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Host-delivered RNAi mediated silencing of *HaAce1*, encoding the major isoform of acetylcholinesterase, imparts resistance against the polyphagous insect-pest, *Helicoverpa armigera*

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Abstract

The polyphagous insect pest, *Helicoverpa armigera,* is a detriment to crop productivity. Host-delivered RNAi has emerged as a potential tool to complement *Bt* technology for controlling insect pest menace. In this study transgenic tobacco (*Nicotiana tabacum* L.; *cv.* Petit Havana) plants expressing dsRNA targeting the *H. armigera* gene, *HaAce1*, encoding the major isoform of *acetylcholinesterase,* were developed. A 643 nucleotide RT-PCR amplified *HaAce1* cDNA fragment was ligated in sense and antisense orientation intervened by *GBSS* intron to develop an inverted repeat (IR) gene construct. The *HaAce1* IR gene construct (IR-*Ace1*) under the transcriptional control of CaMV 35S promoter and *NOS* terminator was used for *Agrobacterium*-mediated transformation of tobacco leaf disc explants. Fourteen *HaAce1*-hpRNA tobacco transgenic lines were obtained after screening of 31 putative transformants by PCR and RT-PCR. Five *HaAce1*-hpRNA tobacco transgenic lines demonstrated high level of resistance against *H. armigera* larvae based on detached leaf insect bioassay. These selected five tobacco transgenic lines carried two to four copies of the transgene. Generation of *HaAce1*-specific siRNA was detected in these tobacco transgenic lines. Semi-quantitative PCR revealed many-fold reductions in the steady-state level of *HaAce1* mRNA in larvae fed on leaves of the selected transgenic tobacco lines compared to that in control tobacco leaf-fed larvae. Our findings demonstrate that the host delivered dsRNA targeting *Ace1* gene could be a promising strategy for controlling insect pest menace in crops.

Keywords: Acetylcholinesterase, *Helicoverpa armigera,*host-delivered gene silencing, RNAi, transgenic tobacco

Key message: Transgenic tobacco delivered hpRNA-mediated silencing of *HaAce1* gene encoding the major isoform of acetylcholinesterase of *Helicoverpa armigera* imparted a high level of resistance against this insect-pest.

1. Introduction

Helicoverpa armigera is a highly polyphagous insect-pest with a host range of more than 180 plant species including chickpea, pigeonpea, and cotton (Pawar 1998; Sharma *et al*. 2005). Crop damage due to *H. armigera* has been estimated to be over US$2 billion annually (Tay*et al*. 2013). High fecundity, nocturnal migratory behavior, and high adaptation to various agro-climatic conditions of this insect pest cause severe damage to crops worldwide. Pesticides have so far been an effective strategy in dealing with the problem of *H. armigera* infestation on crops. But indiscriminate and injudicious use of pesticides has led to the evolution of pest resistance through mutations (Fournier 2005). Moreover, repeated use of chemicals leads to soil-water pollution and toxicity to non-target organisms. Hazardous pesticide residues easily get entry into the food chain causing serious risks to human health (Dirham 1993 and Massee 1958). Transgenic plants expressing *Bt* genes have offered viable insect-pest management strategies, at least for a few crops (James 2011). However, the emergence of resistant insect populations has reduced the efficacy of the *Bt*-mediated insect-pest management strategy (Tabashnik *et al*. 2009; Tabashnik 2015). This urged the need for complementary strategies in preventing and/or delaying the resistance development in insects.

RNA-mediated gene silencing in plants is a collective manifestation of broadly two different functions, first is the defense against molecular parasites like viruses, transposons, and introduced transgenes by either post-transcriptional or transcriptional gene silencing (collectively called RNA interference or RNAi). The second major function is the orchestration of developmental stages by regulation of endogenous gene expression at the post-transcriptional level by microRNA (miRNA) (Pattanayak *et al*. 2013; Jonas *et al*. 2015;

Hung *et al*. 2021). RNAi-mediated gene silencing is an efficient and robust tool to study locus- phenotype correlation (Mansoor *et al*. 2006) and has been exploited in almost all spheres of crop improvement (Saurabh *et al*. 2014; Mamta and Rajam 2018). One such application is controlling insect pest menace to protect yield. hpRNA-expressing transgenic plants have been developed to deliver target-specific dsRNA into the insect system while they feed upon the plants; ultimately leading to the silencing of the target gene (Zhang *et al*. 2017). This strategy, known as host delivered-RNAi (HD-RNAi) has been proven as an efficient strategy of protection from southern corn rootworms and cotton bollworms by introducing dsRNA targeting insect genes in maize and cotton, respectively (Baum *et al*. 2007; Mao *et al*. 2007). Silencing of the chitin synthase gene by RNAi has been found to disrupt the larval development of *Spodoptera exigua* (Chen *et al*. 2008). Whereas knockdown of the ecdysis regulatory gene with a binary UAS/GAL4 RNAi system led to lethal ecdysis deficiency in silkworm (Dai *et al*. 2008). Host-delivered hpRNA-mediated resistance to pod borer was established successfully in transgenic tobacco by the knockdown of the 20-hydroxyecdysone receptor gene, *HaEcR* (Zhu *et al*. 2012). Likewise, RNAi-mediated silencing of the hormone receptor gene, *HaHR3,* disrupted *H. armigera* development (Xiong *et al*. 2013).

Acetylcholinesterase (AChE) is involved in neurotransmission and is highly important for the development and survival of the insect. It catalyzes the breakdown of acetylcholine, a neurotransmitter compound to terminate synaptic transmission between nerve cell junctions. Inactivation of AChE by any means leads to the accumulation of acetylcholine at the receptor end**,** impeding neurotransmission and leading to muscular paralysis, convulsions, bronchial constriction, and ultimately death of pest (Chadwick & Hill 1947). *Ace1* and *Ace2* genes encode two different isoforms of AChE present in most insect species. The relative expression of *Ace1* was found higher in most of the lepidopteran pests, including *H. armigera,* and resistance to insecticides was reported to be associated with a mutation in this allele (Lee *et al*. 2006; Lu *et al*. 2012; Lee *et al*. 2007; Jiang *et al*. 2009; Saini *et al*. 2017). However, a few studies reported AChE2 as the primary catalytic enzyme in some other insect species (Kim and Lee 2013). We previously studied the differential expression of *HaAce1* and *HaAce2* at different developmental stages (larval to adult) of *H. armigera* and observed that the steady-state level of *HaAce1* mRNA was fivefold higher than that of *HaAce2* in adult moths (Saini *et al*. 2017). Bally *et al*. (2016) expressed the *HaAce*-2 dsRNA through the

nuclear as well as chloroplast genome of *Nicotiana benthamiana* and found that a higher accumulation of undiced dsRNA in the chloroplast provided strong resistance against *H. armigera*. AsAChE1 is the predominant isoform in *H. armigera,* it is highly imperative that the efficacy of HD-RNAi mediated silencing of *HaAce-1* be tested for imparting resistance against *H. armigera*. Hence, in the present study, we employed transgenic tobacco-delivered hpRNA-mediated silencing of *HaAce-1*. A high level of resistance in transgenic tobacco lines, as evidenced by detached leaf bioassay, was correlated well with growth retardation and mortality, and down-regulation of *HaAce-1* in *H. armigera* larvae.

