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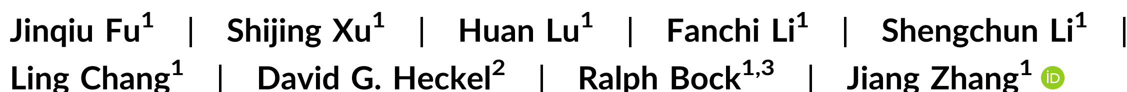
Resistance to RNA interference by plant-derived double-stranded RNAs but not plant-derived short interfering RNAs in *Helicoverpa armigera*



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Abstract

Plant-mediated RNA interference (RNAi) has emerged as a promising technology for pest control through expression of double-stranded RNAs (dsRNAs) targeted against essential insect genes. However, little is known about the underlying molecular mechanisms and whether long dsRNA or short interfering RNAs (siRNAs) are the effective triggers of the RNAi response. Here we generated transplastomic and nuclear transgenic tobacco plants expressing dsRNA against the *Helicoverpa armigera ATPaseH* gene. We showed that expression of long dsRNA of *HaATPaseH* was at least three orders of magnitude higher in transplastomic plants than in transgenic plants. *HaATPaseH*-derived siRNAs are absent from transplastomic plants, while they are abundant in transgenic plants. Feeding transgenic plants to *H. armigera* larvae reduced gene expression of *HaATPaseH* and delayed growth. Surprisingly, no effect of transplastomic plants on insect growth was observed, despite efficient dsRNA expression in plastids. Furthermore, we found that dsRNA ingested by *H. armigera* feeding on transplastomic plants was rapidly degraded in the intestinal fluid. In contrast, siRNAs are relatively stable in the digestive system. These results suggest that plant-derived siRNAs may be more effective triggers of RNAi in Lepidoptera than dsRNAs, which will aid the optimization of the strategies for plant-mediated RNAi to pest control.

KEYWORDS

cotton bollworm, double-stranded RNA, plastid transformation, RNA interference, short interfering RNA, transgenic plants

1 | INTRODUCTION

The cotton bollworm *Helicoverpa armigera* is a major lepidopteran insect pest responsible for severe yield losses in cotton and many other crops. It is highly polyphagous and feeds on over 300 host species from 68 plant families (Wang et al., 2018). It has recently expanded its range from the Old World into South America. Application of chemical pesticides and development of transgenic plant lines expressing *Bacillus thuringiensis* (Bt) toxins are two major strategies for pest management. However, in recent years, evolution of resistance to chemical pesticides occurred, and genes conferring resistance to Bt toxins have been detected in field populations, thus necessitating the development of novel strategies for pest control.

RNA interference (RNAi) is a conserved mechanism in eukaryotes by which double-stranded RNA (dsRNA) triggers gene silencing. Since its discovery more than 20 years ago (Fire et al., 1998), it has

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provided a powerful tool for the study of gene function. More recently, RNAi was applied as a novel strategy for insect pest control. When dsRNAs whose sequences are derived from essential insect genes are delivered into insect cells, downregulation of the target genes through induction of RNAi occurs. dsRNA-mediated gene silencing is triggered by the endogenous RNAi machinery of the insect, and can lead to retarded growth, impaired fecundity, and even death of the insect. Transgenic plants expressing dsRNA targeted against an insect cytochrome P450 gene showed substantial resistance to *H. armigera*, representing the first example of plant-mediated RNAi for pest control (Mao et al., 2007). Similarly, by expressing dsRNA or microRNA against various genes of *H. armigera* in transgenic plants, induction of RNAi and impaired growth of the insects have been observed (Agrawal et al., 2015; Liu et al., 2015; Saini et al., 2018; Wu et al., 2016; Xiong et al., 2013; Zhu et al., 2012). However, full protection of the plants and efficient killing of *H. armigera* have not been achieved so far.

Expressing dsRNAs that target vital genes of the Colorado potato beetle (CPB) from the plastid (chloroplast) genome, we have recently shown that this pest can be efficiently controlled by RNAi. After a few days of feeding on transplastomic (plastid genome-transformed) potato plants expressing dsRNA derived from the *β-Actin* gene (*ACT*) of CPB, all larvae died (Zhang et al., 2015). It has been suggested that the success with controlling CPB by plastid-mediated RNAi is due to (i) the very high expression level of dsRNAs attainable in plastids and (ii) the absence of the Dicer endoribonuclease from plastids, thus enabling accumulation of long dsRNAs. The latter is consistent with the discovery of a length requirement for dsRNA to be effective in pest control (Bolognesi et al., 2012). Long dsRNAs were reported to be much more potent than siRNAs in inducing a strong RNAi response. Systematic tests with dsRNAs of different length in the western corn rootworm (WCR, *Diabrotica virgifera virgifera*, Coleoptera) revealed that dsRNAs with a minimum size of approximately 60 base pairs (bp) are required to trigger a strong RNAi effect (Bolognesi et al., 2012).

Although there is strong evidence supporting the need for long dsRNAs to induce environmental RNAi in beetles (Coleoptera), the generality of these findings and their applicability to other groups of insects is currently unclear. For example, the types of plant-delivered RNAi molecules most efficient in mediating gene silencing in *H. armigera* are not known. Since the plant's own RNAi pathway processes dsRNAs into siRNAs, it has not been possible to distinguish between the effects of long dsRNAs and siRNAs in suppressing target gene expression and inhibiting growth of *H. armigera* when feeding on transgenic (nuclear genome-transformed) plants. Arabidopsis *dcl2dcl3dcl4* mutants (with reduced Dicer activity) expressing dsRNA derived from *HaCYP6AE14* seem to trigger a more pronounced effect than wild-type plants in silencing *HaCYP6AE14* expression in *H. armigera* (Mao et al., 2007). However, feeding of synthetic siRNAs in an artificial diet or expression of microRNAs in transgenic plants also brought about a detectable RNAi response in *H. armigera* (Agrawal et al., 2015; Kumar et al., 2009; Saini et al., 2018). Thus, whether dsRNAs or siRNAs are more efficient in triggering RNAi-induced gene silencing in *H. armigera* and whether different RNA size requirements exist in Lepidopteran versus Coleopteran insects still needs to be clarified.

Thus far, three studies have used plastid transformation technology to test RNAi strategies for controlling Lepidopteran insects. Jin et al. employed a transplastomic approach to express a single 19-nt siRNA from the tobacco plastid genome and showed reduced weight gain of *Helicoverpa zea* larvae that fed on the transplastomic tobacco plants (Jin et al., 2015). A study by Bally et al. showed that transplastomic lines of *Nicotiana benthamiana* that express long dsRNA targeted against the *H. armigera ACE2* gene were less palatable to the insects than nuclear transgenic plants expressing the same dsRNA fragment (Bally et al., 2016). By contrast, transplastomic tobacco plants expressing *ATPaseA* dsRNA did not affect gene expression or survival of the tobacco hornworm *Manduca sexta* (Burke et al., 2019). From these contradictory results, no general conclusion could be drawn about the effectiveness of long versus short dsRNAs and siRNAs in inducing an RNAi response in Lepidopteran insects.

