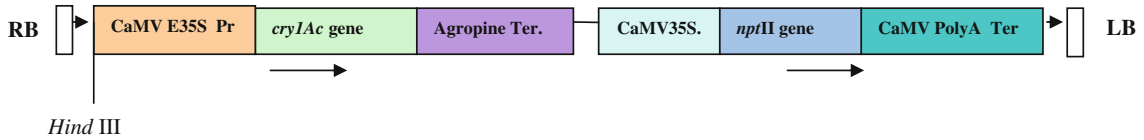

Fig. 1 Schematic representation of T-DNA of plasmid carrying *cry1Ac* gene. *cry1Ac* gene driven by E35S CaMV promoter and *npt*II gene driven by CaMV 35S promoter. *LB* left border, *RT* right border

Table 1 Transformation frequency

Experiment no.	No. of explants used	No. of transgenic plants regenerated	Transformation frequency (%)	Standard error (SE)
1	190	4	2.10	0.96
2	207	7	3.38	1.25
3	237	3	1.26	0.69
4	280	4	1.42	0.67
5	200	3	1.50	0.82

embryos were washed in MSBL medium (MS salts, B5 vitamins, sucrose 3 %, cefotaxime 500 mg/l) to remove the *Agrobacterium* and the plumule portions (~3 mm) were excised from the embryos. These plumules were transferred on MSB2 medium (MS salts, B5 vitamins, Zeatin 2 mg/l, agar 0.8 %, sucrose 3 %, kanamycin 50 mg/l, cefotaxime 500 mg/l) and regenerated green multiple shoot buds were then transferred on fresh MSB2 medium for further development. Subcultures were performed at 3 weeks interval and 16 h photoperiod, 26 ± 2 °C and light intensity of 3,000–4,000 lux were maintained throughout all the steps.

The multiple shoot buds developed from the plumule of the embryos were subcultured on MSB1 (MS salts, B5 vitamins, agar 0.8 %, sucrose 3 %, kanamycin 50 mg/l, cefotaxime 500 mg/l) medium for shoot bud elongation at intervals of 3 weeks. Shoot buds which were more than 2 cm were excised and subcultured on the fresh MSB1 medium for 3 weeks for elongation. Well-grown shoots (>3 cm) were also transferred onto MSB1 medium in bottles for rooting. Rooted plants were transplanted and hardened in foam cups filled with sterile potting mixture. After establishment of the plants in cups, they were shifted to large earthen pots or planted in soil in the greenhouse.

Positive controls for tissue culture, i.e., without inoculating the embryos in *Agrobacterium* were maintained (MSB3 medium) in each of the experiments. The multiple shoot buds developed were transferred on MSB medium for elongation and rooting. The rooted plants were hardened and used as non-transgenic control. The negative controls for transformation were also maintained in each of the experiments. These embryos were incubated on MSB3 for 2 days and then transferred on MSB2 medium.

ELISA analysis for Cry1Ac protein

Putative transgenic plants were tested for the expression of Cry1Ac protein using ELISA plate coated with monoclonal antibodies specific to Cry1Ac protein (DesiGen Diagnostics, Jalna, India). A single leaf disk of 1 cm diameter was taken from putative transgenic plants and control plants. These leaf samples were extracted in 500 µl of 1× PBST buffer. 50 µl of sample was loaded to each well in the precoated plate. After sample loading, 150 µl of polyclonal antibodies specific to Cry1Ac in the ratio 1:20,000 dilution in PBSTO (Phosphate Buffered Saline, Tween-20 and Ovalbumin 0.5 %) was added to each well. This plate was stored at 4 °C overnight. The overnight incubated plate was washed thrice with PBST on the next day. 200 µl per well detection antibody labeled with alkaline phosphatase at 1:6,000 dilution in PBSTO was added to these washed plates. This plate was incubated for 2 h at room temperature and was washed thrice with PBST. Finally, 250 µl substrate buffer containing 1 mg/ml Para nitro phenyl phosphate was added per well, and the color development was recorded at 405 nm wavelength using an ELISA reader. The positive samples were selected on the development of OD (405 nm) value >0.2 (after 30 min incubation) by subtracting the blank value which was compared to the negative non-transgenic control. χ^2 test was performed to validate the data for 3:1 transgenic segregation ratio of *cry1Ac*.

