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Transgenic okra plants expressing a *cry3a* gene for fruit borer (*Helicoverpa armigera*) resistance-an in planta transformation approach

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Abstract
In planta transformation is a novel approach to generate transgenic with a short period of time. Somaclonal variation is a major constraint in tissue cultured dependent genetic transformation in Bhendi (*Abelmoschus esculentus* (L.). Therefore, *agrobacterium tumifaciens* mediated in planta transformation protocols was standardized using sprouted seed explants of bhendi cv. MDU-1, an important vegetable. *agrobacterium* -mediated transformation was performed using LBA4404 strain harboring the binary vector pBinAR carrying *cry3a* gene under the control of CaMV35s promoter and *npt II* gene as a selectable marker. The transformation event consisted of sonicating the explants for 3 minutes, vacuum infiltration (750 mm of Hg) for 2 minutes in *Agrobacterium* (pBinAR-*cry3a*) and co-cultivation for 3 days in MS medium with acetosyringone (100µM) showed transformation efficiency of 12.5%. The presence and integration of *npt II* and *cry3a* transgenes into the bhendi genome was confirmed by polymerase chain reaction (PCR). Work is in progress to grow the T_0 seeds for molecular characterization of the inserted transgene using southern and western blot methods.

Keywords: Bhendi, cotyledon, *npt II*, *cry3a*

Introduction
Bhendi/okra/ lady's finger (*Abelmoschus esculentus* (L.) is an important vegetable crop belongs to Malvaceae family. 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 (Narendran *et al*., 2013) [15]. It green pod contains dietary fiber, minerals, vitamins and oxidation inhibitors like xanthin and lutein (Du¨zyaman and Vural 2003; Kumar *et al*., 2010; Kaur *et al*., 2013) [2, 4, 7]. It is cultivated throughout the world especially in all tropical and subtropical regions of Asia and Africa. Okra is rich in vitamins, calcium, potassium, sodium, and other minerals. Regular consumption of okra prevents hepatitis, cancer, ulcers, and diabetes (Sunilson *et al*. 2008; Kumar *et al*. 2009; Sabitha *et al*. 2011) [19, 5, 17].

Seeds are the rich source of edible oil (13–22%) and protein (20–24%). Okra mucilage, a high molecular weight monosaccharide polymer, can be used as food additive to modify the food quality in terms of food stability, texture and appearance properties by acting as emulsifier, thickener and gelling agent or texture modifier (Noorlaila *et al*., 2015). In pharmaceutical industry, it is being considered as potential hydrophilic polymer and binding agent for tablets. In dairy industry, mucilage could be used as ice cream stabilizer to retard ice recrystallization and melting down of ice cream (Giese, 2014).

As a member of the Malvaceae family, okra is susceptible to a number of pests and diseases. Among of them Yellow Vein Mosaic Virus (YVMV) transmitted by whitefly (*Bemisia tabaci*) is the most serious disease of okra (Ali *et al*., 2000). Okra is severely affected by the shoot and fruit borer (*Helicoverpa armigera*), 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). Frequent use of pesticides by the farmers, without recognizing the vector(s) may create toxic residues in the food chain. Recognizing its economic importance there is a massive scope of utilizing modern biotechnology for further improvement of okra, because conventional plant breeding methods are difficult and time consuming.

Further, 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.

Classical and mutation breeding programs aimed at resistance to diseases have rarely succeeded in okra (Rajamony *et al*. 2006) [16]. 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) [12]. While genes from Bacillus thuringiensis have been successfully engineered into crop plants to get resistance to specific insect pests in a number of species (Kumar and Sharma 1994; Metz *et al*. 1995) [6, 13], this has not been realized in okra due to the absence of a reliable transformation method.

