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Molecular analysis and in vitro evaluation of transgenic Bt tomato plants against the invasive pest, Tuta absoluta (Meyrick)

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ABSTRACT: T_4 generation of transgenic Bt tomato plants (17 Nos.) was established in greenhouse to study the stability in expression of an insecticidal *cry2A* gene (derived from indigenous Bt isolate), over the generations. Screening was done by PCR and ELISA for finding out the presence of lepidopteran toxic *cry2A* gene and protein respectively. Tomato leaf miner (*Tuta absoluta*) is an invasive pest, damages the crop very seriously and cause 100 % yield loss. The plants were evaluated in T_4 generation using first and second instar larvae of *T. absoluta*. The insect bioassay studies resulted in 43.33 to 70.00% and 43.33 to 56.67% mortality against first and second instar larvae of *T. absoluta*, respectively. There was significant reduction in the leaf area damage in the transgenic leaf compared to control leaf. In control, all the larvae survived and the first and second instar larvae of *T. absoluta* weighed approximately 2.42 mg and 3.03 mg respectively, while the surviving first and second instar larvae which fed on transgenic tomato leaves ranged from 0.19 to 0.24 mg and 0.27 to 0.33 mg, respectively. This study emphasizes the potential of the native Bt gene in imparting resistance in tomato plants against *T. absoluta.*

Keywords: *Bacillus thuringiensis*, Cry2A protein, insect bioassay, *Lycopersicon esculentum*, *Tuta absoluta*

INTRODUCTION

Transgenic plants harbouring Bt *cry* gene(s) have the significance in managing the insect pests with reduction of insecticide usage. In this study, the transgenic tomato plants harbouring lepidopteran toxic *cry2A* gene (obtained from an indigenous isolate) were raised and an attempt was made to evaluate the toxicity of the plants against an invasive pest, the tomato leaf miner or pin worm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelichiidae). In 2005, EPPO has reported, it is a major pest of tomato, *Lycopersicon esculentum* as well as other solanaceous crops (EPPO, 2005). It originates from South America and recently has been introduced to the Mediterranean region (Urbaneja *et al*., 2007). Its occurrence in India was reported by different workers in different parts of India *viz*., Bangalore (Sridhar *et al*., 2014); Pune (ICAR, 2015; Shashank *et al*., 2015); Hyderabad (Kalleshwaraswamy *et al*., 2015); Telangana (Kumari *et al*., 2015) and Tamil Nadu (Shanmugam *et al*., 2016). The tomato leaf miner is considered as a key pest of greenhouse and open field tomato and it can cause crop losses up to 100% (Arturo *et al*., 2012).

Plants are damaged by direct feeding on leaves, stems, buds, calyces, young fruit or ripe fruit and by the invasion of secondary pathogens enters through the wounds made by the pest (EPPO, 2005). Percentage of fruit damage by *T. absoluta* ranged between 0.5 - 13.5% in Tamil Nadu, 2.0 - 100% in Karnataka and 5 -12% in Gujarat (Ballal *et al*., 2016). Cost- benefit analysis showed that *T. absoluta* significantly increased costs of pest management, primarily because of increased use of insecticides (Lietti *et al*., 2005). Indiscriminate use of insecticides led to the development of resistance against abamectin, organophosphtes and pyrethroids in Brazil, Argentina and Chile (Siqueira *et al*., 2001; Lietti *et al*., 2005, Salazar and Araya, 2001). Field releases of trichogrammatids also not effective, because of the weak adults emerging from the parasitized *T. absoluta* eggs (Ballal *et al*., 2016).

Alternative to these chemical pesticides, *Bacillus thuringiensis,* is an effective microorganism to control *T. absoluta.* Use of *Bacillus thuringiensis* var. *kurstaki* exhibited a satisfactory efficacy against *T. absoluta* larval infestation in Spanish outbreaks (Medeiors *et al*., 2006). Bt based formulations reduced *T. absoluta* damage up to 90%; however they had low persistence on plants and had to be applied weekly (Gonzalez-Cabrera *et al*., 2010). Cry proteins are specifically toxic to the insect orders Lepidoptera, Coleoptera, Hymenoptera, Diptera and also nematodes, but it is innocuous to humans, vertebrates and plants, and are completely biodegradable

