ISSN: 2278-4632
Vol-10 Issue-6 No. 15 June 2020

AGROBACTERIUM-MEDIATED TRANSFORMATION OF TOMATO WITH CRY1ACF GENEFOR INSECT RESISTANCE

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Abstract:

Tomato plant has been transformed with Cry1AcF gene and 14 putative events were found to be positive for both nptII and cry1AcF. In further advancement of thesepositive events 7 events showninheritance in Mendelian ratio i.e. 3:1 in T1 generation and also these putative events were positive for the ELISA testin T0 and T1 generations..Bioefficacy has also tested against *H. armigera* for T1 events and four putative events shown maximum mean mortality as Bst4(84.5± 4.32%),Bst5(82.5±4.78%),Bst8(96±1.78%) and Bst10(92.5±2.43%). Thus this studyindicates that Cry 1AcF gene effective against *H. armigera*resistance in tomato.

Key words:S. lycopersicum; A. *tumefaciens*; Cry1AcF gene

Introduction

Tomato (*Solanum lycopersicum*) is one of the major vegetable crop cultivated in India for its nutritional and commercial values (Mueller *et.al.,* 2005).India contributes 8.6% of world tomato productionand having 20.70 million tonnes production with yield 25.98 tonnes/ha(FAOSTAT 2017).Insect pests and diseases are serious threats to the tomatoes and may damage up to 45-48% of tomato plants resulting significant loss of tomato yield up to 35- 40% in India (*Bhupendra Koul et.al. 2014*).Many insect-pests, and diseases attacks tomatoparticularly polyphagous lepidopteran insect like *Helicoverpaarmigera,* a fruit borer which mainly damagesthe fruit, while *Spodoptera litura* damages theleaves that affects the tomato productivity(Albajes*et.al.,*1988).Both *H. armigeraand S. litura* are serious pests to several important crops world over and particularly in India, including tomato. Cry genes from *Bacillus thuringiensis*widely being used for development of insect, particularly Lepidoptera, resistant transgenics (Tiwari *et.al.*. 2011, Shelton 2012).Bacillus thuringiensis (Bt), has been successfully transformed in several crop plants like cotton, maize, soybean, rice, canola and potato (Sanahuja

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et.al. 2011; Tabashnik *et.al.* 2011). Various transgenic alfalfa, maize,soybean, oilseed rape, sugar beet, rice,chickpea, tomato, and cotton,Crops have been developed successfully and some of them are commercially produced (James, 2013).In India, chemical control of this pest is not always effective due to resistance development to all major pesticides. Agrobacterium-mediated gene transfer method has beenused successfully for transformation of numerous dicot species (Atkinson 2002). Tomato also has been transformed with various Cry gene(s)from *Bacillus thuringiensis* (Bt) the introduction of insect resistance (H. Kumar *et.al.* 2004, Mandaokar *et.al.* 2000, Fischhoff*et.al.* 1987) The *Bt*-toxins can be effectively used for developing insect pest resistance, being ecofriendly, and highly specific to target insects pest and being non-toxic to beneficial insects and vertebrates lack of the receptors for toxin binding and interaction(Bravo *et.al.* 2011). In India *Bt* brinjal has been developed by National Research Center for plant Biotechnology at the Indian Agricultural Research Institute (IARI),Maharashtra Hybrid Seed Company (MAHYCO),University of Agriculture in Dharwad (Karnataka) and Tamil Nadu Agricultural University, Coimbatore. In 2009 GEAC declared Bt brinjal is safe for the consumption Shelton AM (2010).

Seeds sterilization

Seeds of tomato line Dirty 4 (D4) used for the transformation process. The seeds were surface sterilized by soaking for0.01% streptomycin for 10 min, wash with sterile distilled water for three time, then in 70% ethanol for 1 min, washed with sterilized distilled water, and then soaked in 10% commercial bleach (4% sodium hypochlorite) with a drop of Tween 20 for 10 min, and then rinsed with sterilized distilled water for 4 four times. Seeds were allowed to germinate on half strength MS medium (Murashige 1962)containing 15 g/l sucrose and 8 g/l agar. pH was adjusted to 5.8 prior to autoclaving.