In-planta transformation is an efficient, quick and tissue culture independent system for crop plants improvement. It is a useful system for those plants that lack tissue culture and regeneration system. Therefore, the direct in-planta methods are commonly used for the transformation of many important genes into several plant species such as *Glycine max*, *Arachis hypogeae*, *Arabidopsis thaliana*, *Raphanus sativus*, *Cicer arietinum*, *Beta vulgaris*, *Gossypium hirsutum*, *Solanum lycopersicum* and *Brassica juncea*. The main objective of this study is to transform okra cv. MDU-1 *via* sonication and vacuum infiltration of sprouted seeds with *Agrobacterium tumifaciens* harboring a synthetic *cry3a* gene for resistance against fruit/pod borer.

Materials and methods

Surface sterilization and explant preparation

The healthy MDU1 seeds were acquired from Vegetable Research Station, Palur. These seeds were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 1 min and in 70% (v/v) ethanol for 1 min and then washed thoroughly for 6 times with sterile double distilled water to eliminate the sterilants completely. The surface sterilized seeds were kept overnight in 500 ml Erlenmeyer flask containing 100 ml liquid half strength MS medium (Murashige and Skoog, 1962) [14] in an orbital shaker (120 rpm) under complete darkness (Pre-cultured seeds). The pre-cultured seeds were used as explants for transformation events.

Effect of kanamycin on bhendi seeds of cv. MDU 1

For selection of transformed plants from explants an optimal concentration of kanamycin was determined culturing the seeds on MS medium supplemented with different concentrations of kanamycin (15, 30, 50, 70, 90, 100, 120 mg/l). A total of twenty explants were used with five replications per treatment and the experiment was repeated thrice. For each treatment, data on drying and survival percentage of explants were scored 2 to 3 weeks after initiation of culture.

Bacterial transformation of pBI121-*cry3a* to LBA4404 cells

In the present study, the binary vector pBI121 harbouring *cry3a* gene (1.8 kb) flanked by CaMV35s promoter and OCS polyA terminator and *nptII* as selectable markers was used for transformation. This construct was obtained from Dr. Masoud Tohidfar, Department of Plant Tissue Culture and Gene Transformation, Agricultural Biotechnology Research Institute of Iran (ABRII). This construct was maintained in *Escherichia coli* (DH5α) competent cells by heat shock method.

The binary vector pBI121 harbouring *cry3a* gene was isolated from *E. coli* competent cells was transformed into *Agrobacterium* strain LBA4404 using the freeze-thaw method. The *Agrobacterium* competent cells were prepared in a similar way as for *E. coli* and stored at -80˚C. Thaw the competent *Agrobacterium* cells on ice if it is stored on -70˚C or use the freshly prepared competent cells (use 250 µl per transformation reaction) and add DNA (1-5µl of $CaCl_2$ purified DNA or 10 µl of standard *E. coli* miniprep DNA.) Incubate the mixture on ice for 5minutes. Transfer the mixture to liquid nitrogen and incubate for 5minutes and again, do the same for another 5 minutes in a water bath at 37˚C. Add 1ml of LB broth to each tube, seal it and place the tubes on a rocking table for 2-4 hours at room temperature. Collect the cells by spinning briefly in a micro centrifuge and spread them on two LB agar plates containing antibiotic (kanamycin 50 mg/l) for the T-DNA vector. Incubate the cells for 2 days at 28˚C.

A single colony of *A. tumefaciens strain* LBA4404 harbouring pBI121-*cry3a* gene (1.8kb) flanked by CaMV35s promoter and OCS polyA terminator and *nptII* as selectable markers was used for transformation (Figure 1).