PCR analysis

Genomic DNA was isolated from the young leaves of 19 transgenic lines (Cry1Ac, ELISA positive lines) and non-transgenic controls using DNeasy Plant Mini kit (Qiagen). The PCR amplification was carried out in a 20-µl reaction mixture containing ~50 ng of template genomic DNA, 100 µM of each dNTP, 3 µM of each primer, 1 U Taq DNA polymerase (Promega), 2 mM $MgCl_2$, and 1× DNA polymerase buffer. The sequences of primers for the amplification of 721 bp fragment of *npt*II gene was 5′-TCG GCT ATG ACT GGG CAC AAC AGA-3′ and 5′-AAG AAG GCG ATA GAA GGC GAT GCG-3′. Amplifications were carried out in a thermocycler programmed for 30 cycles. PCR amplification was performed by initial denaturation at 94 °C (5 min) followed by denaturation at

Fig. 2 Transformation and generation of fertile transgenic okra plants. **a** multiple shoot bud induction on selection medium, **b** rooted transgenic plant in vitro ready for hardening, **c** transgenic plants established in green house

94 °C (30 s), annealing at 57 °C (30 s), and extension at 72 °C (30 s), and finally holding at 72 °C (7 min) for extension. The PCR for the amplification 1.36 kb fragment of *cry1Ac* was conducted with a pair of proprietary primer sequence P1A primer: 5′-CTG CTC AGC GAG TTC GTG CC-3′ P1C primer 5′-GGT CTC CAC CAG TGA ATC CTG G-3′. The PCR products were subjected to electrophoresis on a 1 % agarose gel along with molecular weight marker.

Southern hybridization analysis

Genomic DNA (~10 μg) of 10 transgenic plants displaying segregation of transgene in a 3:1 ratio were extracted from young leaves of T_1 plants, and non-transgenic plant samples were extracted using a modified version of the CTAB method described by Doyle and Doyle (1987). DNA was digested with the restriction enzyme *Hin*dIII (New England Bio Labs) at 37 °C overnight. The enzyme *Hin*dIII cuts only once in the T-DNA (Fig. 1). Positive control was plasmid carrying *cry1Ac* gene which was digested with *Hin*dIII enzyme. The digested DNA and plasmid were separated on a 0.8 % Agarose gel for 4 h using 60 V. DNA samples were then transferred by blotting overnight to a positively charged nylon membrane (Roche). The following day, the membrane was hybridized using Digoxigenin labeled *cry1Ac* probe (1.3 kb fragment) at 65 °C overnight. The following day, the membrane was washed, blocked, and fluorescent signal detected according to manufacturer's instruction (Roche).

Insect bioassays

Eleven transgenic plants (T_1) displaying segregation of transgene in a 3:1 ratio were selected and assayed for their efficacy against okra shoot and fruit borer larvae. Young fruits (3 replications per line) from each of the eleven transgenic lines and non-transgenic control plants were used in the insect bioassays. The fruits used for the bioassays were 4–5 days after flowering and 6–8 cm in length. A

laboratory population of the okra shoot and fruit borer was used in the bioassays. Four 2nd instar larvae each were released on to each young fruit of transgenic lines and the control plant. The fruits were placed in a glass bottle, covered with a muslin cloth for aeration and placed in a growth chamber (14 L: 10D), maintained at 28 °C and 60 % relative humidity. Larval mortality was recorded after 5 days by cutting open the fruits. The statistical analysis of the data was done using PROC GLM procedure of SAS software.

Results

Transformation and selection of putative transgenic plants

In the present study, we investigated the possibility of using embryo explants and direct shoot bud initiation from plumule. The explants started enlarging in size during co-cultivation period. On the first selection step, (MSB2—for shoot bud regeneration medium) approximately 20 % plumules produced multiple shoot buds (data not shown); whereas the others did not survive (Fig. 2a). The multiple shoot buds produced on the first MSB2 selection were further subcultured on the same medium. These shoot buds were elongated and rooted on MSB1 medium (MSB1—for shoot elongation and rooting medium), while non-rooted shoots were again subcultured on MSB1 for rooting (Fig. 2b). The whole process of generating transgenic plants took 4–5 months. The transgenic plants were phenotypically normal and fertile (Fig. 2c).