(Bravo *et al*., 2005). The use of *cry* genes to control insects has been less popular in vegetable crops (Selale *et al*., 2017). Bt tomato lines have been developed by different workers which are resistant to tomato fruit borer and root-knot nematodes (Koul *et al*., 2014; Li *et al*., 2007; Mandaokar *et al*., 2000; Saker *et al*., 2011). Bt tomato plants are not yet commercialised in India. In this study, stability in expression of insect resistance by the plants was evaluated in T_4 generation of transgenic tomato plants harbouring *cry2A* gene using different instars of *T. absoluta.* To our knowledge, it will be the first report of evaluating the Bt *cry2A* transgenic tomato plants against the tomato leaf miner, *T. absoluta.*

MATERIALS AND METHODS

The present study was conducted at Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology, Tamil Nadu Agricultural University, Coimbatore. The lepidopteran toxic *cry2A* gene isolated from indigenous Bt isolate was introduced into Arka Vikas tomato plants through *Agrobacterium* mediated transformation by earlier workers and studied up to T_3 generation. T_4 plants were raised from a promising T_3 transgenic tomato progeny in transgenic greenhouse along with control (non- transgenic) plants.

Molecular analysis

Polymerase chain reaction was performed with the genomic DNA isolated from T_4 plants to confirm the presence of *cry2A* and *npt*II genes using gene specific primers to amplify 1183 and 712 bp internal fragments of *cry2A* and *npt*II genes, respectively. PCR was carried out in a 25 µl reaction volume with 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 75 mM dNTPs, 50 ng of forward and reverse primers, 1.5 U Taq DNA polymerase. PCR conditions followed were as initial denaturation at 94 ° C for 1 minute, with 29 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, while extension at 72°C for 1 minute. The final extension was adjusted at 72 ° C for 10 minutes. DNA isolated from non-transformed tomato plants were used as control and the reaction mix without template DNA were used as negative control.

Expression analysis by ELISA

Expression level of Cry2A protein in the T_4 progeny plants was quantified by ELISA kit (Envirologix Cry2A Kit, Portland, USA) as per the manufacturer's instructions. Quantitative ELISA was done on the fully expanded young leaf collected from T_4 plants. Protein extracts were made by grinding 30 mg of leaf tissues in 500 ml of extraction buffer (provided in the kit) and centrifuged at 6000 rpm in 4ºC for 7 minutes. An aliquot of 100 µl of leaf extracts were loaded into the ELISA plate. Negative and positive controls were added to wells of the ELISA plate along with test samples. Optical density (O.D.) of plate was read at 450 nano meters in an ELISA plate reader (Biotek, USA). Quantification of Cry2A endotoxin was done by plotting the absorbance values of Cry2A test samples on the standard curve generated with positive standards and expressed as microgram of Cry2A protein per gram of fresh leaf tissue.

$$\text{µg of Cry2A protein per gram of tissue} = \frac{\text{Concentration on graph (500 µl of extraction buffer/mg of leaf tissue taken) X dilution of sample extract}}{1000}$$

Culturing of *T. absoluta*

The population of *T. absoluta* required for bioassay were mass multiplied in Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology, Tamil Nadu Agricultural University, Coimbatore. Mined leaves with *T. absoluta* larvae were collected from tomato fields and reared on young (20 days old) plants kept in larval cages (60 x 60 x 60 cm) until they reach pupal stage. When the leaves were fully mined fresh tomato plants were provided to the larvae. Then the pupae were collected, placed in a Petri dish and kept in adult emergence cage (60 x 60 x 60 cm). Newly emerged adults were provided with ten per cent sugar solution fortified with multivitamin drops in 5 ml glass vial with cotton swab. Twenty days old tomato seedlings grown in 10 x 10 x 10 cm protray were transferred into paper cups and kept in the adult emergence cage for oviposition. The seedlings with eggs were kept again into the larval cages. The larvae thus hatched were maintained by providing fresh seedlings as and when needed and the culture was maintained continuously.

Insect bioassay

The efficacy of Cry2A protein expressed in T_4 tomato plants against *T. absoluta* was evaluated by insect bioassay. Leaf disc bioassay was carried out using different instars (1st and 2nd instars) of *T. absoluta.* In leaf disc bioassay, leaves from second nodes of T_4 transgenic plants and non- transformed (control) tomato plants was detached and used. Leaf bit of 2.0 cm diameter was made and placed on sterile petriplate lined with wet filter paper. Five larvae of *T. absoluta* were released on each leaf bit overlaid on filter paper using a fine camel hair brush. Each treatment was replicated three times with two plates per replication. Experimental conditions of 26-28ºC and 60 per cent relative humidity were maintained. Larval mortality, leaf area damage and larval weight were recorded after 48 hours at 24 hours interval for six

days. The data were subjected to arcsine/ square root transformation before analysis. Data analysis was done by analysis of variance (ANOVA) following the AGRES statistical package (Version 3.01).