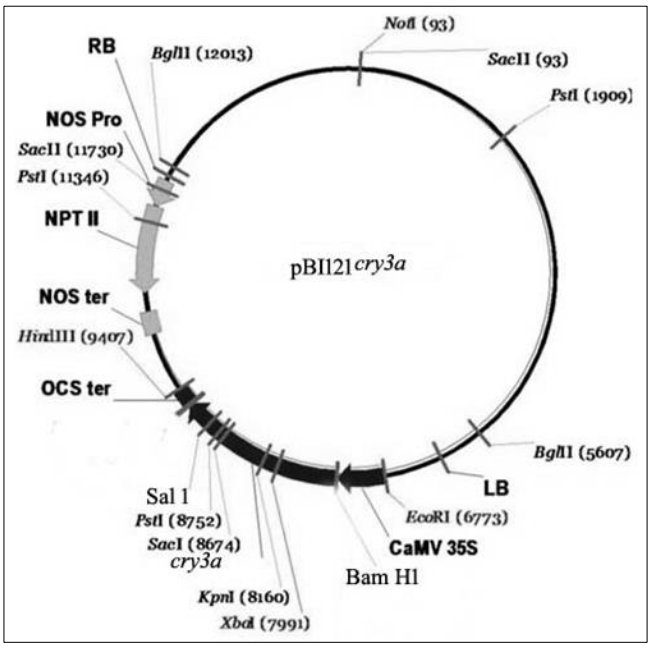

Fig 1: Physical map of pBINAR harboring *cry3a* gene

Preparation of *Agrobacterium* culture for transformation

A Single colony of recombinant *A. tumefaciens* strain was inoculated in 3 ml of YEP broth and allowed to grow overnight in a rotary shaker at 37˚C for 125 rpm. 1ml of overnight grown culture was inoculated in 30 ml of YEP broth and subculture is done. The cell suspension is maintained at 0˚C for 20 minutes by keeping it on ice. Then, it is centrifuged at 5000 rpm for 10 minutes at 4˚C. The supernatant was discarded and the pellet was resuspended in freshly prepared infiltration solution for immediate use.

Transformation via Sonication and Vacuum infiltration

The *in planta* transformation protocols in bhendi using sonication and vacuum infiltration durations, and acetosyringone concentrations were followed according to previous protocols with some slight modifications (Manickavasagam *et al*., 2015) [9]. The sterilized explants were transferred into *Agrobacterium* suspensions containing acetosyringone (100µM). Sonication was carried for 0, 1, 2, 3, 4, 5 and 6-min using a bath sonicator (model 1510 Branson, Branson Ultrasonics, Kanagawa, Japan). Then the seeds were transferred into fresh *Agrobacterium* suspension and subjected to vacuum infiltration for 0, 1, 2, 3, 4, 5 and 6-min

at 750 mm of Hg using a desiccator (Tarsons, Kolkata, India) connected to a vacuum pump (Indian high vacuum pumps, Bangalore, India). The sonicated cum vacuum-infiltrated explants were incubated in *Agrobacterium* suspension medium supplemented with optimized concentration of acetosyringone 100 µM for 1 h under dark for *Agrobacterium* infection. Following this, the *Agrobacterium* infected seeds were blot dried and then co-cultivated in MS medium without hormone containing 100 µM of acetosyringone for 3 days. The infected seeds were washed with sterile distilled water containing 500 mg/l cefotaxime (Alkem laboratories, Mumbai, India) and blot-dried on a sterilized filter paper and inoculated onto solid MS basal medium containing appropriate hygromycin or kanamycin as selectable marker and incubated at 25 ± 2 ˚C under 16 h photo period. The explants were sub-cultured twice at 5 days interval. After 4 weeks, the well rooted survival seedlings were transferred to earthen pots containing the potting mixture (1:1:1 of red soil, sand and farm yard manure) kept in green house and maintained 85% relative humidity at 25 ± 2˚C for acclimatization.

Molecular analysis of putative transgenic plantlets

The antibiotic resistant plants were subjected to molecular confirmation for the transgene integration using PCR with gene specific primers. The primers used for amplification of the *nptII* gene were 5'-GAGGCTATTCGGCTATGACTG-3' and 5' ATCGGGAGGGGCGATACCGTA-3', generating a 750 bp product and the primers for *cry3a* gene were 5'-TAGGATCCATGGCTGCCGACAAC -3' and 5' ATGTCGACTTATGGAGGGTTCACTG -3'resulting in a 1818 bp product.