Agrobacterium Strain

*A tumefaciens*strain, EHA105,with the binary vector, pBinBt8, that carries a chimericcry1AcF gene (1.863 kb) (containing domains from cry1Acand cry1F) cloned at EcoRI and HindIII sites under the controlof CaMV35S promoter and OCS terminator (Fig. 1) was usedfor transformation. The selectable marker, nptII gene, is regulatedby nos promoter and terminator. EHA105/pBinBt8 wasgrown in LB medium (pH 7.0) containing 50 μgml−1 kanamycin.The

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bacterial culture (3 ml) was later re-suspended in100 ml of AB medium (pH 5.2) containing glucose (Winans *et.al.*,1988)and grown for 2 days.

Plant Transformation: Cotyledonexplants of 8–10 day-old seedlings were used as explants,was pre-cultured on pre-culture media containing (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, agar agar 0.25 %, pH 5.8) 2 days. Then explants wereimmersed in a agrobacterial culture suspension having O.D. adjusted to 0.4in MS liquid + 1% sucrose containing 100 um acetosyrngone with gentle agitation for 10 min, blotted to sterilized filter paper. then explants were cocultivated on same medium incubated in dark for 2 days at 25 ±2 _C. After co-cultivation, explants were washed with sterilized distilled water containing 300 mg/l cefotaxime for avoiding bacterial overgrowth and then blotted to sterilized filter paper. The cotyledonary leaf explants were transferred on selection medium containing The cotyledonary leaves were then transferred to (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, Phytoagar 0.25 %, pH 5.8 adjusted before autoclaving) 80mg/l kanamycin, 300mg/l cephotaxime The cultures were incubated at 25°C under 16 hr photoperiod for 3 to 4 weeks, with sub culturing at every 15-20 days. Young shoots were then transferred to MS medium containing 3% sucrose, 0.1 mg/l Zeatin, 0.1 mg/l IAA and kanamycin at 50 mg/l for shoot elongation. The shoots so obtained were transferred to MS medium with 0.05 mg/l of IBA for rooting. Cultures were incubated for 2 to 3 weeks and rooted plants were transferred to pots containing peat and hardened. After 1-2 weeks hardened plants were shifted to green house for further establishment and analysis.

Molecular Analysis:

Putative transformants were characterized and its subsequent generations (Rashid *et.al.,* 1996). Genomic DNA was isolated using C-TAB method (Sambrook *et.al..,* 1989). Fresh leaf sample from T0 and T1 plants grown in greenhouse was taken for DNA isolation and the same DNA isolated from leaf samples was used for PCR analysis with *nptII* and Cry 1AcF gene specific primers.

Enzyme Linked Immunosorbent Assay

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Qualitative ELISA was done for detection of cry protein in transgenic plants. Leaf samples from the putative transgenic plants were collected and analysed for detection for presence of Bt proteom.ELISA kit from Amar immune-dignosticswere used for detection protein expression. The protocol was followed as per the provided with kit. The absorbance of contents from each well was then measured at 450 nm (Thermo ELISA Reader) along with the positive and negative control well.

Molecular characterization of Transformants

The DNA isolated from the leaf of putative transgenic tomato plants by CTAB method protocol (Sambrook *et.al.*, 1989). PCR analysis of Putative T0 as well as T1 generation was carried out with primers for *nptII,* and *cry1AcF* genes. The PCR reaction mixture (20 µl) contained 0.3 U Taq DNA polymerase(B.Genei), 1X assay buffer (10 mM pH 9.0 TRIS–HCl, 50 mM KCl, 1.5 mM MgCl2, 150 µM of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 0.25 µM and 100 ng template DNA. The DNA extracted from non transformed plant plants was used as a negative control, the pBinBt8 vector as a positive control while the reaction mix without DNA as water blank. The PCR reaction cycle wascarried out as initial denaturation of 93°C for 4 min, and 30 cycles of PCR with denaturation of 1 min at 93°C, annealing at 57°C for 1 min,extension at 72°C for 1 min and final extension at72°C for 7 min. Then PCR product was electrophoresis on a 1 % agarose gel, stained with ethidium bromide and finally gel is visualized under gel doc system(alpha imager).