RESULTS

Molecular and biochemical analysis of T_4 transgenic plants

Seventeen T_4 transgenic tomato plants were raised in greenhouse for molecular analyses. Total genomic DNA isolated from T_4 transgenic tomato plants was subjected to PCR analysis with *cry2A* and *nptII* gene specific primers. All the seventeen T_4 plants were found to be positive for the amplification of ~1.2 kbp (Fig.1a) and 712 bp (Fig.1b) internal sequences of *cry2A* and *nptII* gene, respectively. The non- transformed control plant did not show any amplification.

The PCR positive T_4 transgenic tomato plants were further screened by quantitative ELISA kit. All 17 PCR positive plants were found positive for the expression of Cry2A protein. The expression of Cry2A protein in these T_4 transgenic tomato plants ranged 0.247- 0.391 µg/g fresh leaf tissue (Table 1). Based on the Cry2A protein concentration, nine transgenic plants were selected and subjected to *T. absoluta* bioassay.

Detached leaf bit bioassay using different instars

In order to determine the insecticidal activity of Cry2A protein expressed in transgenic tomato plants, detached leaf bit bioassay was carried out using 1st and 2nd instars of *T. absoluta* on the ELISA positive (which expressed relatively higher levels of Cry2A protein) T_4 transgenic tomato plants. Mortality in 1st instar of *T. absoluta* varied from 43.33 to 70.00 per cent in T_4 transgenic tomato plants and control plants showed only 10% mortality (Table 2). Major portion of the leaf mesophyll tissue was consumed by the surviving larvae in control plants. In contrast, larvae did much less feeding on leaf mesophyll tissue of T_4 transgenic tomato plants. There was significant reduction in the leaf area damage ranging between 51.83 to 56.50 mm^2 in the transgenic leaf compared to control leaf (881.50 mm^2). The larval weight also reduced in survivors fed on transgenic leaf, ranged between 0.19 and 0.24 mg, when compared to control plant surviving larval weight 2.42 mg.

Likewise, bioassay against 2nd instar of *T. absoluta* showed 43.33 to 56.67 per cent mortality. The leaf area damage ranged between 54.38 to 59.20 mm^2 in transgenic leaf compared to control leaf 921.08 mm^2 and the larval weight of survivors also reduced in transgenic leaf which ranged between 0.27 to 0.33 mg when compared to control plant surviving larval weight 3.03 mg.

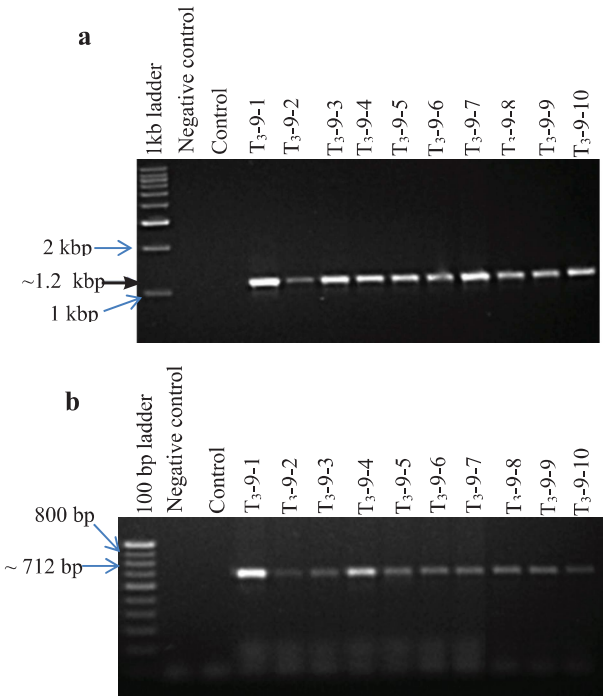

Fig 1. Molecular analysis of T_4 transgenic tomato plants. Amplification of internal sequence of *cry2A* (a) and *nptII* (b) by PCR.