DNA extraction

Extraction of the DNA from the samples was carried out according to the procedures of Doyle and doyle (1990). The DNA pellet was resuspended by using 100 µl TE buffer and kept at -20˚C for long term use. The concentration of DNA was estimated spectrophotometrically. In spectrophotometric analysis, 5 µl of DNA was diluted to 3000 µl of TE buffer. The spectrophotometer readings were recorded at 260 and 280 nm. DNA concentration was calculated using OD values at 260 nm using the following formula concentration of DNA (µl/ml) = OD at 260 nm X 50.

Results and discussion

A single report on genetic transformation of okra requires lot of time to develop transformed okra plants (Narendran *et al*. 2013) [15]. Hence, to establish an efficient, less time consuming in planta transformation method was performed based on our previous reports on sugarcane and brinjal (Mayavan *et al*. 2013; Subramanyam *et al*. 2013). In addition, year round availability, easiness of explant handling, and efficient selection of transformed plantlets raised from seeds motivated us to select seed as an explant for in planta transformation.

In the present study 50 mg/l kanamycin concentrations in the medium caused a drastic decrease in both the frequency of regeneration and number of shoots per explant, hence this concentration was used for the selection of transformed shoots (data not shown). Using npt II as a selectable marker gene and kanamycin as a selection agent is widely used system for screening transformants in a large variety of plants like mulberry (Bhatnagar and Khurana, 2003) [1], chickpea (Mehrotra *et al*., 2011) [11]. Here, also we used the same selectable marker and were able to achieve complete suppression of non-transformed plants with optimized dose of kanamycin (50 mg/l). The identification and development of *cry* genes in transgenic crops for pest management has turned out to be a major accomplishment. A major limitation has been specificity of the *Bt* toxins to only a certain group of lepidopterans pests.

Sonication and vacuum-infiltrated were performed on the seeds of bhendi as explants. The explants pre-incubated with *Agrobacterium* suspension + acetosyringone were sonicated for various time durations (0–60 min) using bath sonicator. After standardization of sonication, the sonicated seeds were further vacuum-infiltrated along with pre-incubated *Agrobacterium* suspension-containing optimized concentration of acetosyringone (100 uM) for different time durations (0–6 min) at a constant pressure (100 mm Hg) using vacuum chamber. Combination of sonication and vacuum infiltration was also tested for transformation efficiency (Table 1).

Table 1: Influences of sonication duration and vacuum infiltration duration on in planta transformation efficiency of bhendi cv. MDU 1

Sonication duration (min)	Vacuum infiltration time (min)	No. of seeds infected	Mean no. of seeds germinated[a]	Mean no. of *cry3a* positive explants	Transformation efficiency (%)
0	0	100	10	-	-
10	-	100	14	-	-
20	-	100	23	-	-
30	-	100	34	2	6
40	-	100	30	-	-
50	-	100	25	-	-
60	-	100	20	-	-
-	1	100	14	-	-
-	2	100	42	-	-
-	3	100	35	-	-
-	4	100	30	2	5.5
-	5	100	24	-	-
-	6	100	20	-	-
30	1	100	18	-	-
30	2	100	48	-	-
30	3	100	31	4	12.5
30	4	100	24	-	-
30	5	100	18	-	-
30	6	100	13	-	-

[a]- explants on section medium (MS+ kanamycin 50 mg/l)

In sonication treatments, the transformation efficiency gradually increased with increasing sonication duration up to 30 min, which resulted in the transformation efficiency of 8.5%. It was hypothesized that a longer duration of sonication has an inhibitory effect on seed germination. The obtained results were accordance with the previous report, where 30 min sonication was found to be optimal to achieve maximum transformation efficiency of 43%, and beyond 30 min sonication, the seed germination rate and the transformation efficiency was reduced in bhendi (Manickavasagam *et al*., 2015) [9].