In five experiments, a total of 1,114 embryo explants were used in transformations. The 64 putative transgenic plants which produced roots on MSB1 were screened by ELISA, and 21 plants were positive for Cry1Ac as assayed by ELISA (OD of >0.200 at 405 nm). Five experiments were performed, and based on the results obtained, the transformation efficiency of these experiments are shown in Table 1. The overall transformation frequency for these five experiments was calculated to be 1.88 %.

Negative controls were maintained in each experiment to ensure that kanamycin effectively suppressed growth of non-transgenic tissue at particular concentrations of antibiotic in the media. None of the embryos from the negative control experiments survived on the initial selection medium, MSB1. This indicated that a kanamycin concentration of 50 mg/l was sufficient to control the growth of non-transformed tissues in these experiments.

Molecular and genetic analysis

These ELISA positive plants were further screened by PCR for the presence of the T-DNA insert. PCR amplifications were undertaken and confirmed the presence of *nptII* and *cry1Ac* genes in ELISA positive lines for *cry1Ac*. This is evidenced by the presence of an amplified product of the expected sizes of amplified PCR products in the case of *nptII* and *cry1Ac* genes (data not shown).

Southern blot analysis was carried out on 10 ELISA (Cry1Ac) positive transgenic lines segregating transgene (*cry1Ac*) in 3:1 ratio and a non-transformed line. DNA samples were digested with *Hin*dIII which cuts only at one place in the T-DNA (Fig. 1). A 1,357 bp (*cry1Ac*) DNA fragment was labeled using DIG and used as probe. Southern hybridization revealed that the plants contain single or multiple transgene integrations and these lines come from independent transformation events. No hybridization was detected in DNA from the non-transformed line (Fig. 3).

Segregation data obtained observed from the selfing of 21 primary transgenic events (T_0) are given in Table 2. Chi square analysis was performed and the events in which the transgene segregated in a Mendelian ratio were identified. Of these plants, the *cry1Ac* gene segregated in Mendelian ratio of 3:1 (positive: negative) in 12 events. In the event OBT3, all 16 T_1 seedlings tested were ELISA positive and this may be due to multiple integrations of the transgene in the genome. Two primary transgenic events (OBT19 & OBT21) did not display inheritance of the transgene into the subsequent generation (T_1). The segregation ratio of 3:1 (presence of Bt protein: absence of Bt protein) was observed in 12 transgenic lines, whereas seven lines did not show segregation in a 3:1 ratio.

Insect bioassays

A total of eleven lines and a non-transgenic control were tested for their efficacy against okra shoot and fruit borer.

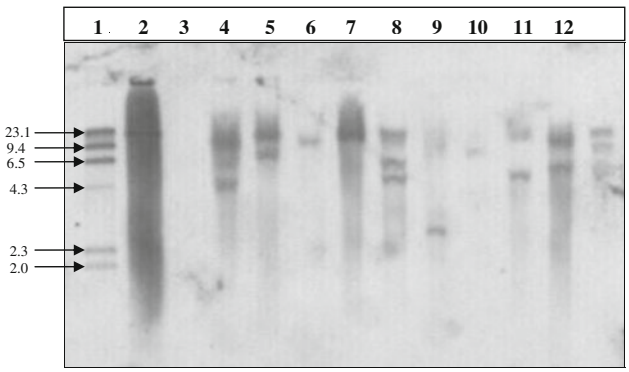

Fig. 3 Southern blot hybridization of non-transgenic and 10 independent transgenic okra lines using *cry1Ac* probe. From *left* to *right*. *Lane 1* M. DNA ladder (λ *Hin*dIII). *Lane 2* Plasmid carrying *cry1Ac* gene- positive control. *Lane 3* Non-Transgenic okra-negative control. Transgenic lines in the order *Lane 4* OBT14-5, *Lane 5* OBT15-1, *Lane 6* OBT12-4, *Lane 7* OBT16-2, *Lane 8* OBT7-2, *Lane 9* OBT6-3, *Lane 10* OBT5-2, *Lane 11* OBT18-6, *Lane 12* OBT2-6 and *Lane 13* OBT9-2

Table 2 Segregation analysis of *cry1Ac* gene in transgenic T_1 plants obtained from selfed T_0 lines