To identify the effective RNA molecules for RNAi induction in Lepidoptera and for the control of *H. armigera*, we generated transplastomic and transgenic tobacco plants that expressed dsRNA targeted against *HaATPaseH*, an essential insect gene encoding a subunit of vacuolar-type proton ATPase (Jefferies et al., 2008). We found that transplastomic plants accumulated dsRNA to levels that were at least three orders of magnitude higher than those in transgenic plants. The presence of siRNAs in nuclear transgenic plants and their absence from transplastomic plants suggests that plastids do not possess an RNAi machinery for dsRNA processing. We further demonstrated that only nuclear transgenic plants caused partial suppression of *HaATPaseH* expression and, therefore, exhibited reduced susceptibility to *H. armigera*. No RNAi effect was observed when *H. armigera* larvae fed on leaves from transplastomic plants expressing dsRNA targeted against *HaATPaseH* or other target genes such as *HaATPaseA* (encoding the A-subunit of vacuolar-type proton ATPase), *HaATPaseD* (encoding the D-subunit of vacuolar-type proton ATPase), *HaCOPB* (encoding the β-subunit of the coatomer protein I vesicle complex) and *HaACT* (encoding the β-subunit of actin, an essential cytoskeletal protein). Moreover, the resistance to plastid-mediated RNAi in *H. armigera* was independent of the conformation of the dsRNA expressed in plastids. Importantly, we found that plastid-expressed long dsRNAs were more prone to degradation upon incubation in intestinal fluid of *H. armigera* than plant-derived siRNAs. These observations suggest that plant-derived siRNAs could be the main effective molecules that induce gene silencing and trigger RNAi responses in *H. armigera*.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Tobacco (*Nicotiana tabacum* cv. Petit Havana) plants were grown under aseptic conditions on agar-solidified MS medium containing 3% (w/v) sucrose (Murashige & Skoog, 1962). Regenerated

homoplasmic shoots were rooted and propagated on the same medium. Rooted plantlets were transferred to soil and grown in a greenhouse under 160 μmol m^{-2} s^{-1} constant light in a 16 h light/8 h dark photoperiod, at 22°C/20°C and 50% relative humidity.

2.2 | Vector construction

To construct a plastid transformation vector for dsRNA expression, two copies of the plastid 16S ribosomal RNA promoter (*Prrn*) were amplified. The first copy was amplified with primer pair Prrn(*Hin*dIII)-F/Prrn(*Sph*I)-R (Table S1), introducing *Hin*dIII and *Sph*I restriction sites with the primer sequences (Table S1). The polymerase chain reaction (PCR) product was digested with *Hin*dIII/*Sph*I (Takara), and cloned into the similarly cut cloning vector pUC19, generating plasmid pJZ11. Subsequently, a *HaATPaseH* fragment (covering nucleotides +204 to +528 of the coding region of the V-type proton ATPase subunit H complementary DNA [cDNA]) was amplified by primer pair ATPaseH(*Sph*I)-F/ATPaseH(*Xba*I)-R (Table S1) and cloned into pJZ11 digested with *Sph*I and *Sal*I, generating plasmid pJZ20. The second *Prrn* promoter copy was amplified using primers Prrn(*Eco*RI)-F/Prrn(*Sac*I)-R (Table S1). The PCR product was digested with *Eco*RI and *Sac*I, and cloned into the similarly cut plasmid pJZ20, resulting in plasmid pJZ33. Finally, the dsRNA expression cassette was excised from pJZ20 as *Eco*RI/*Hin*dIII fragment and inserted into plastid transformation vector pPRV111A (Zoubenko et al., 1994) digested with the same restriction enzymes, generating vector pJZ43 (expressing ds*HaATPaseH*). For targeting other genes of *H. armigera*, *HaATPaseA* (covering nucleotides +2058 to +2402 of the coding region of the v-ATPase A-subunit cDNA, NCBI Sequence ID: XM_021345002.1), *HaATPaseD* (covering nucleotides +265 to +604 of the coding region of the v-ATPase D-subunit cDNA, NCBI Sequence ID: XM_021345863.1) and *HaCOPB* (covering nucleotides +2519 to +2769 of the coding region of the coatomer subunit β cDNA, NCBI Sequence ID: XM_021331819.1), cDNA fragments were amplified with primer pairs ATPaseA(*Sph*I)-F/ATPase(*Sal*I)-R, ATPaseD(*Sph*I)-F/ATPaseD(*Sal*I)-R and COPB(*Sph*I)-F/COPB(*Sal*I)-R, respectively (Table S1). Plastid transformation vectors pJZ41 (expressing ds*HaATPA*), pJZ42 (expressing ds*HaATPD*) and pJZ44 (expressing ds*HaCOPB*) were generated using similar procedures as for pJZ43. The *HaACT* fragment covers nucleotides +660 to +956 of the coding region of the *β-Actin* cDNA (NCBI Sequence ID: XM_021337112.1) and was amplified with primer pair HaACT-F/HaACT-R and cloned into *Sbf*I/*Sac*I-digested plasmid pJZ199 (Zhang et al., 2015), to generate vector pJZ266 (expressing ds*HaACT*). For construction of the hairpin RNA (hpRNA)-expressing vector, the *HaATPaseD* fragment was amplified using primer pair ATPaseD(*Sph*I)-F/ATPaseD(*Sal*I)-R and cloned into pJZ11 digested with *Sph*I and *Sal*I, generating plasmid pJZ19. T*rbcL* (3′-UTR of plastid *rbcL* gene) was excised from a plasmid clone (pZF1; Zhou et al., 2008) as *Hin*dIII/*Eco*RI fragment and inserted into the similarly cut vector pJZ19, generating plasmid pJZ47. The first intron from the potato gibberellin 20 (GA20) oxidase gene was excised from a plasmid clone (pUC-RNAi; Chen et al., 2003) as *Xho*I/*Sal*I fragment and inserted into the similarly cut vector pJZ47, generating plasmid pJZ50. A second copy of the *HaATPaseD* sequence was amplified with primer pair ATPaseD(*Xba*I)-F/ATPaseD(*Sal*I)-R (-Table S1). The PCR product was cloned (in antisense orientation) as *Xba*I/*Sal*I fragment into the similarly cut pJZ50, generating plasmid pJZ52. Finally, the hpRNA cassette was excised from pJZ52 as *Eco*RI/*Hin*dIII fragment and inserted into inserted into plastid transformation vector pPRV111A, generating vector pJZ57 (expressing hp*HaATPaseD*). To assemble vectors designed to express dsRNAs with flanking stem-loop structures (dsRNA-SL), the *HaATPaseA* and *HaATPaseD* fragments were amplified with primer pairs ATPaseA(*Pst*I)-F/ATPaseD(*Sal*I)-R and ATPaseD(*Pst*I)-F/ATPaseD(*Sal*I)-R, respectively (Table S1) and cloned into the similarly cut pJZ10 (a plasmid containing the *Prrn* promoter including a sequence folding into a 24 bp stem-loop structure at the RNA level; Zhang et al., 2015), producing plasmids pJZ12 and pJZ13, respectively. The second *Prrn* promoter copy (also including a sequence folding into a 24 bp stem-loop structure at the RNA level) was amplified with primer pair Prrn(*Eco*RI)-F/PrrnSL2(*Bam*HI)-R (Table S1) and inserted into pJZ12 and pJZ13 digested with *Eco*RI and *Bam*HI, resulting in plasmids pJZ91 and pJZ92, respectively. The dsRNA-SL expression cassettes were then excised from pJZ91 and pJZ92 as *Eco*RI/*Hin*dIII fragments and subcloned into pBluescript KS(−), generating plasmids pJZ96 and pJZ97, respectively. Finally, the dsRNA-SL cassettes were excised from pJZ96 and pJZ97 as *Not*I/*Xho*I fragments and cloned into plastid transformation vector pJZ100, generating vectors pJZ108 (expressing ds*HaATPaseA-SL*) and pJZ109 (expressing ds*HaATPaseD-SL*), respectively.