In infiltration treatments, among various infiltration durations tested, 3 min vacuum infiltration at a constant pressure (100 mm Hg) using vacuum chamber recorded higher transformation efficiency of 6%. Vacuum infiltration creates a negative atmospheric pressure which removes the air and creates empty spaces within the seed. An increase in the pressure allows the *Agrobacterium* suspension into the empty spaces of the explant/seed, and the Agrobacterium infects the meristematic tissue effectively (Subramanyam *et al*. 2011; Mariashibu *et al*. 2013; Jaganath *et al*. 2013) [18, 10, 3]. Beyond 3 min vacuum infiltration, due to the severe *Agrobacterium* infection, the germination rate was reduced, which ultimately led to low transformation efficiency. The obtained results were in concurrence with the previous reports (Subramanyam *et al*. 2011; Mariashibu *et al*. 2013; Jaganath *et al*. 2013) [18, 10, 3]. Combined effects of sonication (30 min) and vacuum infiltration (3 min), enhanced the transformation efficiency up to 12.5% in bhendi.

To confirm the integration of the *cry3a* transgenes into the bhendi genome, PCR was performed on the genomic DNA isolated from randomly selected 25-day-old putatively transformed and control plants. For PCR analysis, the primers were used to amplify the 1200-bp coding region of the *cry3a* gene. The obtained PCR results revealed a 1200-bp amplified fragment from putatively transformed bhendi plants DNA (3a, lanes 3–7). However, no such amplified fragment was observed from the control plant DNA. The obtained results proved that the *cry3a* gene was successfully integrated into the bhendi genome (Figure 2).

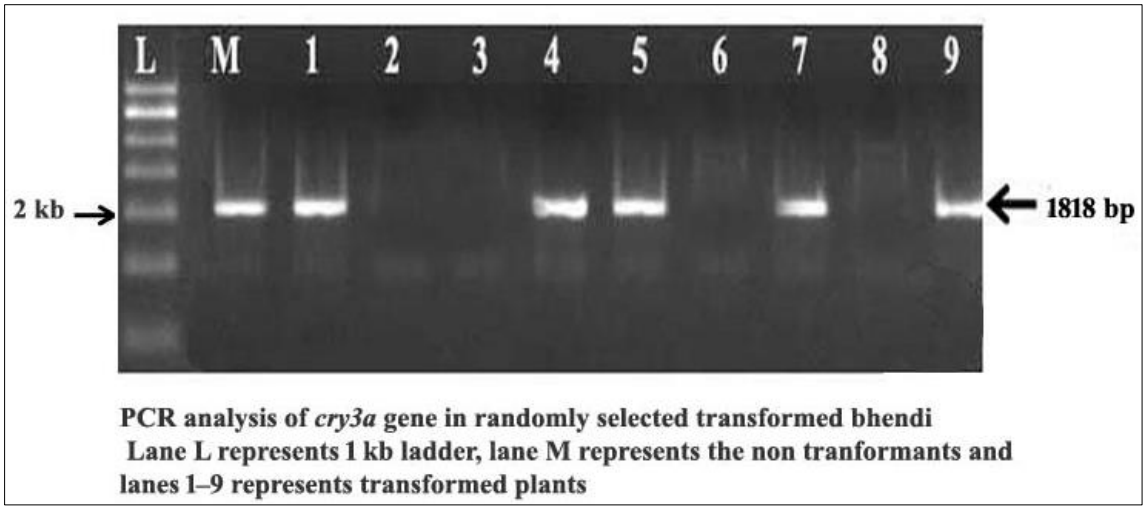

Fig 2: PCR analysis of putative transformed bhendi for amplification of *cry3a* gene

Conclusion

A simple, reproducible, and an improved *Agrobacterium* mediated in planta transformation system has been established for recalcitrant bhendi cv. MDU 1 by optimizing various factors influencing the transformation efficiency. Optimized parameters, sonication (30 min), vacuum infiltration (3 min), acetosyringone (100 µm), and co-cultivation (3 day) enhanced transformation efficiency of 12.% in bhendi when compared with previously published reports.

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