2. Materials and Methods

2.1 Selection of pod borer *Ace1* target region

The HaAce1 gene sequence was retrieved from NCBI through nucleotide search. A sequence length of 2364 bp corresponding to the *HaAce*1 mRNA sequence was obtained (Accession No. DQ064790). The sequence was divided into four regions/fragments and a similarity search was performed through the BLAST search in NCBI to identify the region which has the least homology with *Ace*1 of other non-target organisms. The region/fragment, corresponding to 1258-1900 nt sequence was selected for construct designing.

2.2 Development of *HaAce*1 hpRNA construct

Total RNA was extracted from *H. armigera* larvae by the Trizol method (Sigma-Aldrich, USA) according to the manufacturer's protocol. The cDNA was synthesized from total RNA by using the Affinity Script cDNA synthesis Kit (Stratagene, USA) according to the manufacturer's protocol. A 643 bp sense and antisense fragment of *HaAce*1 were PCR-amplified (Thermal Cycler, Eppendorf, Germany) from cDNA of *H. armigera* using sense and antisense specific primers with specific restriction sites (Supplementary Table S1). The amplified *HaAce1* sense, antisense, and a 105 bp fragment of the thirteenth intron of potato granule-bound starch synthase gene (*GBSS*) (GenBank: X58453.1) were individually cloned into the pGEM T-Easy vector (by employing TA cloning) and confirmed by restriction digestion analysis (sites present in primer) and sequencing. These fragments were further subcloned and assembled into the pUC19 vector where the sense fragment was first taken out from the pGEM T-Easy vector by *Hin*dIII- *Sal*I restriction and cloned onto the pUC19 vector

at the corresponding site to create pUC19- *Ace*1S. Subsequently, the GBSS intron was incorporated into pUC19::*Ace*1S by *Pst*I-*Bam*HI to get *Ace1S*:GBSS-Int and confirmed by restriction analysis. Next, the antisense fragment was subcloned onto pUC19::*Ace*-1S:GBSS-Int at the *Bam*HI-*EcoR*I site to develop the pUC19::*Ace1*S:GBSS-Int:*Ace1*AS. Restriction digestion and sequencing analysis were done to confirm the presence of *Ace1* sense, antisense, and GBSS intron in the right orientation and sequence in the hpRNA construct (pUC19::*Ace1*S:GBSS Int:*Ace1*AS), which was then subcloned onto pBI121 binary vector (at *Xba*1- *Sac*1 restriction sites) under the transcriptional control of CaMV 35S promoter and *NOS* terminator to get the binary vector cassette pBI121::*Ace1*hpRNA. The binary vector was then transformed into *A. tumefaciens* strain EHA105 by freeze-thaw method (Hofgen and Willmitzer 1988). The PCR confirmed colony harboring pBI121::*Ace1*hpRNA was then used for tobacco transformation.

2.3 Tobacco transformation

Leaf discs of *in vitro* grown tobacco plants were used as explants**.** About 100 such leaf explants were cultured on Petriplates containing pre-culture medium (MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA, pH 5.8) for two days in dark conditions. The precultured tobacco leaf discs were infected with acetosyringone-induced *Agrobacterium* culture for 15 min with gentle shaking. After 15 minutes, the *Agrobacterium* culture was drained and the leaf discs were dried on a plate containing a sterile filter paper. These leaf discs were later transferred onto sterile Petriplates containing two sterile filter papers (90 mm Whatman No.3) moistened with 5 ml of liquid MS co-cultivation medium (MS basal salt with 2% sucrose, 1% glucose, 2 mg/l BAP, 0.1 mg/l NAA, and 100mM acetosyringone, pH 5.2), sealed and incubated at 25°C in dark for 3 days. After co-cultivation, the leaf discs were cultured on selection medium (MS basal medium supplemented with 2 mg/l BAP, 0.1 mg/l NAA, kanamycin 100 mg/l, cefotaxime 250 mg/l and carbenicillin 250 mg/l, pH 5.8) at 25°C under 16 h light and 8 h dark condition. Adventitious shoot buds were regenerated from kanamycin-resistant calli. The putative transgenic tobacco plants with well-developed roots were transferred onto soilrite in pots and covered with polybags to maintain humidity and kept under 16 h of light and 8 h of dark conditions till they were transferred to the transgenic net house.

2.4 PCR and RT- PCR confirmation of *Ace1* hpRNA tobacco transgenic lines

Genomic DNA was extracted from fresh leaf tissues of wild-type control and putative transgenic plants using the standard CTAB method (Doyle 1990). PCR analysis was performed to amplify the 790 bp fragment of the *npt*II gene using specific primers (*nptII*-F and *nptII*-R**,** Supplementary Table 2). PCR amplification was carried out in a Thermal Cycler, (Eppendorf, Germany) programmed with a hot start at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec and a final extension at 72°C for 7 min. The amplified PCR products were electrophoresed in 1% agarose gel and visualized on a UV transilluminator and photographed using a gel documentation system. Total RNA was extracted from transgenic tobacco and wild-type control tobacco plants using the Spectrum^{TM} Plant Total RNA Extraction kit (Sigma, USA) according to the manufacturer's protocol. The cDNA was synthesized from 1 µg of total RNA by using the Affinity Script cDNA Synthesis Kit (Stratagene, USA). RT-PCR was done using the *nptII*-specific primer (*nptII*-F and *nptII*-R Supplementary Table 2). The amplified RT-PCR products were electrophoresed on a 1% agarose gel, visualized on a UV- transilluminator, and photographed using a gel documentation system.

2.5 Detached leaf bioassay

H. armigera larvae reared in the lab at 26 ± 2°C, 75% relative humidity (RH), and 16/8h day/night photoperiod, were used for the feeding experiment at their second instar growth stage. Detached leaf bioassay was performed in Petri plates containing transgenic tobacco or wild-type control tobacco leaves placed over well moistened Whatman 1 paper. Six larvae were placed on each leaf. The Petri plates were wrapped and kept at 26 ± 2°C, under a 16 h photo-phase and 75% relative humidity (RH) condition. The insect bioassays were performed in triplicates and observations were taken every 24 h. Mortality and growth impairment in larvae were recorded on daily basis for consecutive three days. The mean percent mortality was calculated using the statistical software GraphPad Prism 7 for the analysis of variance (ANOVA).