Sr. no.	Plant ID	Number of seedlings screened			χ^2 value (3:1)	*P* value
		Total	ELISA + ve	ELISA − ve		
1	OBT 1	17	16	01	3.30^{ns}	0.07
2	OBT 2	26	16	10	2.50^{ns}	0.11
3	OBT 3	16	16	00	5.33*	0.02
4	OBT 4	29	26	03	3.32^{ns}	0.07
5	OBT 5	17	13	04	0.01^{ns}	0.92
6	OBT 6	28	19	09	0.76^{ns}	0.38
7	OBT 7	21	16	05	0.01^{ns}	0.92
8	OBT 8	22	12	10	4.90*	0.03
9	OBT 9	20	17	03	1.06^{ns}	0.30
10	OBT 10	21	11	10	5.72*	0.02
11	OBT 11	22	10	12	10.24*	0.00
12	OBT 12	22	13	09	2.96^{ns}	0.08
13	OBT 13	12	03	09	16.00*	0.00
14	OBT 14	27	17	10	2.08^{ns}	0.15
15	OBT 15	25	03	22	52.92*	0.00
16	OBT 16	24	19	05	0.21^{ns}	0.65
17	OBT 17	22	14	08	1.50^{ns}	0.22
18	OBT 18	21	12	09	3.56^{ns}	0.06
19	OBT 19	28	00	28	GNI	–
20	OBT 20	29	07	22	39.55*	0.00
21	OBT 21	25	00	25	GNI	–

* Significant, *ns* non-significant at <0.05 and segregating in 3:1 and *GNI* gene not inheriting

The analyzed data demonstrated significant differences in survival/mortality between the transgenic and non-transgenic lines used in the assays. The fruits of transgenic line showed a high degree of efficacy against okra shoot and fruit borer larvae as compared to the fruits of non-transgenic line. The survival of okra shoot and fruit borer larvae was observed to be between 0.00 and 16.67 % in the transgenic lines, whereas 100 % survival was observed in the fruits of non-transgenic control (Fig. 4). There was 100 % mortality of the okra shoot and fruit larvae used in the assays, in the fruits of OBT02, OBT04, OBT05, OBT07, OBT09, OBT17, and OBT18 (Table 3). The mortality (%) was observed to be between 83.33 and 100.00 % in the fruits of transgenic lines.

Discussion

Okra (*Abelmoschus* sp.) is one of the most recalcitrant crop plants for genetic transformation and has resisted the genetic engineering efforts of many laboratories. To the best of our knowledge, this is the first report of genetic transformation of okra. The results of the present investigation demonstrate stable transformation of okra and generation of a number of transgenic lines.

Tissue culture based on direct plant regeneration from cotyledon and cotyledonary node explants of okra was reported by Mangat and Roy (1986). Regeneration of plants from callus tissue of okra was also reported by (Roy and Mangat 1989). In our laboratory, these protocols were

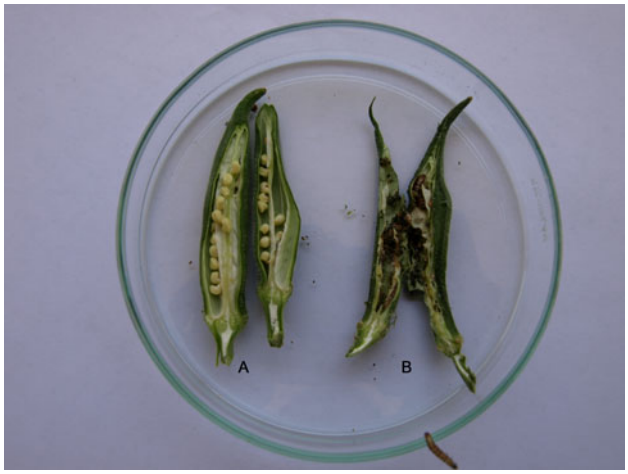

Fig. 4 Insect bioassay using fruits of transgenic *Bt* events and non-transgenic control with larvae of shoot and fruit borer. **a** Longitudinally dissected transgenic *Bt* fruit showing protection from insect damage, **b** longitudinally dissected control fruit showing the internal damage of fruit

Table 3 Effect of different treatments on *Earias vittella* larval survival and mortality