For expression of dsRNAs in the nucleus, the in vivo assembly in *Escherichia coli* (iVEC) approach was employed for cloning as described previously (Wu et al., 2017). Briefly, two copies of the identical *HaATPaseH* fragment as in pJZ43 were amplified. The first copy was amplified with primer pair RiATPH-F-XbaI/RiATPH-R-XbaI (Table S1). The obtained PCR product was mixed with the linearized vector pHELLSGATE8 that had been digested with *Xba*I (PCR fragment and vector mixed in a stoichiometric ratio of 2:1), and the mixture was cotransformed into *E. coli* (XL10-Gold, Agilent Technologies) chemically competent cells (>2 × 10^8 cfu/μg assayed on pUC19). The resulting plasmid clone was named as pSJ9. The second copy of the *HaATPaseH* fragment was amplified using primer pair RiATPH-F-*Xho*I/RiATPH-R-*Xho*I (Table S1), and cloned into the linearized vector pSJ9 that had been digested with *Xho*I, using the iVEC method, generating plasmid pSJ17.

To construct a vector for in vitro synthesis of ssRNA, the *HaATPaseH* fragment was amplified by primer pair ATPaseH(*Xba*I)-F and ATPaseH(*Sal*I)-R (Table S1) using pJZ43 as template. The obtained PCR product was cloned as *Xba*I/*Sal*I fragment into pBluescript KS(−), resulting in plasmid pKS_ATPH.

3 | PLASTID AND NUCLEAR TRANSFORMATION

Tobacco plastid transformation and regeneration of transplastomic lines were carried out as previously described (Zhang et al., 2015). Plasmid DNA for plastid transformation was prepared using the Nucleobond Xtra Plasmid Midi Kit (Macherey-Nagel). Young leaves of

tobacco plants grown under aseptic conditions were bombarded with DNA-coated 0.6 µm gold particles using a PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). Primary spectinomycin-resistant lines were selected on RMOP shoot regeneration medium (Svab & Maliga, 1993) containing 500 mg/L spectinomycin. Several independent transplastomic lines were subjected to two to three additional rounds of regeneration on spectinomycin-containing medium to select for homoplasmy of the transgenic plastid genome. Homoplasmy of transplastomic events was confirmed by Southern blot.

Nuclear transgenic tobacco plants were generated by *Agrobacterium*-mediated transformation as described before (Zhang et al., 2016). Transgenic plants were identified by kanamycin selection and tested for the presence of the transgene by PCR assays.

4 | ISOLATION OF NUCLEIC ACIDS AND GEL BLOT ANALYSES

Total cellular DNA was isolated from leaves of wild type and transplastomic plants by a cetyltrimethylammonium bromide-based protocol (Doyle & Doyle, 1990). Total RNA was isolated using the TRIzol Reagent (Invitrogen) following the manufacturer's protocol. For Southern blot analysis, 5 µg total cellular DNA were digested with *Bam*HI (Takara) for 12 h, separated by agarose gel electrophoresis on 1% agarose gels and transferred onto Hybond nylon membranes (GE Healthcare). To verify plastid transformation and assess the homoplasmic status of the various transplastomic lines, hybridization probes were produced by (i) excision of a 1080 bp fragment (covering a portion of the *16 S rRNA* gene and its promoter region) from pJZ43 with *Apa*I and *Stu*I, and (ii) PCR amplification of tobacco plastid DNA with primer pairs Nt16SP-F/Nt16SP-R and PpsaBrps14-F/PpsaBrps14-R, respectively (Table S1). For RNA gel blot analysis, RNA samples were denatured and separated in formaldehyde-containing 1% agarose gels and blotted onto Hybond nylon membranes (GE Healthcare). For siRNA analysis, samples of 50 µg total cellular RNA were separated in 14% polyacrylamide gels with 0.3 M sodium acetate and 7 M urea as gel buffer and 0.3 M sodium acetate as running buffer. The separated RNA samples were electroblotted onto Hybond nylon membranes in blotting buffer (10 mM Tris-acetate pH 7.8, 5 mM sodium acetate, 0.5 mM Embryonic Testis Differentiation Homolog A [ETDA]) at 40 V for 2 h at 4°C. A 376-bp PCR product generated by amplification of the specific *HaATPaseH* fragment using primer pair ATPaseH(*Sph*I)-F and ATPaseH(*Xba*I)-R (Table S1) was used as probe to determine *HaATPaseH* dsRNA (ds*HaATPH*) or siRNA (si*HaATPH*) accumulation. Similarly, probes to detect *HaATPaseA*, *HaATPaseD*, *HaACT* and *HaCOPB* dsRNA accumulation in plastids were produced by PCR amplification of gene-specific fragments using primer pairs P-HaATPA-F/P-HaATPA-R, P-HaATPD-F/P-HaATPD-R, P-HaACT-F/P-HaACT-R and P-HaCOPB-F/P-HaCOPB-R, respectively (Table S1). Probes were labeled either with [α^{32}P]dCTP using the Multiprime DNA labeling system (GE Healthcare) or with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche), following the manufacturers' protocols. Hybridizations were performed at 65°C for Southern blots and 42°C for northern blots.

4.1 | Quantitative reverse-transcription PCR (qRT-PCR)

qRT-PCR was conducted to analyze relative expression levels of dsRNA in nuclear transgenic lines and *ATPaseH*, *ATPaseA*, *ATPaseD*, *ACT* and *COPB* expression in *H. armigera*. Reactions were performed in a CFX96 Touch™ real-time PCR detection system (Bio-Rad) using SYBR® Premix Ex Taq™ II (Takara). All qRT-PCR assays were conducted in three independent experiments. Three technical replicates were performed for each biological replicate. The tobacco *β-Actin* and the *H. armigera RPL32* genes were used as reference genes (Zhang et al., 2016). Primer sequences for qRT-PCR are listed in Table S1.

4.2 | In vitro ssRNA, dsRNA and siRNA synthesis

For ssRNA synthesis by in vitro transcription from plasmid DNA, pKS_ATPH was linearized with *Xba*I and purified. In vitro transcription was carried out with T3 RNA polymerase (Thermo Scientific). The amount of ssRNA obtained was determined with a NanoDrop ND-1000 spectrophotometer. dsRNA was synthesized with the T7 RiboMAX Express RNAi System (Promega) according to the manufacturer's instruction. The minimal T7 promoter sequence (5′-TAATACGACTCACTATAGG-3′) was added to the 5′-end of forward and reverse primers (Table S1). A gene-specific siRNA, si*HaATPaseH* (sense: 5′-ACCUGUGCUGAGGUGUUCAdTdT-3′; antisense: 5′-UGAACACCUCAGCACAGGUdTdT-3′), was designed as dsRNA and chemically synthesized (GenePharma). To prepare siRNAs for in vitro feeding assay, dsRNAs were digested with ShortCut® RNase III (New England Biolabs, catalog No. M0245L) according to the manufacturer's protocol.