2.6 Genomic Southern Analysis

Approximately, 15µg of genomic DNA per sample was digested completely with 5 units of *Hin*dIII (10 U/ µl) and resolved on a 0.8 % agarose gel (without ethidium bromide) in 1X TAE buffer. Southern blot preparation was done according to Sambrook and Russell (2001) using a positively charged nylon membrane (Hybond N^+, Amersham, UK). After the transfer, the set-up was carefully disassembled leaving the hybridization membrane in contact with the gel. The membrane was carefully removed from the gel, rinsed in 2X SSC (0.3 M NaCl, 0.03 M trisodium citrate, pH 7.0), and placed on Whatman 3 paper for air-drying. The membrane was fixed at 220,000 microjoules/cm^2 for 2 min using a Stratalinker UV crosslinker (Stratagene, Sigma-Aldrich, St. Louis, Missouri, USA). A 790 bp DIG-labeled probe was PCR amplified using *npt*II primers. Probe preparation, prehybridization, hybridization, washing, and detection were performed following the protocol provided by DIG Luminescent Detection Kit (Roche, Basel, Switzerland). The hybridized membrane was exposed to X-ray film (Fujifilm, Kodak) in an intensifying cassette under dark conditions. The cassette was placed for 2 h and the exposed X-ray film was developed to visualize the results.

2.7 Detection of siRNA production through Northern Blot analysis

Total RNA was isolated from the selected transgenics lines according to the standard protocol by TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Approximately, 90 µg of total RNA per sample was resolved in 15% denaturing urea PAGE in 0.5 X TBE buffer. Electroblotting was performed onto the positively charged nylon membrane (N^+, Bright Star-Plus, Applied Biosystem, California, USA) according to the protocol described by Rio *et al.* (2014). The RNA blotting was carried out at 4 $^{\circ}$C for 4 h. After the transfer, the blot was treated with EDC as described by Pall and Hamilton (2008) for efficient fixing of the RNA onto the membrane through cross-linking. A 643 bp of *Ace1* DNA fragment was PCR amplified by using *HaAce1*-specific primers to use as a probe after DIG-labeling. Probe preparation, pre-hybridization, hybridization, and band detection were performed following the protocol provided by DIG Luminescent Detection Kit (Roche, Basel, Switzerland). Hybridization was carried out using ULTRA hyb-oligo hybridization buffer (Ambion, Austin, Texas, USA) and 0.3 nM of DIG end-labeled probe at 37˚C for 16 h, and the membrane was subsequently washed under low stringency conditions (2X SSC + 0.1% SDS) at 28-32˚C. The subsequent steps were carried out according to the instructions

provided by DIG Luminescent Detection Kit for nucleic acids (Roche, Basel, Switzerland; version 07), and the blot was developed by exposing the membrane to the X-ray film in an intensifying cassette under dark conditions. The cassette was placed for 2h and the exposed X-ray film was developed to visualize the results.

2.8 Analysis of *HaAce1* expression in *H. armigera* larvae

Total RNA was isolated from *H. armigera* larvae fed on *Ace*1 RNAi transgenic tobacco leaves and non-transgenic tobacco leaves. About 1µg of RNA was taken for cDNA synthesis by using the Affinity Script cDNA synthesis kit (Stratagene, USA). Subsequently, the cDNA synthesized was quantified and the expression level of the internal control (ß- actin) was used as a normalizer. The primers specific to the *HaAce1:Sense* sequence (Table 3.3) were used to measure the extent of down-regulation of *HaAce1* expression in larvae fed upon *Ace*1 RNAi transgenic lines through semi-quantitative PCR reaction. PCR amplification was carried out in a Thermal Cycler, (Eppendorf, Germany) programmed with a hot start at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 45 sec and a final extension at 72°C for 7 min. The amplified PCR products were electrophoresed in 1.5% agarose gel and visualized on a UV transilluminator and photographed using a gel documentation system.

3. Results

3.1 Selection of target region and development of *Ace*1 RNAi binary vector

The *HaAce1* cDNA sequence (GenBank Accession No. DQ064790) was retrieved from GenBank, NCBI. It comprised of 2364 nt cDNA sequence and an ORF of 1875 nt coding for a protein of 624 amino acids. The 643 nt long target region corresponding to the 1258-1900 nt sequence was found to be the least homologous with other *acetylcholinesterase* sequences of non-target organisms as revealed through BLAST search (data not shown). Therefore, this region of *HaAce1*mRNA was targeted for the construction of an inverted repeat gene construct. *in silico* analysis of the target region using an online web tool (http://jura.wi.mit.edu/bioc/siRNAext/) predicted 31 potential siRNAs out of which 21 siRNAs showed negative thermodynamic values (Supplementary Table 3). The selected 643 nt long target region was PCR amplified from *HaAce1*cDNA and used for developing the

inverted repeat gene construct. A 105 nt genomic DNA sequence corresponding to 3821-3925 nt of potato *GBSS* gene, comprising the 13th intron and a few nucleotides of both the exons, was used as the intervening sequence for the inverted repeat (IR) gene construct. The recombinant pUC19 vector harboring *HaAce1* hpRNA gene construct (pUC19::*Ace1*-S:*GBSS*-Int:*Ace1*-AS) was developed by individually amplifying and cloning of *HaAce1* sense, GBSS intron, and the *HaAce1* antisense sequence onto pGEM-T Easy vector and then assembling onto the pUC19 vector. The *HaAce1* hairpin assembly in pUC19 vector was further subcloned onto the binary vector pBI121 by replacing the GUS coding region under the transcriptional control of the CaMV 35S promoter and *NOS* terminator to express dsRNA (Fig.1). The binary vector containing *HaAce1* hpRNA was mobilized into the *Agrobacterium tumefaciens* strain EHA105 and used for tobacco transformation.

3.2 Generation and molecular characterization of tobacco transformants

A total of 31 putative transformants were obtained from 320 leaf discs co-cultivated with *A. tumefaciens* EHA105 harboring pBI121::*Ace1* hp RNA gene construct (Fig. 2A). Fourteen transgenic tobacco lines were obtained through PCR and RT-PCR screening (Fig. 2B & 2C) using *nptII* specific primers which amplified a 790 bp fragment. All the selected transgenic lines appeared phenotypically similar to the wild-type control plant. Therefore, the selected lines were used for detached leaf insect bioassay.

3.3 Performance of the tobacco transgenic in response to deliberate *H. armigera* challenge

Detached leaf insect bioassay was conducted to determine the effectiveness of the selected 14 *HaAce1* hpRNA transgenic tobacco lines. Five out of 14 transgenic tobacco lines demonstrated a high to moderate level of resistance against *H. armigera* larvae based on leaf damage and insect mortality (Line 4, 8, 10, 18, and 20) (Fig.3A&3B). The average visual leaf damage caused by the larvae on the selected lines was less than 10%. The least damage was observed in Line 4 and 8 while slightly higher leaf damage was observed in Line 10, 18, and 20. As expected, the extent of damage in the wild-type control leaf was around 3 times more than that of transgenic lines. The feeding of *HaAce1* hpRNA transgenic leaves caused varying impacts on the growth and survival of the larvae. A significant difference in growth was

observed between *H. armigera* larva fed on leaves of *HaAce1* hpRNA transgenic lines and those that fed on leaves of wild-type control. The size of larva fed upon transgenic lines was less than half of the larva fed upon wild-type control plants (Fig.3C). After three days of feeding, the least leaf damage and the highest level of resistance against *H. armigera* larvae were observed in lines, 4 and 10 (Fig.3A). Larvae fed on leaves of both, Line 4 and 10 demonstrated stunted growth, and significantly higher larval mortality of 55.5%, compared to the larvae that fed upon wild-type control plant (Fig. 3B).