Bioassay

The larval population of *H. armigera* were reared on an artificial diet (Gupta *et.al.*, 2004) on condition at 26 ± 2°C, about 70% relative humidity and day period of 14 h. Bioassays for *H. armigera*bioassays were carried out by placing neonate on freshly detached tomato leavesof putative PCR positive transformants. 40 to 50 days old plants leaves were used for the assay. The leaf petiole was plugged with wet cotton and transferred individually to plastic plates having insects.Two replica were maintained from each plant in all bioassays. Five neonate larvae were introduced into each plastic container and the observations recorded at an interval of 24 h for at

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least 4 days. % mortality of the released larvae and the extent of leaf damage were recorded in case of bioassays. The most tolerant plants were selected for further advancement.

Results:

PCR analysis of transgenic To and T1 plants

Putative 20T0 plants from the transformation experiment were tested for the presence of Cry1AcF gene &*nptII* gene (Marker gene). Out of these, 14 events werefound positive for the both genes. Positive plants are raised for advancement of T1 generation. 40 plants of each T1 generation developed from 12 To events were analyzed for PCR analysis with *npt II* specific primers and Cry1AcF gene, while further positive plants, tested for bioassay.302 plants were found positive for both *npt II* and Cry1AcF genes.Results showed inheritance pattern in the ratio3:1 for 7 putative events in T1 generation, which is in Mendelian ratio for segregation of *nptII* gene and Cry1AcF gene.

Expression analysis of cry1AcF: In ELISA analysis 12 plant was ELISA positive out of 20.While from 156 T1 transgenic plants 120 plants shown detectable level of Cry protein. All To transgenic plants were subject to bioassay. Transgenic T1 plants generated from expressing high cry1AcF protein further tested against *H. armigera* in Bioassay.

Bioefficacy of cry1AcF transgenic plants against *H.armigera*:

All 12 To putative events were analysed for Bioefficacy against *H. armigera*out of which 4 events shown highest mean mortality Bst4 (90%),and Bst5,Bst8,Bst10 (100%)T1 plants from events Bst4,Bst5,Bst8,Bst10 were raised.All the T1 transformants which are PCR and ELISA positive, were subjected to bioassayby releasing 05 neonate (larvae) larvae of *H.armigera*ineach replication separately. Observations were made on %larval mortality and % leaf damage. The mean %larval mortality of the putative transformants for*H.armigera* wasBst4(84.5± 4.32%),Bst5(82.5±4.78%),Bst8(96±1.78%),Bst10(92.5±2.43%) and 13.05±2.12,7.5±1.06,3.35±0.29, and 3.3±0.58 among the different plants studied, while non-transformants tomato plants shows 1.5±1.07 average leaf mortality and 40.75±1.68 % average leaf damage.

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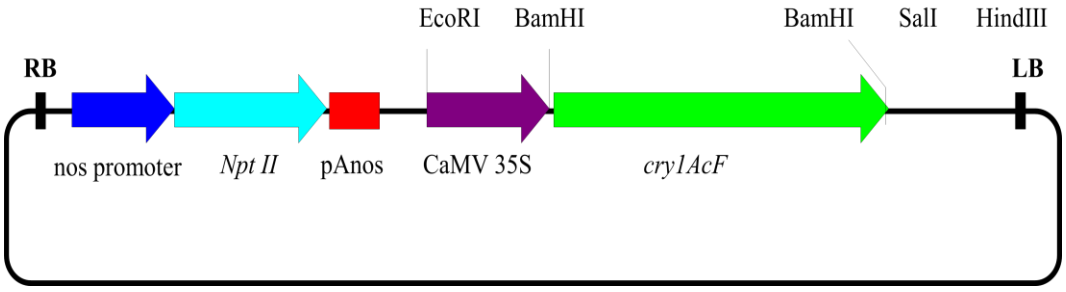

