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Transgenic Tobacco Expressing a Novel *Bt* gene, *Cry1AcF*, showed Resistance against *Helicoverpa armigera*

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ABSTRACT: *Helicoverpa armigera* is a notorious insect-pest that causes massive yield losses to several agriculturally important food crops every year. *Helicoverpa armigera* decimates food crops like tomato, potato, and brinjal as well as cotton, soybean in India. Overexpression of a novel *Bacillus thuringiensis* (*Bt*) cry (crystal) gene is being explored in the development of *H. armigera* resistant crops. In this study, we showed that the chimeric *Bt* gene cry1AcF, which was developed by swapping the domains of the cry1Ac and cry1F genes, is effective against *Helicoverpa armigera*. The recombinant binary vector pCAMBIA2300::CaMV35s: *cry1AcF*: OCST harbouring *cry1AcF* gene was introduced into (*Nicotiana tabacum* L. cv. Petit Havana) using *Agrobacterium* mediated tobacco transformation. We have developed in-vitro transgenic plants and characterized ten putative transgenic tobacco lines by using PCR and strip tests. An insect bioassay was performed with detached leaves to evaluate the extent of leaf damage and larval mortality in order to select those transgenic events with superior performance. Further insect bioassay, conducted using selected transgenic lines, resulted in 85-90% larval mortality, indicating high efficacy against *H. armigera*. The efficiency of the novel chimeric *Bt* gene, cry1AcF, was demonstrated by the high level of resistance in transgenic tobacco against the insect pest *H. armigera*. The *cry1AcF* gene can be an extremely valuable asset towards resistance management against *H. armigera* in other susceptible crops.

Keywords: *Helicoverpa armigera, cry1AcF,* durable resistance, transgenic tobacco.

INTRODUCTION

Global population growth has led to increase the demands and supplies for agricultural products. A number of factors are making ensuring food security in a sustainable manner increasingly difficult. Globally, insect-pests cause upto 40% yield losses in crop plants, making them one of the most important biotic concerns. *Helicoverpa armigera* is an important Lepidopteran polyphagous insect-pest that feeds on more than 350 plant species. In India, this noctuid pest causes serious problems to a number of economically important crops such as cotton, maize and some vegetable crops (Navik *et al.,* 2021). Due its Polyphagous nature and migratory activity, it has been developed resistance against many insecticides. Chemical pesticides are ineffective due to this insect's evolved resistance against many insecticides from different chemical classes and are also hazardous to conventional breeding imparting resistance against *Helicoverpa armigera* is so far not successful due to lack of resistant gene pool in the crop germplasms which is difficult to introgress resistance genes into elite varieties. Under such situation the, bacteria *Bt* emerged as solution. Using transgenic technology for plant protection in crop plants was made possible by the introduction of plant genetic engineering and *Bt* insectisidal proteins (Karthik *et al.,* 2021). Transfer of *Bt* insecticidal crystal protein (Cry) coding genes into susceptible crops is the most common method of combating insect resistance. However the long term efficacy of *Bt* toxins is eventually deteriorated due to the evolution of resistance by pests (Badran *et al.,* 2016; Moar *et al.,* 2017). Both synthetic insecticides and several *Bt* toxins are no longer effective against *Helicoverpa armigera*. The evolution of insect resistance against *Bt* was due to alterations in midgut proteases and a number of major mutations in the insect gene that codes for the receptor protein. Insects have evolved resistance to *Bt*, resulting in a substantial reduction in the binding specificity of a Cry protein. Various approaches have been used to improve toxicity against certain target species, including those which have developed resistance to *Bt*. The most approaches include refugia and gene pyramiding (Ren *et al.,* 2019). Although gene pyramids delay cross-resistance, but still there is a lack of diversity and combinations of resistance genes that possibly increase resistant alleles in insects over time and space as the proportion of homozygous individuals increase in insect populations (Dale *et al.,* 2002; Manyangarirwa *et al.,* 2006). Recently, protein-engineering methods have been emerged to develop next generation *Bt* toxins