Table 1. Quantitative ELISA on T_4 transgenic tomato plants expressing *cry2A* gene

Progeny	DAS	Cry2A concentration in fresh leaf tissue (µg/g)* Mean± SD
T_4-1	61	0.299 ± 0.019
T_4-2	61	0.276 ± 0.005
T_4-3	61	0.281 ± 0.010
T_4-4	61	0.338 ± 0.015
T_4-5	61	0.391 ± 0.002
T_4-6	61	0.327 ± 0.012
T_4-7	61	0.317 ± 0.001
T_4-8	70	0.259 ± 0.005
T_4-9	70	0.281 ± 0.014
T_4-10	70	0.247 ± 0.000
T_4-11	70	0.271 ± 0.002
T_4-12	70	0.268 ± 0.000
T_4-13	70	0.277 ± 0.001
T_4-14	70	0.308 ± 0.018
T_4-15	70	0.264 ± 0.006
T_4-16	70	0.304 ± 0.004
T_4-17	70	0.261 ± 0.001

* Mean of two replications

DISCUSSION

Development of insect-resistant plants through expression of insecticidal protein gene of *Bacillus thuringiensis* (*Bt*) is documented successfully in several crop plants like cotton, maize, soybean, rice, canola and potato (Sanahuja *et al*., 2011; Tabashnik *et al.,* 2011). Bt formulations are used as sprays by organic farming farmers for managing some devastating pests and they are considered as eco-friendly as they do not harm people and other non-target organisms (Sanahuja *et al*., 2011; Tabashnik *et al.,* 2011). *T. absoluta* is a major insect pest of tomato, and it causes crop losses upto 100% in open fields and greenhouses. In this study, stability of expression of insect resistance was evaluated in T_4 transgenic tomato plants using different instars of *T. absoluta* under *in vitro* conditions. Seventeen T_4 transgenic tomato plants were raised in greenhouse for molecular analyses and all are positive to *cry2A* and *nptII* gene. The expression of Cry2A protein ranged from 0.247 to 0.391 µg/g fresh leaf tissue. Based on the Cry2A protein concentration nine transgenic plants were selected and subjected to *T. absoluta* bioassay using 1st and 2nd instars. Hanur *et al*., (2015) reported that, *cry2A* transgenic tomato plants causing 100% mortality in neonate larvae of *H. armigera* and proved that Cry2A protein is highly effective at early stages of *H. armigera*. In 2017, first transgenic tomato lines developed specifically for resistance to *T. absoluta* using *cry1Ac* gene and observed 38 to 100% larval mortality (Selale *et al*., 2017). To our knowledge, the present study is the first report for *T. absoluta* bioassay using different instar larvae in transgenic *cry2A* tomato plants. Mortality of 43.33 to 70.00% and 43.33 to 56.67% were recorded in 1st and 2nd instars larvae, respectively. Whereas, Sivasupramaniam *et al*. (2008) observed feeding of leaf discs from Cry1Ac/ Cry2Ab2 cotton resulted in mortality of second instars of *S. frugiperda* ranging from 69 to 93 per cent depending on plant age. A significant variation was reported in mortality of first and second instar European corn borer in Bt cotton expressing Cry1Ac. The survival of second instars was more when compared to first instars (Hallad *et al*., 2011).

Laboratory bioassays reported by earlier workers indicated that Cry protein showed high level of resistance to (younger than 3rd instar) larvae, and the efficacy of resistance decreased significantly with increasing age of the insects. It may be due to the quantity of toxins required to kill later instars is higher than that of earlier

Table 2. Detached leaf bit bioassay on T_4 transgenic tomato plants expressing *cry2A* gene using 1st instar larvae of *T. absoluta*

T_4 progeny	DAS	Mortality[a] (%)* (1st instar) Mean ± SD	Leaf area damage[b] (mm^2)* Mean ± SD	Surviving larval weight[c] (mg)* Mean ± SD
T_4-1	99	63.33 ± 5.77 (52.78)[b]	51.83 ± 1.72 (7.20)[g]	0.22 ± 0.01 (0.47)[bcd]
T_4-4	99	50.00 ± 0.00 (45.00)[c]	55.83 ± 2.14 (7.47)[bc]	0.21 ± 0.01 (0.45)[de]
T_4-5	99	63.33 ± 5.77 (52.78)[b]	54.50 ± 2.66 (7.38)[cde]	0.22 ± 0.01 (0.47)[bcd]
T_4-6	99	43.33 ± 5.77 (41.16)[d]	53.83 ± 1.72 (7.31)[def]	0.19 ± 0.01 (0.44)[e]
T_4-7	99	53.33 ± 5.77 (46.92)[c]	52.00 ± 1.41 (7.21)[fg]	0.24 ± 0.01 (0.49)[b]
T_4-9	99	60.00 ± 0.00 (50.77)[b]	51.83 ± 1.72 (7.26)[efg]	0.23 ± 0.01 (0.48)[b]
T_4-13	99	50.00 ± 0.00 (45.00)[c]	53.00 ± 0.89 (7.28)[efg]	0.21 ± 0.01 (0.46)[cd]
T_4-14	99	70.00 ± 0.00 (56.79)[a]	55.50 ± 1.52 (7.45)[bcd]	0.21 ± 0.01 (0.46)[d]
T_4-16	99	70.00 ± 0.00 (56.79)[a]	56.50 ± 1.87 (7.52)[b]	0.23 ± 0.00 (0.48)[bc]
Control	99	10.00 ± 0.00 (18.44)[e]	881.50 ± 3.33 (29.69)[a]	2.42 ± 0.10 (1.55)[a]
CD (P=0.05)		4.74	0.18	0.00
SEd		2.27	0.08	0.01