5 | INSECT BIOASSAY

Cotton bollworm eggs were hatched on artificial diet, and larvae were reared in a growth chamber at 27°C under a 14 h light/10 h dark photoperiod and 70% relative humidity. Synchronous second-instar larvae were selected and divided into groups of 36 individuals. Young and fully expanded leaves from 4-week-old and 8-week-old tobacco plants were used for feeding assay and replaced daily during the whole bioassay. The larvae were weighed individually at the indicated time points, and photos were taken. For in vitro dsRNA and siRNA feeding assays, 16 synchronous second-instar larvae were fed with tobacco leaves that had been painted with 200 ng/cm^2 dsRNA or siRNA. After larvae had been allowed to feed for 72 h, total RNA was isolated from individual larvae with the Trizol Reagent (Invitrogen) and used for cDNA preparation and quantitative real-time PCR. All the bioassays were repeated at least for three times.

5.1 | In vitro RNA degradation assays

Hemolymph from third instar *H. armigera* larvae was collected through an incision made on one of the legs at room temperature and immediately chilled on ice. *H. armigera* larvae spit intestinal fluid after stimulation. To stimulate spitting, the larva was gently fixed between fingers, and softly touched by a pipette tip at the larval mouth cavity. The intestinal fluid sample was then collected into a 2 ml sterile Eppendorf tube on ice. Hemolymph and intestinal fluid samples were cleared by centrifugation at 13,000 rpm for 10 min, followed by transfer of the supernatant to a fresh tube. Samples of 200 ng ds*HaATPaseH* or si*HaATPaseH* (in 1 µl water) were incubated with 5 µl hemolymph or intestinal fluid (diluted 15-fold) at 25°C for the indicated times. The reactions were then analyzed by electrophoresis in 1% agarose gels. For *in planta* degradation assays, 1.5 g leaf materials from transplastomic or nuclear transgenic tobacco plants were homogenized with a grinder in 2 ml phosphate-buffered saline buffer. Samples of 200 µl of the homogenate were incubated with 200 µl intestinal fluid (diluted 15-fold) at 25°C for the indicated times. Subsequently, the RNA was isolated and analyzed by northern blotting.

5.2 | Data analysis

Statistical analyses of the data for larval weight, pupal weight and relative transcript expression were performed by *t* tests for comparison of two groups or one-way analysis of variance (ANOVA) coupled with Dunnett's tests for multiple comparisons using the SPSS software (IBM). Two-way ANOVA was used to analyze the effects of in vitro synthesized dsRNAs and siRNAs on *H. armigera* larvae.

6 | RESULTS

6.1 | Generation of transplastomic and nuclear transgenic plants expressing dsRNA against an essential cotton bollworm gene

To compare the efficiency of the RNAi responses induced by dsRNA expressed from either the plastid or the nuclear genome of tobacco plants, a 376-bp fragment of the *HaATPaseH* cDNA from *H. armigera* was cloned into both nuclear and plastid transformation vectors harboring dsRNA expression cassettes (Figure 1A,B). The *ATPaseH* encodes the H subunit of vacuolar-type proton ATPase, an electrogenic proton pump that generates energy to pump protons across the plasma membrane by hydrolyzing adenosine triphosphate to adenosine diphosphate and phosphate (Jefferies et al., 2008). Plastid transformation vector pJZ43 was constructed to facilitate integration of the dsRNA expression cassette into the *trnV-3'rps12* region of the tobacco plastid genome via homologous recombination. Vector pJZ43 consists of the left (containing the 5′-part of *rrn16* and *trnV*) and right (containing the 3′-part of *rps12*) flanking plastid DNA regions, and two expression cassettes that harbor the spectinomycin resistance gene *aadA* and the *ATPaseH* fragment for dsRNA expression, respectively. Two convergent plastid P*rrn* promoters drive dsRNA synthesis in plastids (Figure 1a). The pJZ43 construct was introduced into the plastid genome of tobacco by biolistic bombardment, followed by selection for spectinomycin resistance conferred by the chimeric *aadA* marker gene (Svab & Maliga, 1993). Homoplasmic transplastomic lines were isolated by conducting additional cycles of regeneration on spectinomycin-containing medium, and the homoplasmic status was ultimately confirmed by Southern blot analyses (Figure 1c) and seed assays (Figure S1). To be able to compare the level of resistance to *H. armigera* in transplastomic and nuclear transgenic plants, the binary vector pSJ17 was constructed for expression of hpRNA targeting the identical region of the *ATPaseH* messenger RNA (mRNA) of *H. armigera*. Construct pSJ17 was introduced into nuclear genome by *Agrobacterium*-mediated transformation (Figure 1b). Total RNA from 10 transgenic lines was extracted, and the expression level of the hpRNA was determined by qRT-PCR. Two transgenic lines displaying high transgene expression levels (Nt-SJ17#2 and Nt-SJ17#11) were selected according to analysis of a Dunnett's test relative to Nt-wt, homozygous lines were produced (Figure S1), and used for all subsequent analyses (Figure 1d).

6.2 | Comparison of dsRNA and siRNA accumulation in transplastomic and nuclear transgenic plants

To assess the stability and accumulation levels of ds*HaATPasH* in chloroplasts of transplastomic tobacco plants, northern blot experiments were conducted. Comparison to a dilution series of in vitro synthesized RNA, ds*HaATPasH* accumulation levels in leaves were determined to be as high as ~0.4% of the total cellular RNA (Figure 2a). By contrast, dsRNA accumulation in nuclear transgenic tobacco plants was at least three orders of magnitude lower than in transplastomic plants, as revealed by qRT-PCR (Figure 2b). Consistent with these data, hybridization signals were hardly detectable by northern blot analysis (Figure 2c). Moreover, siRNAs were abundant in nuclear transgenic plants, but were completely absent from transplastomic plants (Figure 2d). In agreement with previous observations (Zhang et al., 2015), this suggests that the processing of dsRNA into siRNAs does not occur in plastids, presumably because no RNAi machinery is present in this compartment.