through domain swapping which is a viable approach to combat resistance against broad host range. *Cry1AcF* is one such chimeric *Bt* protein created by swapping domains of *cry1Ac* and *cry1F*, that showed synergistic effect and increased specificity against *H. armigera* with improved durability (Kumar *et al.,* 2011; Rathinam *et al.,* 2019). The first and second domains of this protein are derived from cry1Ac and the third domain is derived from cry1F. The chimeric Cry1AcF showed increased toxicity against *Helicoverpa armigera*, revealing synergistic effects (Chakrabarti *et al.,* 1998). This toxin has been shown effective against *Helicoverpa armigera* and *Spodoptera litura* in previous studies (Keshamma *et al.,* 2012; Keshavareddy *et al.,* 2013; Muralimohan *et al.,* 2020). In this study, we tested the efficacy of transgenic tobacco plants expressing *cry1AcF* against *Helicoverpa armigera*. The larvae of *H. armigera* fed on transgenic tobacco leaves expressing the chimeric *cry1AcF* gene showed growth retardation and a significant mortality rate.

MATERIALS AND METHODS

Construction of binary vector cassette harbouring *cry1AcF* for tobacco transformation. The cry1AcF cassette was transferred on to the binary vector pCAMBIA 2300 from the pET expression vector carrying the cry1AcF cassette. The complete *cry1AcF* cassette contains CaMV 35S promoter, cry1AcF gene and OCS terminator. The complete cassette was excised out from pET vector by *Eco*RI and *Hind* III digestion and ligated onto linearized pCAMBIA 2300 get pC::*cry1AcF*. Using heat shock transformation, the ligated circularized vector was transformed into *Escherichia coli* HiPurA™ DH5α competent cells. Both colony PCR and restriction analysis confirmed the presence of positive bacterial colonies. The vector cassette pC::cry1AcF was introduced into the *Agrobacterium* strain EHA 105 using the freeze thaw technique. The EHA105 cells harboring the pC::*cry1AcF* binary vector was then subsequently transferred to tobacco according to the procedure described by Saini *et al.,* (2018). The putative transgenic plants were transferred into pots containing sterile soilrite and protected properly with polythene cover made with holes to maintain humidity. Transgenic plants along with vector control plants were then transferred to a transgenic nethouse for hardening.

PCR screening of putative transformants. Plant genomic DNA was isolated using the CTAB method (Doyle and Doyle, 1990) from transgenic tobacco plants and vector control tobacco plants. PCR was performed to amplify the transgene fragment using gene-specific primers (Table 1). Plasmid of pC::*cry1AcF* was used as a positive control in all PCR reactions. A PCR reaction mixture of 25 µl was prepared for each sample comprising 100 ng plant genomic DNA, 10X PCR buffer (100 mMTris-HCl (pH 8.3), 500 mMKCl, 15 mM $MgCl_2$), dNTP mixture (2.5 mM each), 1µM each of forward and reverse primers and 1 U of *Taq* DNA Polymerase (Takara, India). PCR aamplification was performed with initial denaturation at 95°C for 5 min followed by 35 cycles, each consisting of denaturation at 95° C for 40 sec, annealing at 55° C for 40 sec and extension at 68°C for 40 sec followed by final extension for 10 min at 68°C (ABI Veriti™ PCR cycler). The amplified PCR product was electrophoresed on a 0.8 % agarose gel (Biorad) and a gel documentation system (Azure 600 gel imaging system) was used to capture the image of amplified DNA fragments.

Cry1AcF lateral flow strips (LFS) test for confirmation of protein expression. Cry1Ac LFS specific for Cry protein was used to test the PCR tested tobacco transformants for expression of Cry protein (Eurofins Amar Immunodiagnostics Pvt Ltd, Hyderabad). Cry1AcF expression was detected from transgenic plants' leaf tissue using the immunological kit "Cry1Ac LFS". The leaf samples from 10 transgenic events and vector control plant were collected in a sterile microfuge tube (1.5 ml). Extraction buffer of 500 µl (provided along with the kit) was added and crushed with the help of micro pestle. Then the LFS strips were placed in the tube containing the leaf extract. The results were observed after incubating for 1-2 minutes at room temperature.