* Mean of three replications
Figure in parentheses are arcsine transformed value (a) & square root transformed value (b&c)
Means sharing the same letters denote non-significant differences while different letters denote statistically significant differences with $p<0.05$

instars. The mortalities of 1st to 6th instar larvae of *C. suppressalis* in 7-day bioassays feeding on rice expressing *cry1Ac* + *CpTI* genes were 89.6%, 87.1%, 72.37%, 50.0%, 26.6%, and 0%, respectively (Hu *et al*., 2005). The decreased sensitivity in late instar population may be due to increased physiological resistance to Cry toxins, or reduced binding sites in mid gut epithelium (Hallad *et al*., 2011). Selale *et al*. (2017) reported that, damage was limited to small scars in the leaves of the transgenic plants which were caused by the initial feeding attempts of the larvae. Similarly in our study, there was significant reduction in the leaf area damage in the transgenic leaf compared to control leaf. Oliveira *et al*. (2016) reported that, surviving larvae of *S. frugiperda* fed on cotton transgenic plants (using *cry1Ia12* gene) were short in length and were extremely weak (2-15 mg), compared to larvae fed on non- transformed plants (60 mg). Likewise, in the present study, the weights of the 1st and 2nd instar surviving larvae fed on transgenic tomato plants ranged from 0.19 to 0.24 mg and 0.27 to 0.33 mg, respectively, whereas the larvae survived in control plants weighed 2.42 mg and 3.03 mg for 1st and 2nd instar larvae of *T. absoluta*, respectively. We conclude that, the *cry2A* transgenic tomato plants used in the present study have the potential to control *T. absoluta* up to 2nd instar larva.

Table 3. Detached leaf bit bioassay on T_4 transgenic tomato plants expressing *cry2A* gene using 2nd instar larvae of *T. absoluta*

T_4 progeny	DAS	Mortality[a] (%)* (2nd instar) Mean ± SD	Leaf area damage[b] (mm^2)* Mean ± SD	Surviving larval weight[c] (mg)* Mean ± SD
T_4-1	102	53.33 ± 5.77 (46.62)^{ab}	55.86 ± 0.82 (7.47)^{e}	0.32 ± 0.02 (0.57)^{bc}
T_4-4	102	43.33 ± 5.77 (41.16)^{c}	58.62 ± 0.55 (7.66)^{b}	0.29 ± 0.01 (0.54)^{de}
T_4-5	102	53.33 ± 5.77 (46.92)^{ab}	54.99 ± 0.31 (7.42)^{fg}	0.27 ± 0.01 (0.52)^{e}
T_4-6	102	43.33 ± 5.77 (41.16)^{c}	58.55 ± 0.52 (7.65)^{bc}	0.28 ± 0.01 (0.53)^{de}
T_4-7	102	46.67 ± 5.77 (43.08)^{bc}	54.38 ± 0.48 (7.37)^{g}	0.28 ± 0.01 (0.53)^{de}
T_4-9	102	50.00 ± 0.00 (45.00)^{abc}	57.38 ± 0.43 (7.57)^{d}	0.32 ± 0.02 (0.56)^{bc}
T_4-13	102	43.33 ± 5.77 (41.16)^{c}	55.17 ± 0.21 (7.43)^{ef}	0.33 ± 0.01 (0.57)^{b}
T_4-14	102	56.67 ± 5.77 (48.85)^{a}	59.20 ± 0.27 (7.69)^{b}	0.27 ± 0.01 (0.52)^{e}
T_4-16	102	50.00 ± 0.00 (45.00)^{abc}	57.73 ± 0.50 (7.60)^{cd}	0.30 ± 0.01 (0.55)^{cd}
Control	102	0.00 ± 0.00 (0.52)^{d}	921.08 ± 1.57 (30.35)^{a}	3.03 ± 0.08 (1.74)^{a}
CD (P=0.05)		3.66	0.12	0.02
SEd		1.75	0.06	0.01

* Mean of three replications

Figure in parentheses are arcsine transformed value (a) & square root transformed value (b&c)

Means sharing the same letters denote non-significant differences while different letters denote statistically significant differences with $p<0.05$

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