3.4 Molecular analysis of the selected transgenic lines:

The mRNA level of *HaAce-1* in larvae fed on hpRNA expressing transgenic tobacco leaves was determined by semi-quantitative RT-PCR with the ß-*actin* gene as the internal control. About two-fold (73%) reduction in the steady-state level of *HaAce1* mRNA was observed in larvae that fed on transgenic tobacco leaves compared to that of larvae fed on wild-type control tobacco leaves (Fig. 3D). Southern analysis carried out with *nptII* specific probe revealed that the selected tobacco transgenic lines carried two to four copies of the transgene (Fig 4A). Expression and processing of *HaAce1*-hpRNA in the selected transgenic tobacco lines were detected using Northern hybridization. DIG-dUTP labeled probe was synthesized by PCR using the *HaAce1* target sequence as a template. Hybridization of the DIG-dUTP labeled probe to the fractioned small RNA showed the production of *HaAce1* specific siRNA in all the selected transgenic lines. As expected, no band was detected in the wild-type control line (Fig 4B).

4. Discussion:

In the past, several transgenic crops expressing different insecticidal biomolecules (e.g. Cry proteins) had been developed and commercialized to minimize the loss caused by insect infestation. Crop plants expressing *Bt* genes offer a high degree of protection against various insect pests (Qaim and Zilberman 2003; Wu *et al*. 2008). At present, the *Bt*-based strategy is the only commercially effective technology for agricultural pest control. However, the threat of resistance development to *Bt* toxins in field populations of insects challenges the sustainability of the strategy (Gahan *et al*. 2001; Bravo and Soberon 2008; Tabashnik *et al*. 2008; Monsanto 2010; Kranthi 2015; Gassmann 2021). Moreover, *Bt* insecticidal proteins have been found to have little or insignificant effects on homopteran sap-sucking pests such

as aphids, leafhoppers, and whitefly (Price and Gatehouse 2008; Lawo *et al*. 2009; Romeis and Meissle 2011). In this context, the utilization of RNA interference (RNAi) in pest control offers a complementary strategy to the *Bt* technology.

RNAi is a mechanism of regulation of gene expression which is highly conserved among higher eukaryotes (Carthew *et al*. 2009). The effective utilization of this pathway to target the expression of specific genes holds considerable promise for the development of novel RNAi-based insect-pest management strategies (Gordon and Waterhouse 2007; Price and Gatehouse 2008; Zotti and Smagghe 2015; Mamta and Rajam 2017; Yan *et al*. 2020). Significantly, crop plants could be equipped with dsRNA targeting almost any gene of insect-pests in the transgenic background as a resistance management strategy against those insect-pests. The main advantage of an insecticidal RNAi strategy is that it entices an endogenous gene silencing mechanism normally present in plants and insect-pests. This feature makes RNAi a strategy that may finally provide a safe, truly area-wide insect pest and disease management approach. The feasibility of an insecticidal RNAi approach in plants was first demonstrated using dsRNA expressing corn and tobacco (Baum *et al*. 2007; Mao *et al*. 2011).

Host-delivered (HD) –RNAi has widely been used to impart resistance in plants against viruses (Duan *et al*. 2012), insects (Huvenne and Smagghe 2010), parasitic plants (Alakonya *et al*. 2012), and fungi (Nunes and Dean 2012). Since HD-RNAi does not introduce any additional protein into the transformed plant, it gives an advantage on biosafety ground. HD-RNAi-mediated *H. armigera* resistance was demonstrated successfully in transgenic tobacco by targeting a number of insect-pest genes including the 20-hydroxyecdysone receptor gene, *HaEcR* (Zhu *et al*. 2012), hormone receptor gene, *HaHR3* (Xiong *et al*. 2013; Han *et al*.2017), etc. There are two isoforms of Acetylcholinesterase (AChE) in insects, AChE1 and AChE2, encoded by *Ace1* and *Ace2*, respectively. The AChE1 has been proposed as the major neuro-regulatory isoform with a higher level of expression than AChE2 (Lee *et al*. 2006; Lu *et al*. 2012; Lee *et al*. 2007; Jiang *et al*. 2009; Saini *et al*. 2018). Targeted down-regulation of *Ace1* has been found to severely affect the growth and development of several insects including *H. armigera* (Lepidoptera: Noctuidae) (Kumar *et al*. 2009; Lu *et al*. 2012; Revuelta *et al*. 2009; Bally *et al*. 2016). Therefore, we hypothesized that targeting *HaAce1* through HD-RNAi could impart resistance in plants against *H. armigera* as AChE1 is the major neuro-regulatory

enzyme, and would complement *Bt* technology for durable resistance against this notorious polyphagous insect-pest.

The RNAi–mediated gene silencing is sequence-specific, but the underlying environmental risk of plant-mediated RNAi still exists because of potential off-target silencing effects of homologous genes that share a high identity with the target insect gene (Xiong *et al.* 2013; Casacuberta *et al.* 2015). Hence, the 2364 nt long Ha*Ace1* cDNA sequence was retrieved from GenBank and was divided into four regions/blocks of 600 nt each. Each region/sequence block was used as a 'query' for the BLAST search of GenBank to ascertain the degree of homology of *HaAce*1 nt sequences with non-target organisms. The 600 nt cDNA regions, corresponding to 1200-1800 nt of *HaAce1* cDNA sequence, exhibited the least homology with sequences of non-target organisms. Therefore, the 643 nt cDNA region corresponding to the 1258-1900 nt cDNA sequence of *HaAce1* was targeted for the development of an inverted repeat gene construct. *In silico* analysis of this 643 nt *HaAce1* cDNA region predicted to generate 31 potential siRNAs, out of which 21 siRNAs had net negative thermodynamic values based on the energy difference of 5′ end of sense and antisense strand of siRNA duplex (supplementary table S3). It implies that 31 siRNAs could potentially be generated in *Ace1* RNAi transgenic tobacco lines. However, only 21 siRNA antisense strands of duplex siRNAs having net negative thermodynamic values are to be preferentially loaded on RISC as their 5′ ends are thermodynamically less stable compared to the 5′ ends of sense strands (Schwarz *et al*. 2003). Therefore, *HaAce1* mRNA could be targeted for silencing by antisense strands of these potential 21 siRNAs. The presence of 21 types of potential siRNAs in the insect cellular system increases the frequency of interaction between the target mRNA and siRNAs. This could lead to the efficacious silencing of the target gene. Apart from the types of potential siRNAs generated inside the plant, the concentration of dsRNA is one of the important factors which determine RNAi efficiency. "Transitive RNAi" is the phenomenon reported in plants, fungi, and nematodes that involves a strong amplification of the RNAi signal (Lipardi *et al*. 2001; Sijen *et al.* 2001; Vaistij *et al.* 2002). This phenomenon is yet to be identified in any insect genome sequenced so far (Scott *et al*. 2013). Since "Transitive RNAi" uses target mRNA as a template, it could not be harnessed in HD-RNAi particularly if the targeted gene is of insect origin. Moreover, since the unfavorable condition of the insect digestive system can degrade the dsRNAs/siRNAs