Insect feeding bioassays. Insecticidal activity of transgenic tobacco lines expressing Cry1AcF was evaluated by detached leaf feeding bioassays for 96 hours. Ten PCR tested tobacco transgenic lines were examined for the level of resistance to *Helicoverpa armigera*. Three plants from each transgenic line were taken, and two leaves from each plant were fed second instar larvae. A total of 30 larvae were fed to each transgenic line, with five larvae per leaf. Tobacco transgenic lines transformed with pCAMBIA2300 vector without any transgene served as control. From the top, second or third healthy leaves were taken from each line, cut into size, and placed on moist filter paper kept in a petridish (90 ×15 mm size). Five second instar larvae were released with each leaf for feeding. In order to maintain relative humidity, autoclaved double distilled water was applied to the filter paper at regular intervals. Para film was used to seal the plates, and tiny holes were drilled to allow for aeration. All the plates were kept in the insect culture room and the culture room was maintained at 27^0C at 80% relative humidity and 16 hours of sunlight and 8 hours of darkness. Larval mortality was recorded and analyzed every 24 hours until 96 hours. Further, three best performing lines were selected based on mortality rate and extent of leaf.

RESULTS AND DISCUSSION

Complete c*ry1AcF* gene construct was transferred from pET expression vector system to binary vector pCAMBIA2300 developed as pC::CaMV35SP:*cry1AcF*:*Nos*T (pC::AcF). pET vector harbouring cry1AcF cassette was restricted with *Eco*RI and *Hin*dIII enzymes to take out the gene construct, which was then ligated onto pCAMBIA2300 vector backbone linearized by *Eco*RI and *Hin*dIII restriction to the binary vector cassette, pC::AcF as shown schematically in Fig. 1 (A). Different enzyme

combinations were used for restriction analysis to confirm that all components of pC::AcF are in the right orientation (Fig. 1B). pC::AcF was digested with *Hind* III to yield a DNA fragment of about 10 kb fragment was linearized. After restriction with *Eco*RI and *Hind* III, 2.65 kb of DNA corresponding to cry1AcF was released.

The binary vector cassette pC::AcF was transformed into the EHA105 *A. tumefaciens* strain after confirmation by restriction analysis, and colony PCR was performed using *nptII* gene specific primers. The PCR positive bacterial colonies were selected for tobacco transformation (Fig. 2).

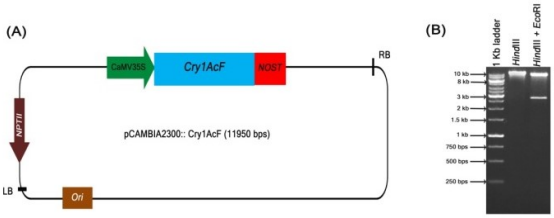

Fig. 1. **(A)** Schematic representation of pC::AcF recombinant vector used for tobacco transformation. **(B)** Restriction confirmation of pC::AcF binary construct: *Hin*dIII digestion linearized the pCAMBIA2300: 35S P: *cry*1*Ac*F:*OCS* T binary construct.

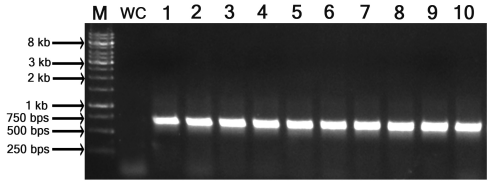

Fig. 2. Colony PCR of *Agrobacterium* colonies with *nptII* specific primers containing recombinant binary vector pC::AcF. From the left 1 kb ladder, Water Control, Lanes 1-10 positive bacterial colonies.