Fig.1.: Gene construct of Cry1AcF

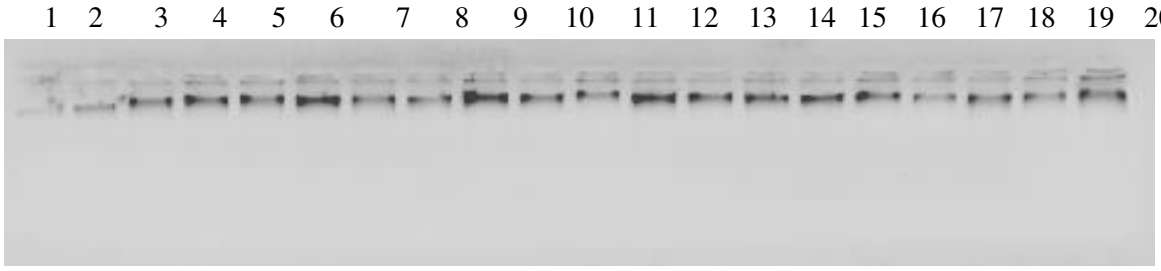

Fig 2(a)

PCR NptII

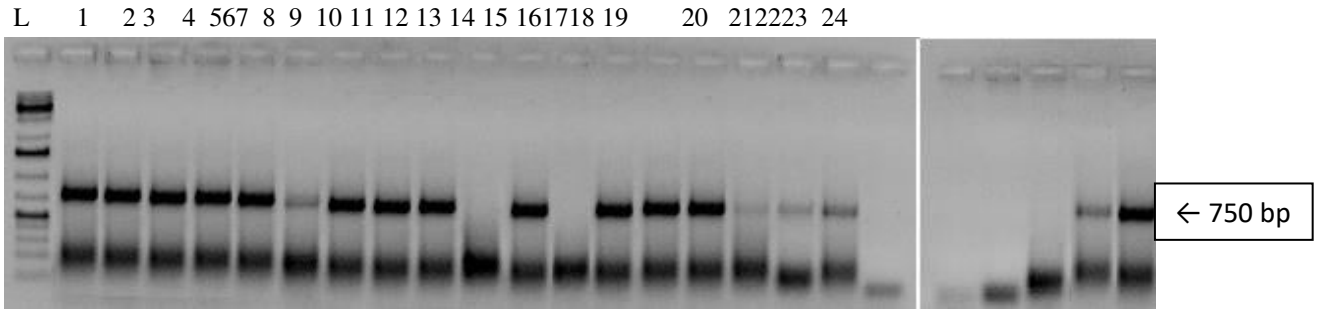

Fig 2(B)