6.3 | Assessment of the effect of transplastomic and nuclear transgenic plants on *H. armigera*

Having obtained the transplastomic and nuclear transgenic plants, we next tested whether dsRNA expression has a measureable effect on *H. armigera*. To this end, groups of larvae were fed with leaves

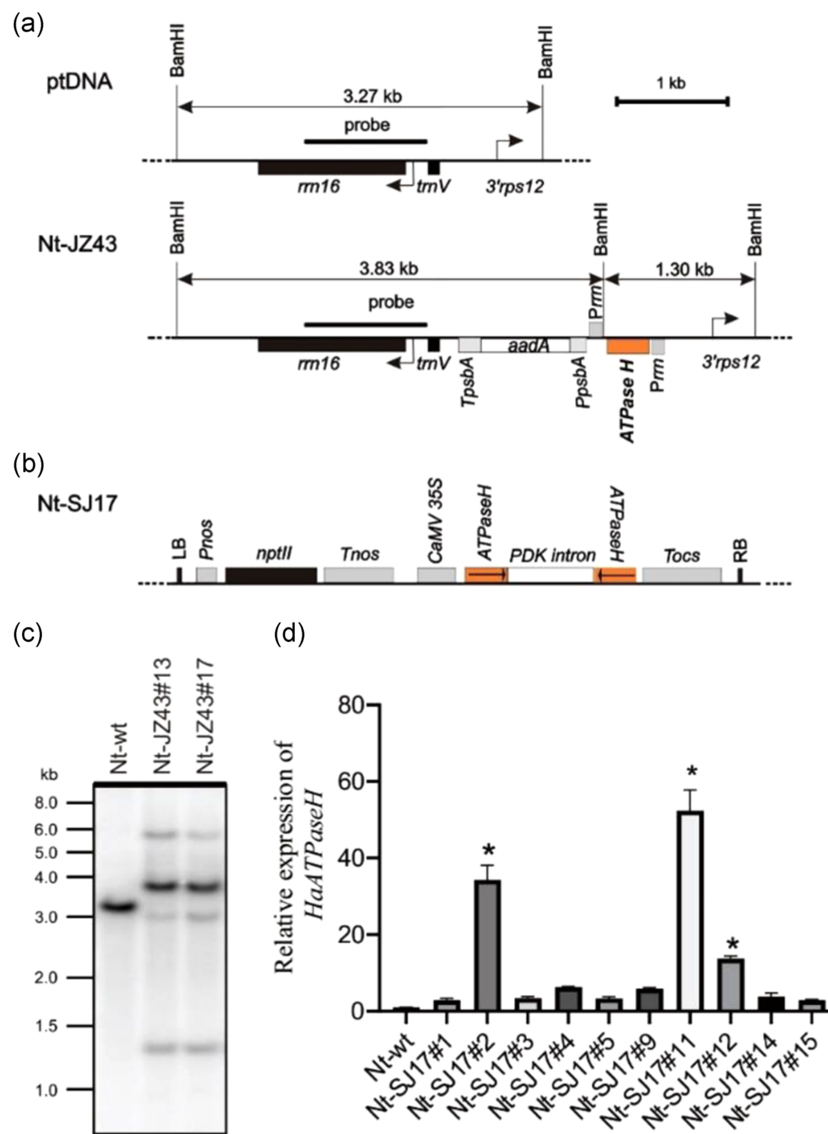

FIGURE 1 Generation of transplastomic and nuclear transgenic tobacco plants expressing dsRNA against the *ATPaseH* gene of *Helicoverpa armigera*. (a) Physical map of the targeting region in the plastid genome (ptDNA) to which the transgenes are targeted, and map of the transgenic region in Nt-JZ43 transplastomic lines. The dsRNA is generated by two convergent copies of the 16S rRNA promoter (P*rrn*). The location of restriction fragment length polymorphism (RFLP) probe is shown as a black bar. Genes above the line are transcribed from left to right, genes below the line are transcribed in the opposite direction. The *Bam*HI restriction sites used for RFLP analysis of transplastomic lines are indicated, and the sizes of the restriction fragments detected in southern blot analyses are given. The location of the hybridization probe is also shown (black bar). The selectable marker gene *aadA* is driven by the *psbA* promoter (P*psbA*) and the 3′-UTR (T*psbA*) from tobacco. (b) Map of the T-DNA locus in nuclear transgenic tobacco lines (Nt-SJ17 lines) transformed with a hairpin construct for expression of *HaATPaseH* dsRNA. CaMV 35S: 35S promoter from the Cauliflower mosaic virus; Pnos: nopaline synthase gene promoter; Tnos: nopaline synthase gene terminator; PDK intron: intron from pyruvate orthophosphate dikinase gene from *Flaveria trinervia*; T_{OCS}: octopine synthase gene terminator. (c) Southern blot analysis of transplastomic tobacco plants. Note replacement of the wild type-specific 3.27 kb band with the 3.83 kb band characteristic of the transplastome in both Nt-JZ43 lines (cf. panel a). Minor hybridizing bands in the Nt-JZ43 lines likely originate from flip-flop recombination at the duplicated plastid promoter sequences (Rogalski et al., 2006). (d) Analysis of the relative expression of the hpRNA in Nt-SJ17 transgenic lines by qRT-PCR. The tobacco *β-Actin* gene was used as an internal standard. Data are shown as means ± SEM ($n = 3$ biological replicates). $^{*}p < 0.05$, Dunnett's test. dsRNA, double-stranded RNAs; hpRNA, hairpin RNA; rRNA, ribosomal RNA; UTR, untranslated region.

of 8-week-old wild-type, transplastomic or nuclear-transgenic plants, and the weight of larvae was measured to follow their growth. These assays revealed that neither transplastomic plants nor nuclear transgenic plants induced mortality of *H. armigera* larvae. However, leaves of nuclear transgenic plants caused substantially reduced growth of *H. armigera* larvae (Figure 3). By contrast, we did not observe any effect on the growth of *H. armigera* larvae that were fed with transplastomic leaves (Figure 3a).

To confirm that the growth reduction was due to downregulation of the target gene, *HaATPaseH* expression was measured by qRT-PCR in *H. armigera* larvae. While larvae fed on nuclear transgenic plants showed a significant reduction in target gene expression, no such effect was seen in larvae fed on transplastomic plants. This result strongly suggests that the growth suppression observed in *H. armigera* larvae feeding on the nuclear transgenic plants was due to induction of an RNAi response (Figure 3b). In line with these findings, the leaf area consumed by *H. armigera* larvae was significantly lower in nuclear transgenic plants than in wild-type and transplastomic plants (Figure 3c).

To test whether the observed effects of *H. armigera* could be related to the presence of toxic compounds (such as nicotine) in tobacco plants, we also used young tobacco plants (4 weeks old) for the bioassays. Previous work has shown that, due to their lower contents of secondary metabolites, the leaves of young plants have