A well-matured second leaf was excised aseptically from an in vitro cultured tobacco plant, cut into small discs using scalpel and blade and then cultured for 48 hours on a pre-culture medium. *Agrobacterium* strain EHA105 infected leaf discs and incubated on co-cultivation media for two days under the dark condition. Co-cultivated leaf discs were transferred into petri-plates containing MS selection medium containing antibiotics and growth hormones (Fig. 3A). After co-cultivation, carbenicillin (500 mg/l) and cefotaxime (500 mg/l) were used to eliminate the growth of *Agrobacterium*. For the selection of the transformants, kanamycin (100 mg/l) was added to the selection medium. The selection medium was kept at a high BAP (2 mg/l) to NAA (0.1 mg/l) ratio to aid in the development of adventitious shoots from the explants.

The preliminary screening of transformants on selection medium was facilitated by kanamycin-induced selection pressure on explants. The explants were developed into adventitious shoots after kanamycin selection. Transformed explants gave rise to callus (Fig. 3B), shoot primordial (Fig. 3C) and shoot in a sequential manner (Fig. 3D). Well-developed shoots of about 5 cm height were excised from regenerating compact callus and transferred to rooting medium. After proper development of roots in rooting media (Fig. 3E), plants were hardened by shifting them to pots for hardening (Fig. 3F) and were grown in transgenic net house. PCR screening was performed using genomic DNA extracted from leaves of each putative transformant.

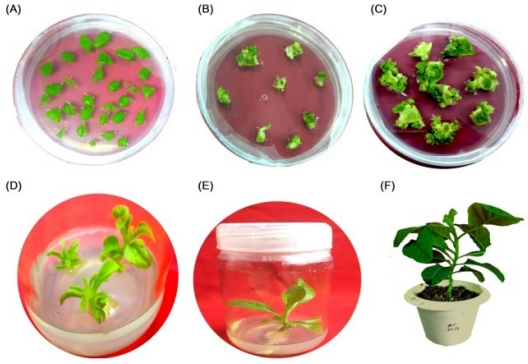

Fig. 3. Transformants of tobacco observed during tissue culture at different stages. (a) Tobacco leaf discs pre-culturing, (b) callus initation from leaf discs, (c) Differentiation of callus, (d) Well developed shoot, (e) Regenerated plantlet with well-developed root, (f) Transferred plants into pots.

PCR analysis with gene specific primers resulted amplification of a 450bp fragment of *cry1AcF* gene from 10 plants (Bt-1 to Bt-10), but no amplification from vector control. pC::AcF DNA was used as positive control (Fig. 4A). Immunological Cry1Ac LFS analysis was done on ten tobacco transgenic lines to determine the expression of Cry1AcF (Eurofins Amar Immunodiagnostics Pvt Ltd, Hyderabad). A highly intense upper blue line on the strip indicates the control band and the lower blue line specify the expression of Cry1AcF protein in transgenic samples. Cry1Ac protein expression was recorded in all the 10 transgenic tobacco lines (Fig. 4B).