during the feeding process, a higher amount of dsRNAs/siRNAs present in the host plant are required for optimal knockdown of the target gene (Garbutt *et al.* 2013). Therefore, maintaining an ample concentration of dsRNA inside the host plant becomes more critical which was achieved by the use of a strong promoter (*i.e.* CaMV 35S) in the current study. Chloroplast transformation is another attractive avenue for generating a high amount of dsRNA and thus developing plants' resistance to herbicides and insect-pests (Block *et al*. 2007, Block *et al*. 2015; Kota *et al*. 1999; De Cosa *et al*. 2001; Lutz *et al*. 2001; Ye *et al*. 2001; Chen *et al*. 2014). High level of gene expression, absence of RNAi machinery, and, consequently, gene silencing in chloroplast ensure higher accumulation and availability of intact full-length dsRNA (Bally *et al*. 2009; Zhang *et al*. 2015; Zhang *et al*. 2017). In a recent study, transplastomic tobacco lines expressing dsRNA specific to lepidopteran *chitin synthase* (*Chi*), *cytochrome P450 monooxygenase* (*P450*), and *V-ATPase* demonstrated a strong RNAi effect against *H. armigera* (Jin *et al*. 2015). In a similar study, Bally *et al.* (2016) reported that the transplastomic expression of hpRNAs derived from a 189 nt stem sequence of the *Ace2* (AF369793) in *N. benthamiana* conferred a comparatively higher level of resistance against *H. armigera* larvae as compared to that of nuclear-transformed counterpart. The 189 nt stem sequence was selected in such a way that about 95% of every possible processed 21-mer siRNA would also recognize *Ace1* (AY142325) of *H. armigera* and *H. zea*, with 0 to 1 mismatch, as claimed by Bally *et al.* (2016). Contrary to the reports by Bally *et al.*2016 our sequence analysis revealed that both the sequences (AF369793 and AY142325) showed high similarity (98.07%) with the *HaAce2* (JF894119.1), and no significant similarity was found with *HaAce1* (DQ064790.1) indicating that only *HaAce2* was targeted and silenced and not the *HaAce1* (Bally *et al.*2016). In our study, none of the 31 potential siRNAs generated from the 643 nt regions of *HaAce1* (DQ064790.1) was found to have more than 7 nt match with the *HaAce2* (JF894119.1).

In the present study, we intend to check the efficacy of silencing *HaAce1* through nuclear transformation so that this strategy can be combined with *Bt* technology as an immediate solution to the resistance development by the targeted insect-pest. It has already been demonstrated that pyramiding of *Bt* and RNAi provides durable resistance and delays resistance development in the target insect-pest against the *Bt* protein (Ni *et al.* 2017; Head *et al.*2017). The key issue is to find a number of insect-pest genes silencing of which by HD-

RNAi will cause considerable growth retardation and mortality of the targeted insect-pest. *HaAce1* has been reported as a major neuro-regulatory gene in *H. armigera* playing a vital role in insect's growth and development (Saini *et al*. 2018). Hence, it was perceived that silencing of *HaAce1* would affect the life of *H. armigera*, and damage to a particular crop would be less to a great extent. In the previous studies, it was reported that *H. armigera* larvae showed developmental deformities and mortality after feeding on an artificial diet containing *HaAce1* siRNAs (Kumar *et al*. 2009; Zha *et al.* 2011; Xiong *et al*. 2013). In the current study, high mortality and stunted growth were observed in larvae fed on *HaAce*I-hpRNAi tobacco leaves but not in larvae fed on control tobacco leaves (Fig. 3A & 3B). The highest mortality rate of 55.5% was observed in two selected *HaAceI*-hpRNAi tobacco lines (Line 4 & 10). The overall rate of *H. armigera* larval mortality caused by feeding on leaves of the transgenic tobacco lines varied from 55.5- to 38.9%. The larvae that survived after three days of feeding showed stunted growth indicating the potency of the HD-RNAi strategy adopted in the present study. siRNA accumulation was detected in the selected hpRNA tobacco transgenic lines but not in the control tobacco plant. The reduction of *HaAce1* mRNA level in larvae fed on transgenic tobacco leaves compared to that fed on control tobacco leaves, as revealed by semi-quantitative PCR (Fig. 3D), was due to the silencing of *HaAce1* by siRNAs generated from hpRNA by the endogenous RNAi machinery of the transgenic tobacco lines (Fig 4B).

Considering the efficacy and biosafety concerns, transplastomic expression of hpRNA is preferred as reported by Bally *et al.* (2016, 2020). However, the higher magnitude of target knockdown and greater stability of siRNAs derived from nuclear-transformed lines in the digestive system of *H. armigera* as compared to the transplastomically produced dsRNA, suggest that host-delivered siRNAs could be a more effective trigger of RNAi in Lepidoptera than host-delivered dsRNAs (Fu *et al*. 2022). Moreover, the lack of efficient transformation, selection, and regeneration protocols for engineering fertile homoplastic lines across different crop species is the major limitation to the immediate application of this approach in major food crops (Wani *et al*. 2015). Hence, the strategy of nuclear transformation is at present a relevant approach for testing its efficacy alone or in combination with *Bt* technology.

In conclusion, the present study proves the effectiveness of imparting resistance against the notorious insect pest, *H. armigera*, through HD-RNAi-mediated silencing of the insect *HaAce*-1 gene using tobacco as a model system.

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6. Declaration of interest statement:

Authors hereby declare that no conflict of interest exists

7. Authors contribution Statement:

Conceiving and designing the experiment were done by DP. Data generation, curation, and analysis were done by SJ, RPS, KVR, and JT. The experiment was conducted by SJ, KVR, GD, and RPS. Development and characterization of transgenic plants were performed by SJ, KVR, and RS. Supervision of the research work was done by DP. Writing of the original draft was by SJ; review and editing of the manuscript were done by RS and DP. All authors read and approved the final manuscript.

8. Data Availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

9. References:

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Figures' description:

Fig.1. Construction strategy of RNAi binary vector, pBI121:: IR-*Ace1*.