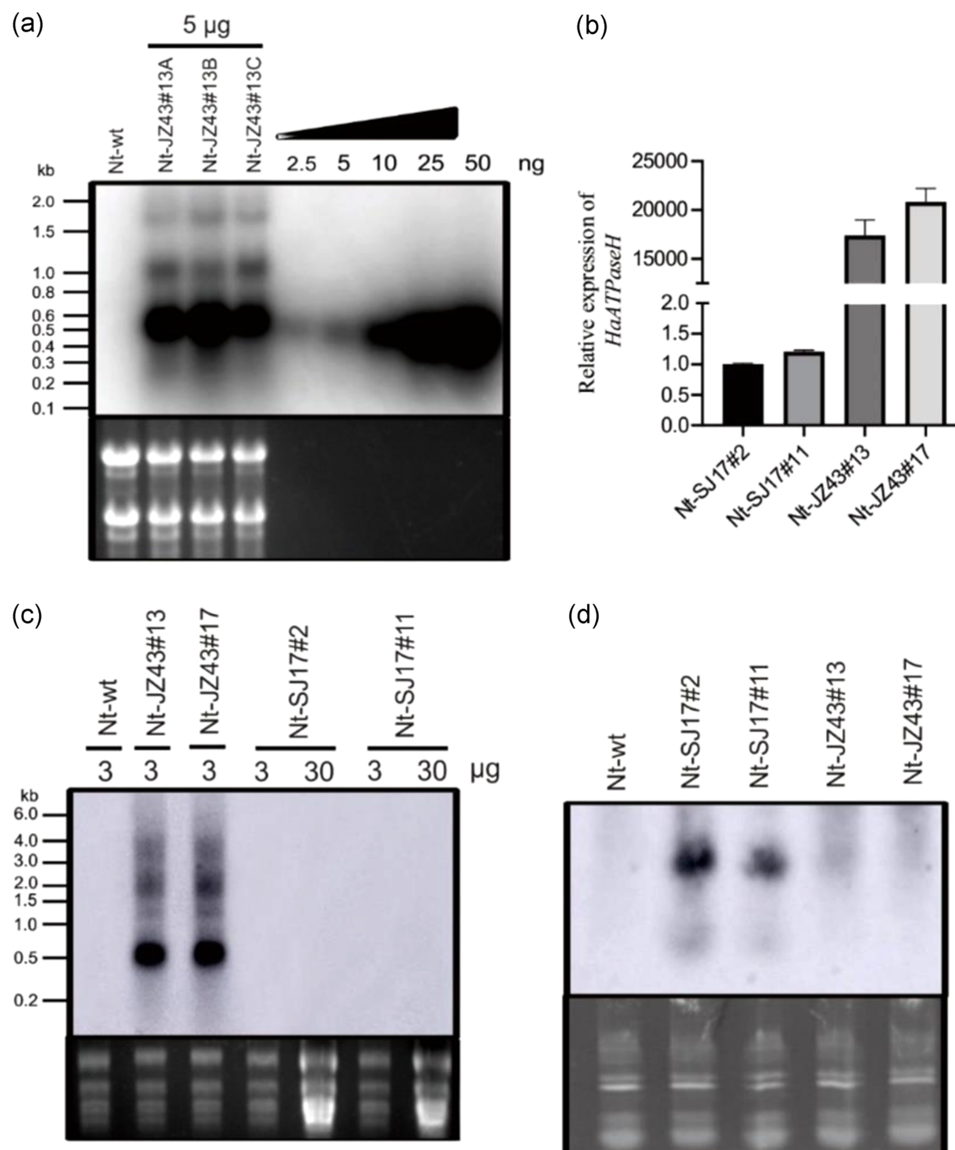

FIGURE 2 Quantification of dsRNA accumulation levels in transplastomic tobacco lines by northern blot analyses, and comparison of ds*HaATPH* and si*HaATPH* accumulation in transplastomic and nuclear transgenic tobacco plants. (a) Northern blot analysis of dsRNA accumulation. Five micrograms of total cellular RNA were loaded from three individual plants of transplastomic line Nt-JZ43#13 (a–c) and the wild-type control (Nt-wt). For semi-quantitative analysis, a dilution series of in vitro synthesized ssRNA was loaded. (b) Analysis of ds*HaATPH* expression levels by qRT-PCR. (c) Analysis of ds*HaATPH* accumulation by northern blotting. The amount of total RNA loaded in each lane is given (in μg). The GelView-stained gels before blotting are shown below the blot as a loading control. Note that 10 times more RNA was loaded for the Nt-SJ17 nuclear transgenic lines. (d) Analysis of si*HaATPH* accumulation by northern blot. Note that si*HaATPH* accumulate only in the Nt-SJ17 nuclear transgenic plants, but not in the Nt-JZ43 transplastomic plants. Thirty micrograms of total cellular RNA were loaded in each lane of the siRNA blot. The GelView-stained gels before blotting are shown below the blot as a loading control. dsRNA, double-stranded RNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; siRNA, short interfering RNA; ssRNA, single-stranded RNA [Color figure can be viewed at wileyonlinelibrary.com]