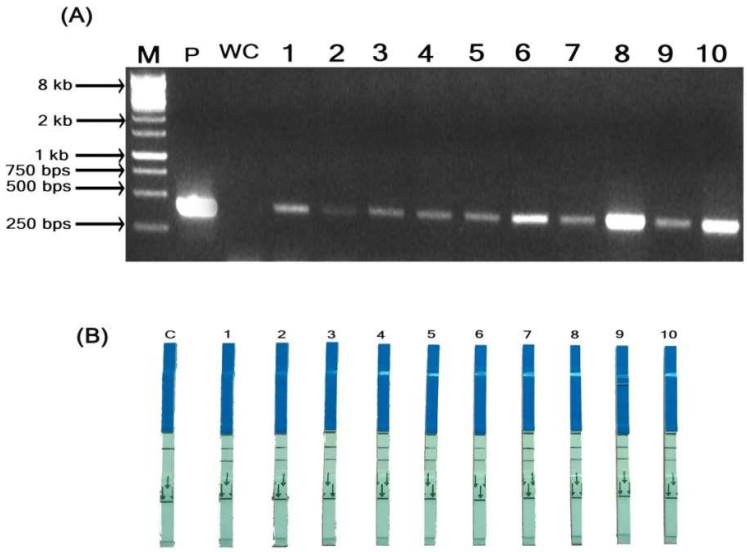

Fig. 4 (A) PCR amplification using gene specific primers which amplified a 450 bp region of *cry1AcF* gene. Lane M: 1 kb ladder; Lane WC: Water Control; Lane P: pC::AcF plasmid control; Lanes Bt-1 to Bt-10: Transgenic lines. **(B)** Expression analysis of the *cry1AcF* gene by Cry1Ac LFS strip test for transgenic lines (1-10) compared to the vector control plant.

The effectiveness of tobacco transgenic lines against *H. armigera* was tested by detached leaf feeding bioassays with second instar larvae. Detached leaf insect bioassay was performed till 96 hours to access the extent of leaf damage and percentage of larval mortality for the selection of superior transgenic events. Observations like larval growth retardation, and mortality were recorded regularly till the completion of 96 h. Ten transgenic lines were lines were subjected to insect bioassay and recorded the mortality rates, out of which three transgenic events showed 85-90 average % mortality after 96 h of continues feeding as compared with vector control (6.67 % mortality) (Fig. 5). It was observed that only 6-11% leaf damage caused by *H. armigera* in three transgenic plants expressing *cry1AcF* gene (Fig. 6 (A) and (B)). During bioassays, the larvae that fed transgenic leaves behaved erratically and frequently switched the sites where they were feeding, in contrast to the typically acting vector control larvae.

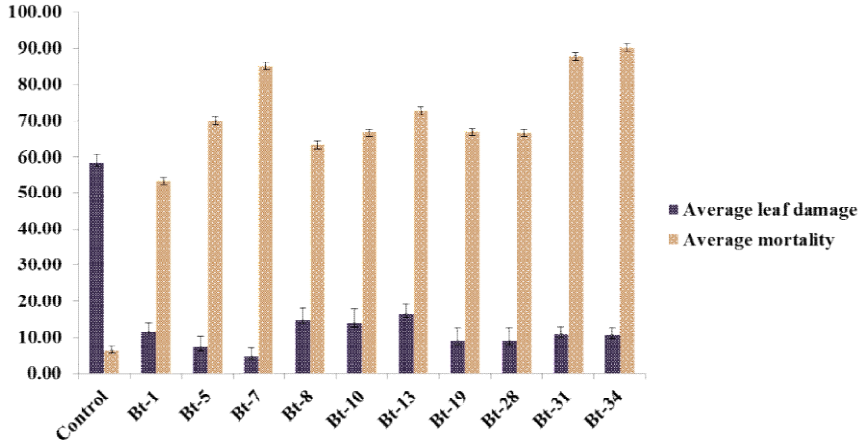

Fig. 5. Graph showing average larval mortality and Leaf damage in percentage determined by the transgenic plants compared with vector control. Standard error represented in bars.