PCR Aacf

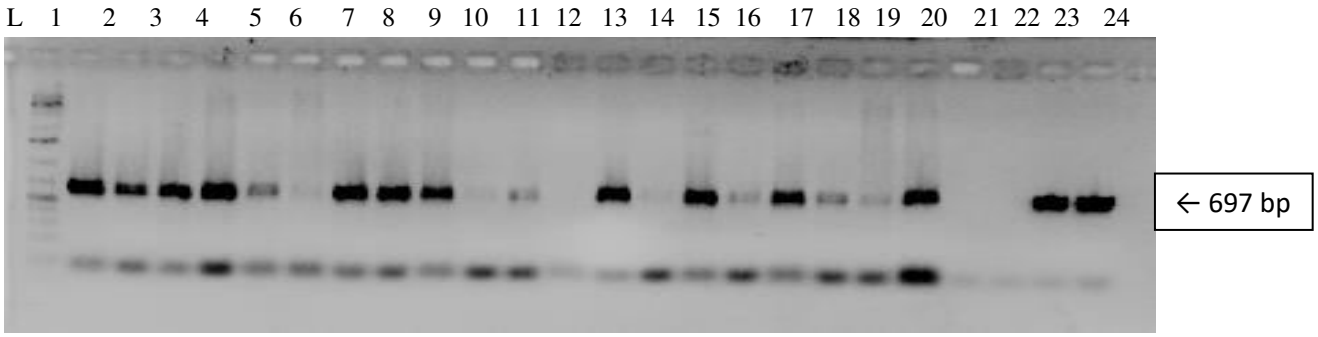

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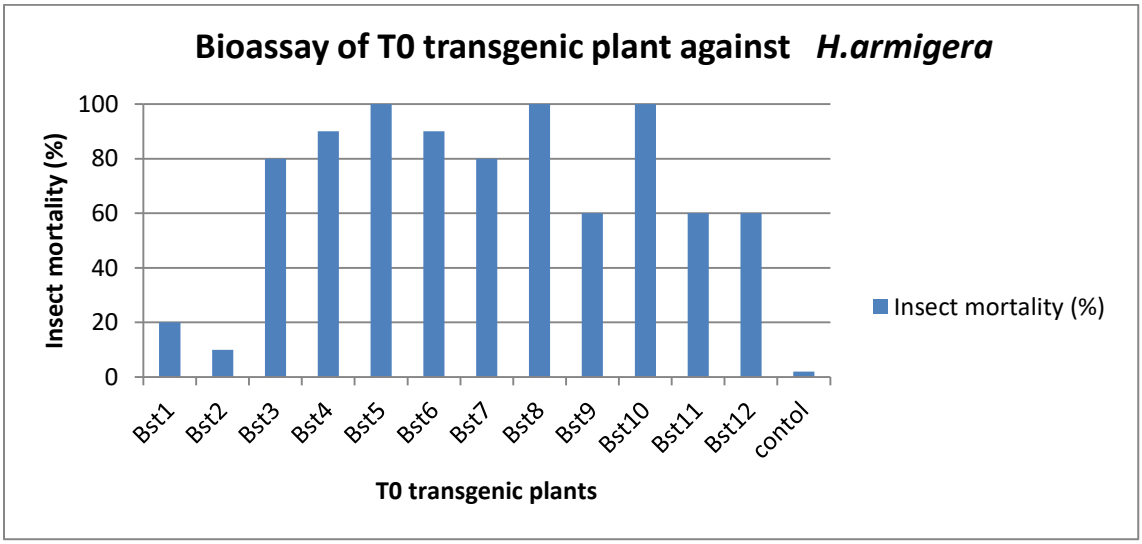
Fig 2(C)

Fig 2(D)

Fig. 2 a)gel showing amount of DNA. B) gel showing PCR analysis in T_1 generation plants with nptII 750 bp primers C. gel showing PCR analysis of samples with cry1Aacf gene with band of 697 bp primers in T_1 generation. L-ladder 1kb.Lane 1–20 21-22: -ve control, 23-24: positive control. D) Bioassay of tomato fruit with *Helicoverpa armigera*