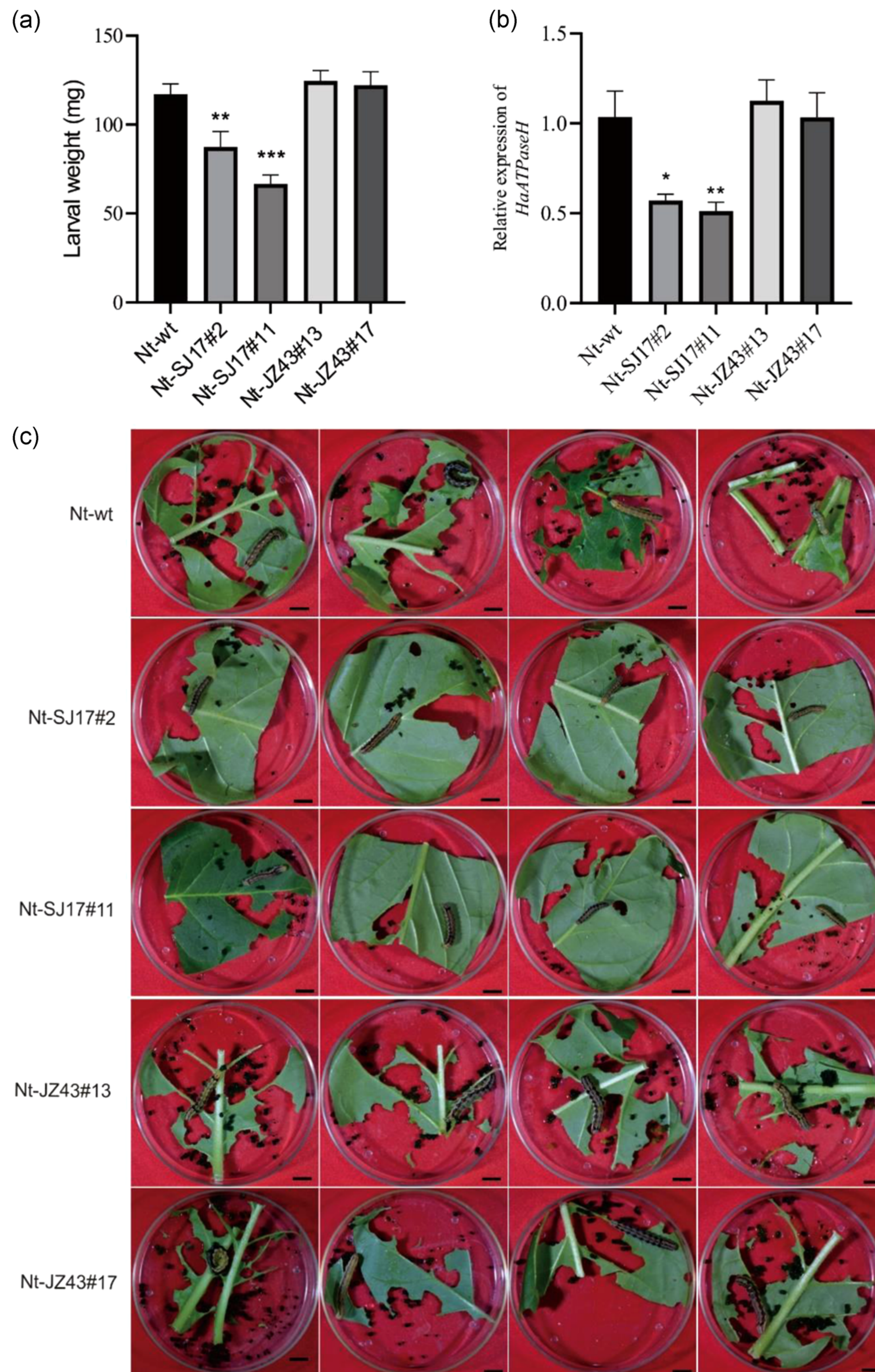

FIGURE 3 Feeding assays of *Helicoverpa armigera* larvae with transplastomic and nuclear transgenic tobacco plants. (a) Larval weight after 6 days of feeding. Data are means ± SEM ($n = 36$). Significant differences to the wild-type control were identified by Dunnett's test. $^{**}p < 0.01$, $^{***}p < 0.001$. (b) Analysis of *ATPaseH* mRNA accumulation in *H. armigera* after 6 days of feeding by qRT-PCR. Significant differences to the wild-type control were identified by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$. (c) Representative example of bioassays with detached leaves of wild-type tobacco plants (Nt-wt), nuclear transgenic plants (Nt-SJ17#2 and Nt-SJ17#11) and transplastomic plants (Nt-JZ43#13 and Nt-JZ43#17). Leaves were exposed to second-instar *H. armigera* larvae, replaced with fresh young leaves every day, and the photograph was taken at Day 6. mRNA, messenger RNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction. [Color figure can be viewed at wileyonlinelibrary.com]

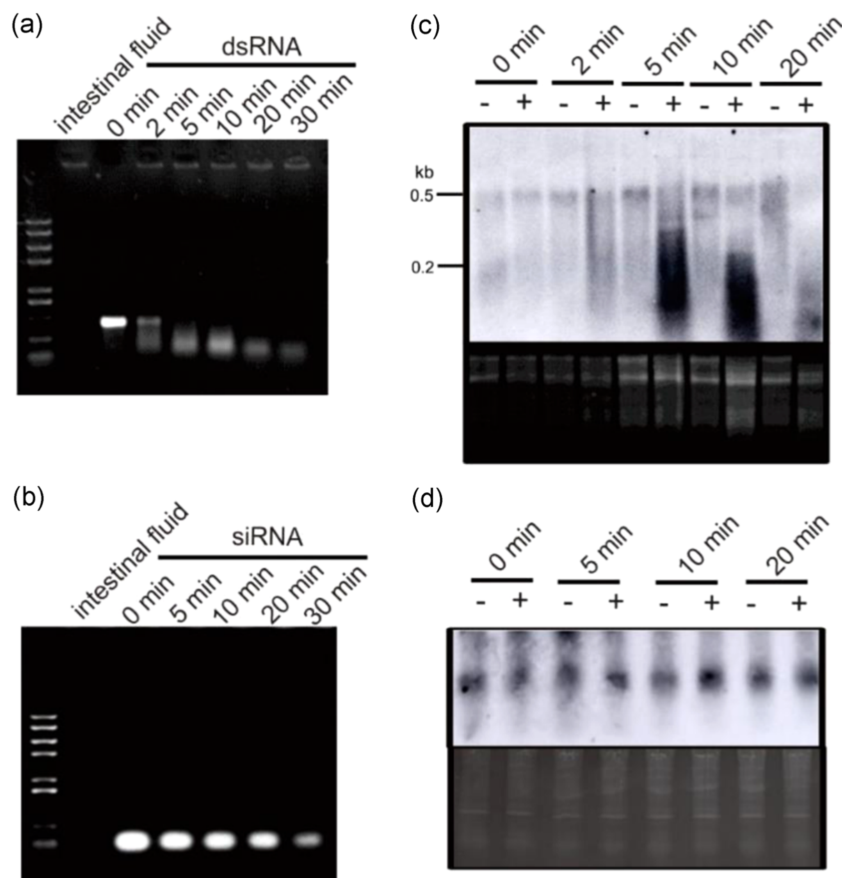

FIGURE 4 In vitro degradation of dsRNA and siRNA incubated with intestinal fluid of *Helicoverpa armigera*. (a) Image of an agarose gel showing samples of 200 ng of in vitro-synthesized ds*HaATPH* incubated for the indicated times with 5 μl intestinal fluid (diluted 15-fold) of *H. armigera* at room temperature. (b) Image of an agarose gel showing similarly treated samples of 200 ng si*HaATPH*. The samples were separated in 1% agarose gels. (c) Northern blot analysis of ds*HaATPH* degradation in transplastomic tobacco line Nt-JZ43#13 upon incubation with intestinal fluid (diluted 15-fold) of *H. armigera*. (d) Northern blot analysis of si*HaATPH* stability in nuclear transgenic tobacco line Nt-SJ17#11 upon incubation with intestinal fluid (for details, see Materials and Methods). dsRNA, double-stranded RNA; siRNA, short interfering RNA [Color figure can be viewed at wileyonlinelibrary.com]

no serious effects on the growth of *H. armigera* (Bally et al., 2016). When bioassays were conducted with *H. armigera* fed on 4-week-old tobacco plants, reduced growth of larvae and reduced pupal weight were observed only for *H. armigera* fed on nuclear-transgenic plants. No such effect was seen upon feeding with transplastomic plants, which were indistinguishable from wild-type plants in these bioassays (Figure S2).

To examine whether the inefficiency of transplastomic plants expressing dsRNA targeted against *H. armigera* is due to the target genes selected or the conformation of the dsRNA (hpRNA vs. dsRNA), we generated additional transplastomic tobacco plants that express dsRNAs targeted against the *ATPaseA*, *ATPaseD*, *COPB* and *ACT* genes of *H. armigera*, and also plants that express hpRNA targeted against the *ATPaseD* gene of *H. armigera* (Figures S3 and S4). We found that none of these transplastomic plants could induce detectable RNAi responses in *H. armigera* (Figure S5). These results suggest that the resistance to RNAi by plastid-derived dsRNAs is independent of the targeted genes and also independent of the dsRNA conformation.

Since siRNAs were only produced in nuclear transgenic plants but not in transplastomic plants, this led us to test whether siRNAs could be the trigger of RNAi in *H. armigera*. We then applied in vitro synthesized dsRNAs and RNaseIII generated siRNAs directly onto tobacco leaves that were fed to *H. armigera* larvae. *HaATPaseH* mRNA levels were not affected more strongly by RNAs derived from *HaATPaseH* than by RNAs derived from encoding green fluorescent protein (*GFP*), and also not suppressed more strongly by siRNA than dsRNA (Figure S6). Therefore, siRNAs produced *in planta* have a more potent effect than in vitro produced siRNAs on *H. armigera* gene expression.

6.4 | Rapid degradation of plastid-expressed dsRNAs but not plant-derived siRNAs in the digestive system of *H. armigera*

To determine the cause of the low efficacy of transplastomic plants in conferring resistance to *H. armigera*, we isolated hemolymph and intestinal fluids from *H. armigera* and incubated them with either in vitro synthesized ds*HaATPaseH* or si*HaATPaseH*. The results indicate that both ds*HaATPaseH* and si*HaATPaseH* are stable in the hemolymph (Figure S7). Interestingly, ds*HaATPH* was rapidly degraded upon incubation with intestinal fluid. Strong degradation was already visible after 2 min, and the dsRNA was nearly completely degraded after 5 min (Figure 4a). By contrast, si*HaATPaseH* was relatively stable in the intestinal fluid, and remained largely intact for over 20 min. Although upon extended incubation, some degradation of si*HaATPaseH* was also evident, the overall degradation rate of si*HaATPaseH* was much slower than that of ds*HaATPaseH* (Figure 4b).