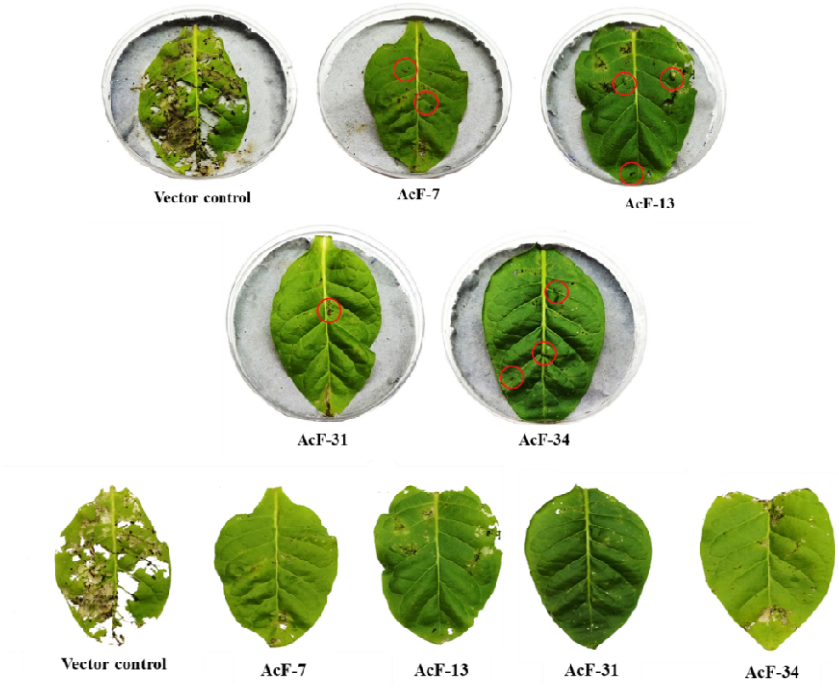

Fig. 6. (A) The leaf detached insect bioassay of Cry1AcF-expressing transgenic tobacco leaves (AcF-7, AcF-31, AcF-34) against *S. frugiperda.* (B) The extent of leaf damage in transgenic lines (AcF-7, AcF-13, AcF-31, AcF-34) compared with vector control tobacco leaves.

To meet the demands of a growing population, crop production must be increased. Insect pests are responsible for the greatest limitation of agricultural productivity. Developing and deploying insect-resistant varieties is one of the methods of reducing yield loss and thereby increasing production (Popp *et al.,* 2013). *Helicoverpa armigera* emerged as the most dangerous and lethal pest for many agriculturally important crop plants, leading to severe economic losses (Makgoba *et al.,* 2021). Varietal resistance to *H. armigera* is either very moderate or negligible, and cannot be deployed to control infestation by this destructive insect-pest. Two approaches such as chemical management and transgenic technology have so far proved successful in controlling *Helicoverpa armigera*. Despite its effectiveness in controlling inscet-pest, chemical management is expensive and harmful to the environment and human health. Genetic engineering of transgenic crops has been successfully applied to control insect-pest attacks. In modern agriculture, insecticidal proteins encoded by the genes of *B. thuringiensis* (*Bt*) are more effective and are used successfully to control most of the lepidopteran pests (Pinheiro and Valicente 2021). However, the efficacy of *Bt*-strategy has been questioned due to cross-resistance among insect-pests. In fact, numerous insect species are already resistant to *Bt* toxins in the field population which is a major concern for the long-term durability of *Bt* crops (Baum *et al.,* 2015). Therefore, it has become an emergency task to develop new pest management strategies. Insect resistance can be slowed down in several ways by using alternative strategies which include refugia (Bates *et al.,* 2005), tissue specific expression of toxin encoding genes (Zaidi *et al.,* 2005; Wang *et al.,* 2016), temporal and spatial rotations of transgenic varieties (Bates *et al.,* 2005). However, there have been many reports of pink bollworm resistance to *Bt*-cotton in India as a result of poor refuge plantings for many years (Mohan, 2020). It has been suggested that gene pyramiding is the most effective way to manage *Bt* resistance. The motive behind the use of gene pyramiding was to delay the development of resistance by using two or more toxins that bind distinct positions of receptors of the epithelial cells in midgut of the larvae. Hence, developing resistance to different toxins requires mutations in distinct sites in receptor genes at the same time, which is extremely rare in insects. The second generation dual-*Bt* cotton varieties, Bollgard II (Cry 1Ac + Cry 2Ab) and Wide Strike (Cry 1Ac + Cry 1F) reduced *Helicoverpa zea* infestation which could not be adequately controlled with the Cry1Ac alone (Manyangarirwa *et al.,* 2006). In a study, *Pectinophora gossypiella* larvae have shown resistance to the two widely used *Bt* toxins Cry1Ac and Cry2Ab under laboratory conditions. The same strain of larvae survived when tested on cotton bolls expressing solely Cry1Ac toxins, however there was mortality when tested on cotton bolls expressing both Cry1Ac and Cry2Ab toxins, indicating asymmetrical cross-resistance (Liu, *et al.,* 2017). Although gene pyramids delay cross-resistance, the distribution of homozygous individuals in insect populations may increase resistance over time because the proportion of homozygous individuals in insect populations increases, despite the existence of gene pyramids to delay cross-resistance (Dale *et al.,* 2002; Manyangarirwa *et al.,* 2006). In addition, pyramided *Bt* toxins are become