Fig 2A. Various stages of tobacco transformation with IR-*Ace1* RNAi gene construct. i). Co-cultivation; ii). Selection and callusing; iii). Adventitious Shooting; iv). Shoot elongation and rooting; v) Fully developed plant transferred to the pot.

Fig.2B. PCR confirmation of *Ace*1 RNAi transgenic tobacco lines. Lane 1 & 18, 1 kb DNA ladder; lane1- 14, IR-*Ace*1 RNAi transgenic tobacco lines; lane 16, non-transgenic tobacco; lane 17, pBI121::IR-*Ace*1 RNAi vector. Arrow indicates amplification of 790 bp fragment of *npt*II.

Fig.2C. RT-PCR analysis for nptII expression in *Ace*1 RNAi transgenic tobacco lines. Lane 1 & 18, 1 kb DNA ladder; lane1- 14, *Ace*1 RNAi transgenic tobacco lines; lane 16, non-transgenic tobacco plant; lane 17, pBI121-*Ace*1 RNAi vector. Arrow indicates the amplification of the 790 bp fragment of the *npt*II gene in transgenic tobacco lines.

Fig.3A. Detached leaf bioassay of *Ace*1 RNAi tobacco lines with 2^{nd} instar larvae of *H. armigera.* 4, 8, 20, 10, and 18 are independent *Ace*1 RNAi tobacco lines and C, non-transformed control tobacco leaves.

Fig. 3B. The *H. armigera* larvae ($2n^{d}$ instar) were fed with transgenic lines expressing *ace1* hpRNA. The larval mortality % was calculated and compared with the control-fed larva. C, non-transformed control tobacco; Lines 4, 8, 10, 18, 20, transgenic tobacco lines.

Fig.3C. Comparison of larval growth during insect bioassay. 1, larvae fed on control leaves; 2, larvae fed on *Ace*1 RNAi tobacco leaves.

Fig.3 D. Semi-quantitative RT-PCR analysis for *Ace*1expression in *H. armigera* larvae. a. *Ace*1 gene expression in *H. Armigera*; b. Amplification of internal control gene ß- actin; M –

1 kb ladder, C- Control *H. armigera,* Ri- *H. armigera fed* on *Ace1* RNAi tobacco leaves. Arrows show 700 bp amplification of *Ace1* transcript and 150 bp of *ß- actin* transcript specific amplification respectively.

Fig.4A. Southern analysis of *Ace*1 RNAi transgenic tobacco lines. WT, non-transformed control tobacco; Lines 4, 8, 10, 18, 20, transgenic tobacco lines. The numbers on the left indicate the size of the band in kb.

Fig.4B. Northern analysis of *Ace*1 RNAi transgenic tobacco lines. Control, non-transformed control tobacco; Lines 4, 8, 10, 18, 20 transgenic tobacco lines.

Figures

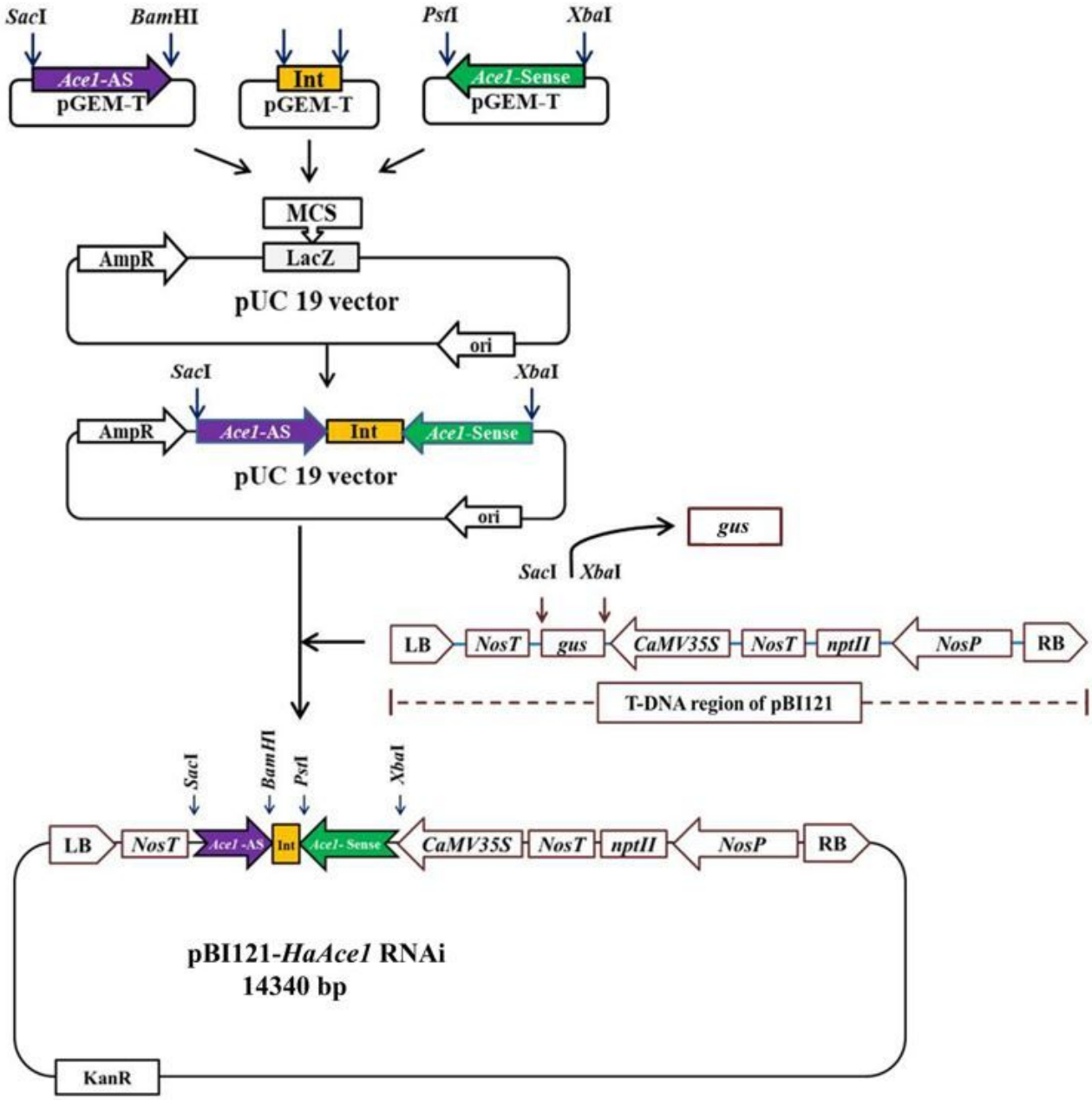

Figure 1

Construction strategy of RNAi binary vector, pBI121:: IR Ace1.