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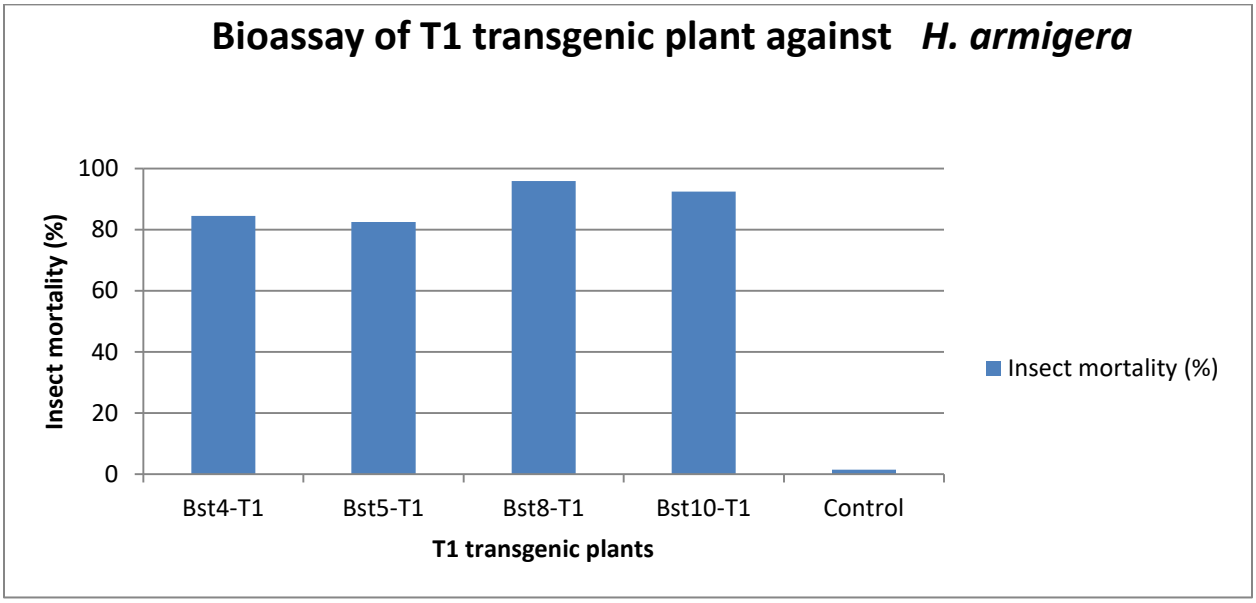

The tomato fruit bioassay against *H. armigera.* The mean % larval mortality of the putative transformantswasBst4(90.00± 3.16%),Bst5(86.5±3.75%),Bst8(97±1.43%),Bst10 (91.5±2.85%)

Discussion:

Because of climatic changes, number of insect pest becoming more devastating for tomato and other crops. DifferentC*ry* genes from *Bacillus thuringiensis* inculcated through transgenic technology in various commercial crop plants for getting insect resistance and increase in the yield through effective eco-friendly control of insect pests (Sanahuja *et.al.*. 2011; James 2012). But single Bt gene not effective in givinglong term resistance to the insect. Stacking of two or more Bt genes be a possible way to achieve insect resistance tomato.Chakrabarti *et.al.*. (1998,)reported that EC50 of Cry1Ac reduced by 13 times when Cry1Ac and Cry1F toxins mixed, and will be used in transgenic plant for control against *H. armigera.*Keshavareddy *et.al.*(2013) found partial resistance of Cry1AcF gene against *S. litura* when transformed in Groundnut (*Arachis hypogaea L*).In present study, tomato plant has been transformed with Cry 1AcF gene to its efficacy against *H.armigera.* PCR ananlysis shown stable integration of Cry 1AcF gene in To and T1 generation with transformation efficiency2.28% intomato D4 cultivar. Seven putative events shown segregation ratio of 3:1 in T1 generationFurther Detached leaf bio-assays of plants with detectable cry1AcF expression in T0 generation under laboratory conditions showed significant larval mortality of *H.armigera* compared to wild type plants

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especially in events Bst4 Bst5 Bst8 Bst10 shown 100% mortality. Further T1 generation of events showing 100 % mortality in T0 generation raised. In T1 generation Bst8(97%), shown maximum average mean mortality of 96% in leaf and 97% in fruit bioassay against *H.armigera* while, Bst4 (84.5% and 90.00%), Bst5(82.5% and 86.5%), Bst10 (92.5% and91.5%). Thus Bst8 is best promising putative event giving maximum resistance to *H. armigera*. In future, further these transgenic events to be check for bioefficacy against different insect in further generation advancement. Present study shows that Cry1AcF is effective against insect *H. armigera*

Acknowledgment

We thank **Dr.P. Ananda Kumar,** Principal Investigator(Ex), National Research Centre on Plant Biotechnology(Indian Council of Agricultural Research), Pusa Campus New, New Delhi, India for kindly providing the bacterial strain harboring the Cry AcF gene used in this study.

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