significantly less effective due to antagonisms and cross-resistances (Ni *et al.,* 2017). Consequently, it is necessary to create or search for Bt proteins with higher toxicity and broader host ranges. It is possible to increase the binding affinity of Cry proteins by changing their domains, or by adding non-Cry protein fragments or peptides (Deist *et al.,* 2014). An investigation demonstrated that expression of domain swapped chimeric proteins made host plants more resistant to a wide range of insect-pests and considerably prevented the introduction of insect-pest incidence (Muralimohan *et al.,* 2020). eCry3.1Ab, a chimeric *Bt* protein targeted at both lepidopteran and coleopteran insect pests, was developed by integrating domain III of Cry1Ab into Cry3A. The chimeric protein showed acute toxicity and caused 94% mortality against Western corn rootworm (Walters *et al.,* 2010). The chimeric toxin Cry1AcF developed as a result of swapping cry1Ac and cry1F domains to increase potency against lepidopteran insects especially against *H. armigera* (Kumar *et al.,* 2013). It has been reported that the Cry1AcF is safe and allergen free (Rathinam *et al.,* 2017). In the present study, we developed transgenic tobacco expressing the chimeric Cry1AcF *Bt* protein to test its efficacy against *H. armigera*. Ten transgenic tobacco lines were obtained after screening of putative transformants by gene specific PCR. Strip test confirmed Cry1AcF expression in all of these 10 transgenic tobacco lines. Detached leaf insect bioassay was conducted with second instar showed varied resistance level in these 10 transgenic lines as evidenced by growth retardation and mortality of *H. armigera* larvae. The transgenic tobacco lines were confirmed by PCR and strip test and grown in a controlled environment until they reached maturity. The phenotypic comparison of transgenic plants and wild-type controls revealed no phenotypic difference in plant growth. The effectiveness of the cry1AcF gene against *H. armigera* in transgenic tobacco plants was assessed using leaf detached insect bioassay. Leaf detached insect bioassay demonstrated that the transgenic plants expressed the transgene were exhibited higher mortality of 90% against *H. armigera* larvae with less leaf damage as compared with vector control. Our study obtained highest mortality rates of 72-80% after 96 hrs of continues feeding second-instar larvae to the plants that express *cry1AcF* gene. Furthermore, the best performing superior lines were fed with fifth instar larvae to check the resistance against larvae. In one transgenic line (*Bt*-7), the larvae died after 96 hrs of continuous feeding where as in vector control the fourth instar larvae was developed into pupa. This clearly indicates that this insecticidal gene can be more effective against *H. armigera* second instar as well as for higher instar larvae.

CONCLUSION

A large group of insects have developed resistance against insecticides and *Bt* to overcome the selection pressure. But there is enough scope to enhance the efficacy of Cry toxin through the usage of next generation *Bt* toxins. Cry1AcF is a chimeric gene that has been successfully validated for efficacy against *H. armigera*. Next generation *Bt* technology is a potent and efficient approach for insect-pest by developing resistant transgenic plants. Combining the different *Bt* toxins using domain swapping and developing transgenics with *Bt* technology could be a potential strategy for delaying insect resistance. Results have shown fruitful in terms of efficacy of Cry1AcF toxin and promise to be an extremely valuable approach for durable resistance against *H. armigera*.

FUTURE SCOPE

The designed binary vector cassette pCAMBIA2300::cry1AcF can be further used to transfer other *H. armigera* susceptible crops such as cotton, pigeonpea, maize, tomato and potato and the long term efficacy of *Cry1AcF* need to be further analysed.

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Conflict of Interest: None.

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