Treatment	Status of larvae	
	Percent live larvae	Percent dead larvae
Control	100.00 (1.57)^{a}	0.00 (0.00)^{c}
OBT02	0.00 (0.00)^{c}	100.00 (1.57)^{a}
OBT04	0.00 (0.00)^{c}	100.00 (1.57)^{a}
OBT05	0.00 (0.00)^{c}	100.00 (1.57)^{a}
OBT06	8.33 (0.17)^{bc}	91.67 (1.39)^{ab}
OBT07	0.00 (0.00)^{c}	100.00 (1.57)^{a}
OBT09	0.00 (0.00)^{c}	100.00 (1.57)^{a}
OBT12	16.67 (0.35)^{b}	83.33 (1.22)^{b}
OBT14	8.33 (0.17)^{bc}	91.67 (1.39)^{ab}
OBT16	8.33 (0.17)^{bc}	91.67 (1.39)^{ab}
OBT17	0.00 (0.00)^{c}	100.00 (1.57)^{a}
OBT18	0.00 (0.00)^{c}	100.00 (1.57)^{a}
P value	0.0001	0.0001
Significance	**	**

Values in parentheses corresponds to angular transformed values. Values followed by the same letter in the same column are not significantly different (LSD, $P = 0.05$)

** Statistical significance at 1–5 % level of significance

used in the transformation experiments, and transgenic calli positive for *GUS* gene expression were generated. However, our attempts to induce shoots from these calli failed. We identified embryo explants for tissue culture from the plumule portion which regenerated at a high frequency without interfering with the callus phase. Our results indicate that selection of embryo explants and the tissue culture-based transformation protocol are crucial for the successful transformation of okra.

Okra and cotton (*Gossypium* sp.) belong to the Malvaceae family. A majority of the protocols for the genetic transformation of cotton are based on somatic embryogenesis and such protocols are genotype-dependent and take relatively longer time periods for the regeneration of transgenic plants (Finer and McMullen 1990; Sunilkumar and Rathore 2001; Kumar et al. 2004). Many of such protocols developed for cotton are genotype-dependent, and long duration in the in vitro conditions increases chances of somaclonal variation. A protocol for the somatic embryogenesis in okra was reported by Ganesan et al. (2007). The time taken to generate transgenic okra plants in our experiments was 4–5 months. This protocol enabled direct regeneration of plants in short period, and thereby avoiding long periods of in vitro culture for somatic embryogenesis and increased chances of somaclonal variation.

The plant parts of the *Abelmoschus* are highly mucilaginous and DNA extraction is difficult using mini-preparations. Kochko and Hamon (1990) devised a method using dark-grown seedlings for yielding restrictable total DNA of okra. We addressed this issue using DNA extraction kits (DNeasy-Qiagen), and the DNA quality was suitable for the PCR amplifications of fragments of interest.

Our study also demonstrates the inheritance of the *cry1Ac* gene into the subsequent generation (Table 2). The tissue culture system developed for the delivery of T-DNA after injury of plumule portion of the embryo and direct regeneration of shoot buds from this portion enables high frequency and efficient genetic transformation of this crop. The transformation efficiency in these experiments was achieved as high as 3.38 % (Table 1).

The introduced genes were found to be inherited in the subsequent generation in a ratio 3:1 in 57 % of transgenic *Bt* okra lines indicating potentially useful single insertions or multiple insertions at one locus (Table 2). In one of the okra lines (OBT13), all the T_1 seedlings were positive for *cry1Ac* gene expression (ELISA) indicating possible multiple transgene integrations in the genome. In two transgenic events (OBT19 and OBT21), all T_1 seedlings screened were negative in ELISA indicating that the transgene did not inherit into the T_1 generation. Eleven *Bt* lines expressing *cry1Ac* gene were analyzed in insect bioassays. Seven events showed complete protection against okra shoot and fruit borer in feeding experiments.

Insect-resistant okra holds a promise as a strategy for durable resistance against the target pests. An efficient genetic transformation system will accelerate the ability to engineer okra with a number of value-added traits.