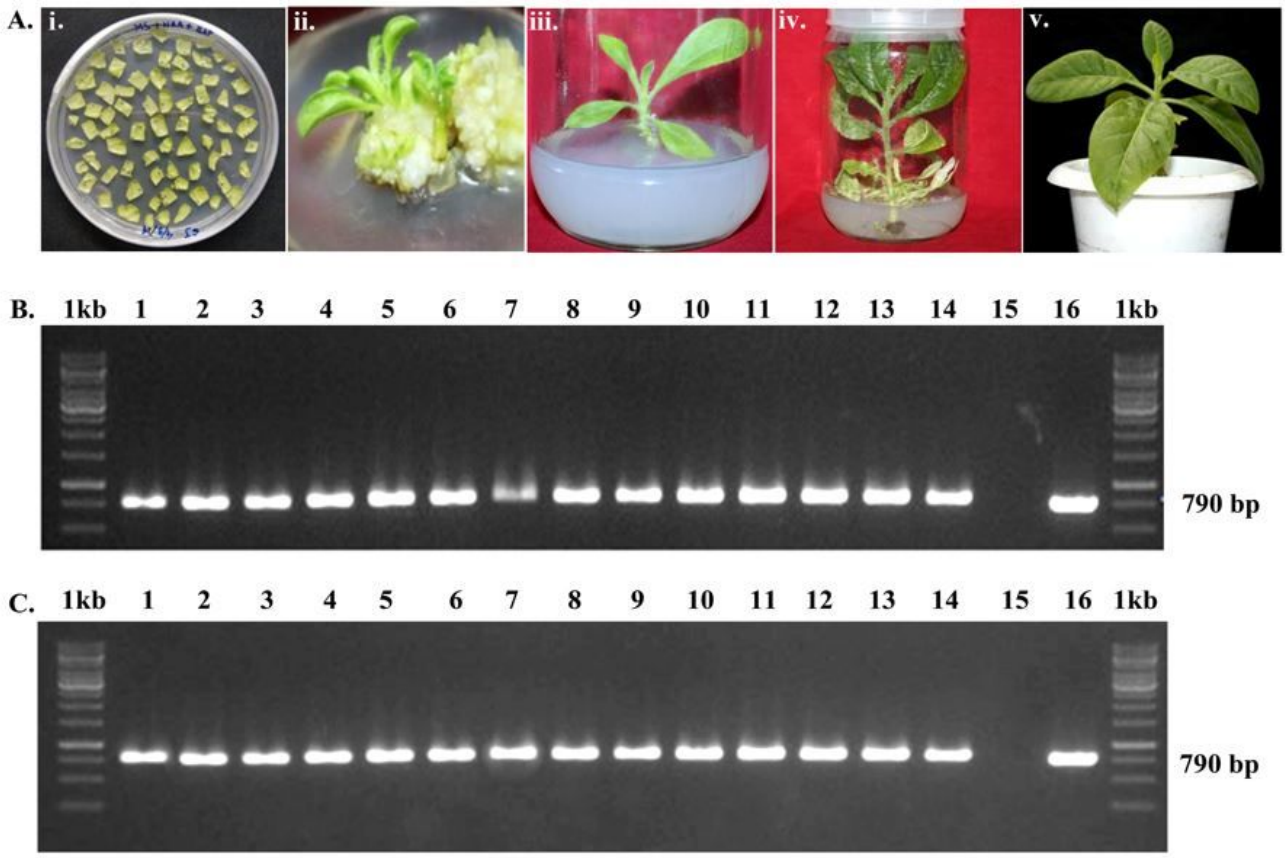

Figure 2

A. Various stages of tobacco transformation with IR-Ace1 RNAi gene construct. i). Co-4 cultivation; ii). Selection and callusing; iii). Adventitious Shooting; iv). Shoot elongation and 5 rooting; v) Fully developed plant transferred to the pot.

B. PCR confirmation of Ace1 RNAi transgenic tobacco lines. Lane 1 & 18, 1 kb DNA ladder; lane1 14, IR Ace1 RNAi transgenic tobacco lines; lane 16, non transgenic tobacco; lane 17, pBI121::IR Ace1 RNAi vector. Arrow indicates amplification of 790 bp fragment of 10 nptII.

C. RT PCR analysis for nptII expression in Ace1 RNAi transgenic tobacco lines. Lane 1 & 18, 1 kb DNA ladder; lane1 14, Ace1 RNAi transgenic tobacco lines; lane 16, non transgenic tobacco plant; lane 17, pBI121 Ace1 RNAi vector. Arrow indicates the amplification of the 790 bp fragment of the nptII gene in transgenic tobacco lines.