References

Ali M, Hossain MZ, Sarkern NC (2000) Inheritance of yellow vein mosaic virus (YVMV) tolerance in a cultivar of okra (*Abelmoschus esculentus* (L.) Moench). Euphytica 111:205–209

Brar KS, Arora SK, Ghai TR (1994) Losses in fruit yield of okra due to *Earias* spp. as influenced by dates of sowing and varieties. J Insect Sci 7:133–135

Dhankhar BS, Singh R, Kumar R, Kumar S (2009) Genetic improvement. In: Dhankhar BS, Singh R (eds) Okra handbook: global production, processing and crop improvement. HNB Publishing, New York, pp 125–158

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Düzyaman E, Vural H (2003) Evaluation of pod characteristics and nutritive value of okra genetic resources. Acta Hort (ISHS) 598:103–110

Fatokun CA (1987) Wide hybridization in okra. TAG Theor Appl Genet 74:483–486

Finer JJ, McMullen MD (1990) Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep 8:586–590

Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. Bio Tech 5:807–812

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Ganesan M, Chandrasekar R, Ranjitha Kumari BD, Jayabalan N (2007) Somatic embryogenesis and plant regeneration of okra (*Abelmoschus esculentus* (L.) Moench) through suspension culture. Biol Plant 51(3):414–420

Hamon S, van Sloten DH (1995) Okra. In: Smartt J, Simmonds NW (eds) Evolution of crop plants. Longman Scientific and Technical, Longman Group, UK

Jambhale ND, Nerkar YS (1981) Inheritance of resistance to okra yellow vein mosaic disease in interspecific crosses of *Abelmoschus*. Theor Appl Genet 60:313–316

Joshi AB, Hardas MW (1956) Alloploid nature of okra, *Abelmoschus esculentus* (L.) Moench. Nature 178:1190–1191

Joshi AB, Singh HB, Joshi BS (1960) Is yellow vein mosaic disease a nuisance in your bhendi, then why not grow 'Pusa Sawani'. Indian Frm 10:6–7

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299–1303

Kochko A, Hamon S (1990) A rapid and efficient method for the isolation of restrictable total DNA from plants of the genus *Abelmoschus*. Plant Mol Biol Rep 8:3–7

Krishnakumar NK, Srinivasan K (1987) Efficacy and economics of pest control of okra with conventional and synthetic pyrethroid insecticides. Indian J Plant Prot 15:81–83

Kumar PA, Sharma RP (1994) Genetic engineering of crop plants with *Bacillus thuringiensis* δ-endotoxin genes. J Plant Biochem Biotechnol 3:3–9

Kumar S, Dhingra A, Daniell H (2004) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol 56:203–216

Mangat BS, Roy MK (1986) Tissue culture and plant regeneration of okra (*Abelmoschus esculentus*). Plant Sci 47:57–61

Memon AJ, Abro GH, Syed TS (2004) Varietal resistance of okra against *Earias* spp. J Entomol 1:1–5

Metz TD, Roush RT, Tang JD, Shelton AM, Earle ED (1995) Transgenic broccoli expressing a *Bacillus thuringiensis* insecticidal crystal protein: implications for pest resistance strategies. Mol Breed 1:309–317

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nwangburuka CC, Kehinde OB, Adegbite OA, Denton OA (2011) Mitotic chromosomes in *Abelmoschus esculentus* (L.) *Moench esculentus* (L.) Moench. Ann Biol Res 2:85–90

Perlak FJ, Oppenhuizen M, Gustafson K, Voth R, Sivasupramaniam S, Heering D, Carey B, Ihrig RA, Roberts JK (2001) Development and commercial use of Bollgard cotton in the USA—early promises versus today's reality. Plant J. 27:489–501

Rajamony L, Chandran M, Rajmohan K (2006) In Vitro embryo rescues of interspecific crosses for transferring virus resistance in okra (*Abelmoschus esculentus* (L.) Moench). Acta Hort (ISHS) 725:235–240

Roy MK, Mangat BS (1989) Regeneration of plants from callus tissue of okra (*Abelmoschus esculentus*). Plant Sci 60:77–81

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 82–84

Satpathy S, Rai S (1998) Influence of sowing date and crop phenology on pest infestation in okra. Veg Sci 25:175–180

Shukla A, Agrawal RK, Pathak SC (1997) Seasonal incidence of okra shoot and fruit borer, *Earias vittella* (Fab.) and effect of temperature on its infestation level. Adv Plant Sci 10:169–172

Sunilkumar G, Rathore KS (2001) Transgenic cotton: factors influencing *Agrobacterium*-mediated transformation and regeneration. Mol Breed 8:37–52

Verma RS, Upadhyay KD, Gupta SP, Singh R, Viswanath, Lodhi PS (1984) Interaction between root-knot nematode, *Meloidogyne javanica* and spotted bollworm. *Earias vittella* on okra and effects on yield. Indian J Plant Prot 12:131–133