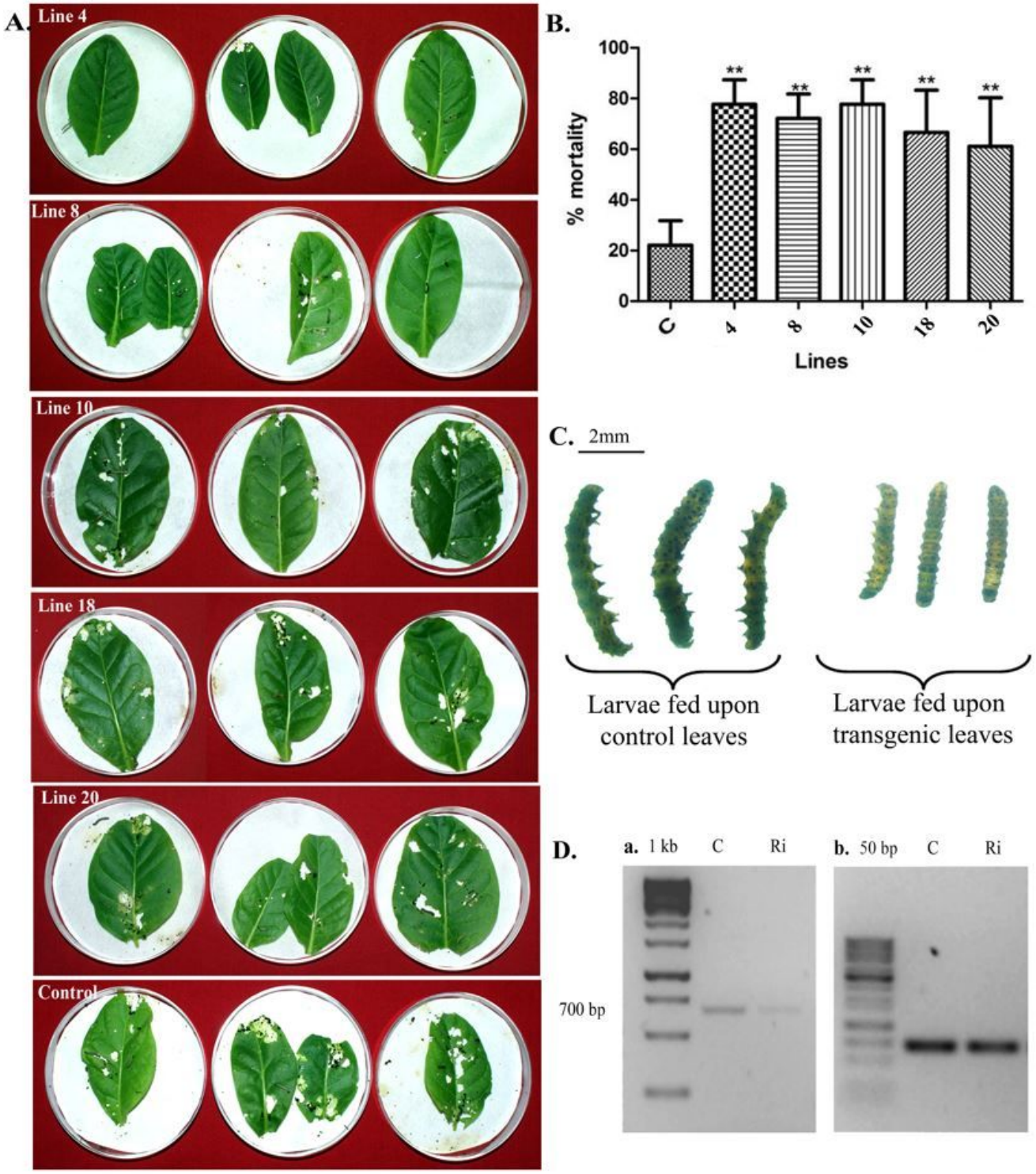

Figure 3

A. Detached leaf bioassay of Ace1 RNAi tobacco lines with 2nd instar larvae of H. armigera. 4, 8, 20, 10, and 18 are independent Ace1 RNAi tobacco lines and C, non transformed control tobacco leaves.

B. The H. armigera larvae (2nd instar) were fed with transgenic lines expressing ace1 hpRNA. The larval mortality % was calculated and compared with the control fed larva. C, non transformed control tobacco;

Lines 4, 8, 10, 18, 20, transgenic tobacco lines.

C. Comparison of larval growth during insect bioassay. 1, larvae fed on control leaves; 2, larvae fed on Ace1 RNAi tobacco leaves.

D. Semi quantitative RT PCR analysis for Ace1expression in H. armigera larvae. a. Ace1 gene expression in H. Armigera; b. Amplification of internal control gene ß actin; M 1 kb ladder, C– Control H. armigera, Ri– H. armigera fed on Ace1 RNAi tobacco leaves. Arrows show 700 bp amplification of Ace1 transcript and 150 bp of ß– actin transcript specific amplification respectively.

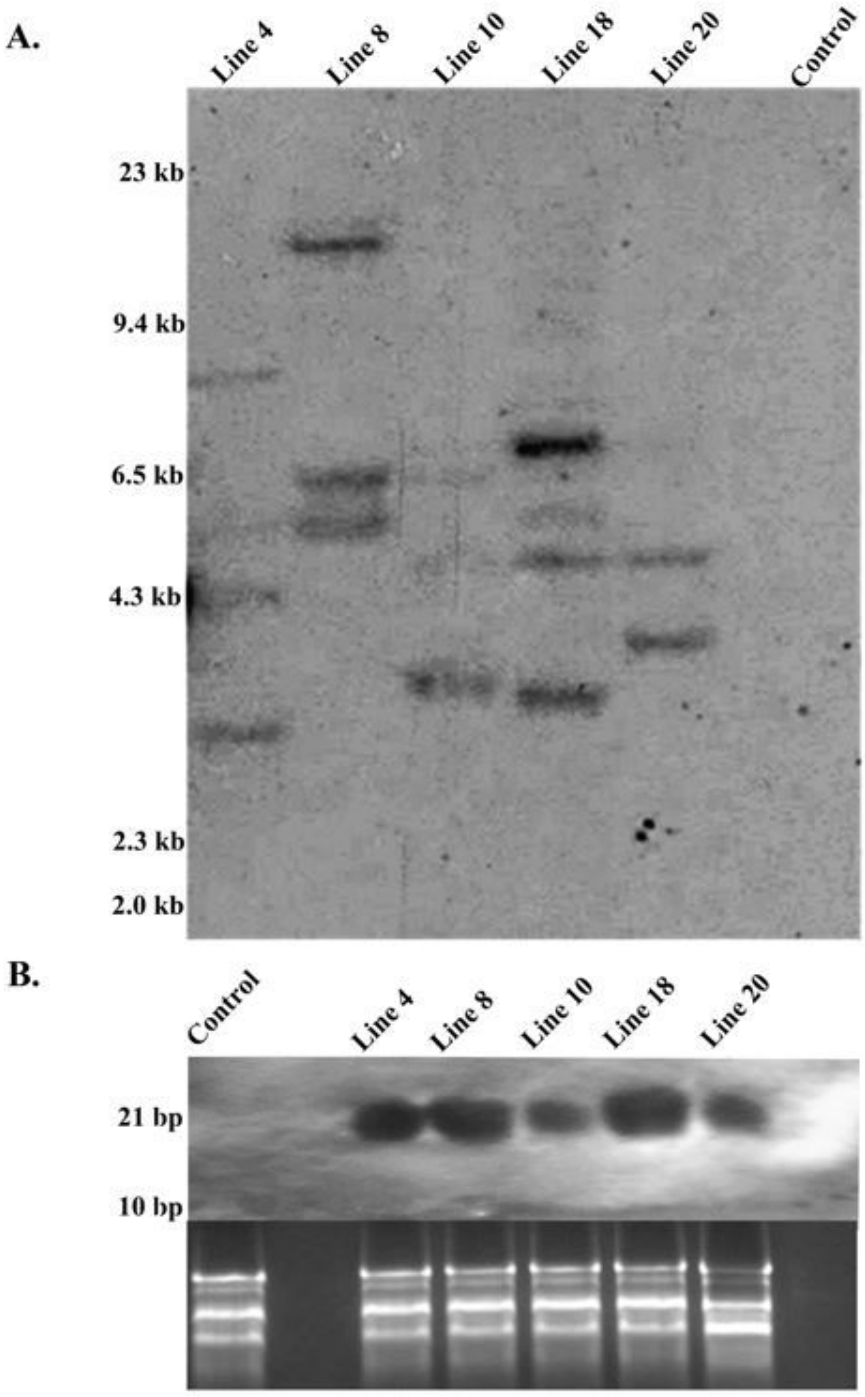

Figure 4

A. Southern analysis of Ace1 RNAi transgenic tobacco lines. WT, non transformed control tobacco; Lines 4, 8, 10, 18, 20, transgenic tobacco lines. The numbers on the left 6 indicate the size of the band in kb.

B. Northern analysis of Ace1 RNAi transgenic tobacco lines. Control, non transformed control tobacco; Lines 4, 8, 10, 18, 20 transgenic tobacco lines.

Supplementary Files

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- SupplementarytablesAce1.pdf