To further assess degradation of ds*HaATPaseH* produced in transplastomic plants versus si*HaATPaseH* produced in nuclear

transgenic plants, leaf homogenates of wild-type plants, nuclear transgenic plants and transplastomic plants were incubated with intestinal fluid of *H. armigera* in time course experiments. The RNAs from the samples were then isolated and analyzed by northern blot analyses. The results confirmed that the dsRNAs produced in transplastomic plants were rapidly degraded by the intestinal fluid (Figure 4c), while the siRNAs generated in nuclear transgenic plants were very stable and almost unaffected by treatment with intestinal fluid (Figure 4d).

7 | DISCUSSION

More than 10 years ago, a ground-breaking study indicated the feasibility of controlling *H. armigera* by plant-mediated RNAi through silencing the P450 monooxygenase gene *CYP6AE14* (Mao et al., 2007). Since then, substantial efforts have been made to improve the RNAi efficiency for pest control by expressing dsRNAs or microRNAs against essential genes of *H. armigera* (Agrawal et al., 2015; Liu et al., 2015; Saini et al., 2018; Wu et al., 2016; Xiong et al., 2013; Zhu et al., 2012). However, although some growth suppression could be observed, efficient pest control and killing of *H. armigera* were not achieved. Moreover, some aspects of the pioneering studies are difficult to reconcile with more recent data. Silencing the *CYP6AE14* gene was believed to increase mortality induced by high concentrations of gossypol in cotton, yet two independent studies have shown that heterologously expressed, catalytically active CYP6AE14 does not metabolize gossypol at a detectable level (Krempl et al., 2016, Wang et al., 2018). CRISPR/Cas9 knockout of the P450 gene cluster containing *CYP6AE14* did not increase susceptibility to dietary gossypol, although it did confer susceptibility to insecticides (Shi et al., 2018).

We previously showed that long dsRNAs accumulated to high levels in plastids of transplastomic potato plants and induced an efficient RNAi response in the CPB, killing the larvae (Zhang et al., 2015). In this study, we examined whether a similar transplastomic approach would increase the efficiency of plant-mediated RNAi in controlling *H. armigera*. We found that ds*HaATPaseH* accumulated to high levels in transplastomic lines in the absence of Dicer-like enzyme activity, while ds*HaATPaseH* accumulation in nuclear transgenic lines was almost undetectable (Figure 2). Yet the dsRNA of transplastomic lines had no effects on gene expression or growth of *H. armigera* larvae, independently of the genes targeted, the dsRNA conformation and plant age (Figures S2–S5).

Only the nuclear transgenic plants showed detectable RNAi effects in suppressing larval growth and reducing *HaATPaseH* expression (Figure 3). Since si*HaATPaseH* accumulated only in nuclear transgenic lines, we propose that plant-produced siRNAs are more effective than plastid-produced dsRNAs in inducing an RNAi response in *H. armigera*. Moreover, since direct feeding with RNaseIII generated siRNAs produced insignificant RNAi responses in *H. armigera* (Figure S6), plant-derived siRNAs are more effective in inducing RNAi than synthetic siRNAs. Plants are known to protect their siRNAs from degradation by 2′-*O*-methylation on the 3′ terminal ribose (Ji & Chen, 2012).

A recent comprehensive review has outlined several significant differences between Coleoptera and Lepidoptera, including dsRNA degradation in the digestive tract of the insect, cellular uptake

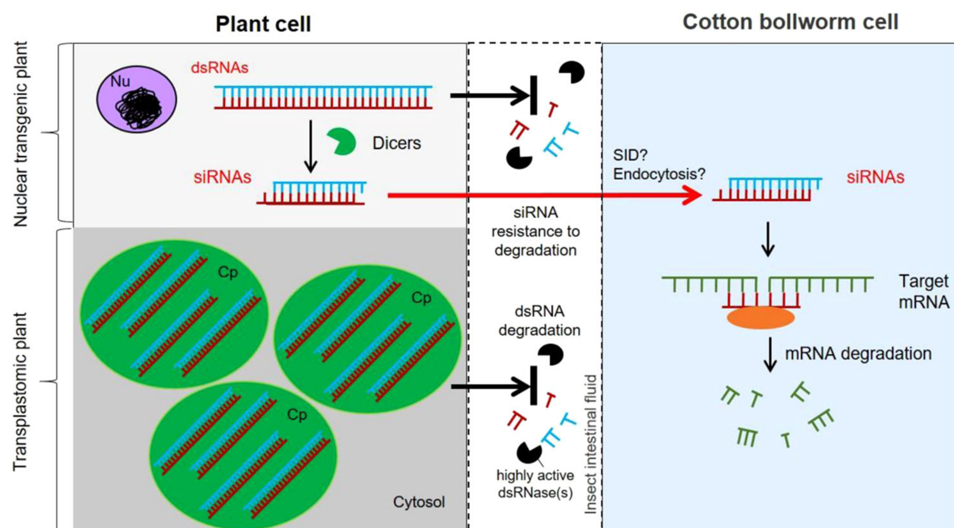

FIGURE 5 A model demonstrates the siRNAs are the effective RNA molecules in plant-mediated RNAi for controlling *Helicoverpa armigera*. Nuclear transgenic plants accumulate two types of RNA molecules (dsRNAs and siRNAs as processing products of dsRNAs), while transplastomic plants only express long dsRNAs in plastids. Rapid degradation of dsRNA by highly active dsRNase(s) in the intestinal fluid of *H. armigera* may be responsible for the low efficacy of transplastomic plants. By contrast, siRNAs are relatively stable in the digestive system of *H. armigera* and may escape degradation. Subsequently, siRNAs may enter epithelial cells and/or the hemolymph and trigger the RNAi responses of *H. armigera*, presumably by homologs of dsRNA transport protein SID and/or by endocytosis. Cp, chloroplasts; dsRNA, double-stranded RNA; Nu, nucleus; RNAi, RNA interference; siRNA, short interfering RNA [Color figure can be viewed at wileyonlinelibrary.com]

efficiency of dsRNAs, endosome capture, and expression levels of the core RNAi machinery of the insect (Zhu & Palli, 2020). Some of these factors may explain the inefficiency of the transplastomic plants in inhibiting the growth of *H. armigera*. The mechanisms involved in siRNA and dsRNA uptake in *H. armigera* are currently not well understood. Whether RNA-binding proteins similar to the dsRNA-translocating protein SID (Feinberg & Hunter, 2003; Li et al., 2015) and/or the endocytosis pathway mediate the uptake of siRNAs by *H. armigera* gut epithelial cells (Huvenne & Smagghe, 2010), remains to be investigated.

It was proposed that dsRNA-degrading enzymes have a major impact on the efficiency of environmental RNAi. Recently, dsRNase homologues were found in almost all groups of insects (Singh et al., 2017). However, the efficiency of dsRNA degradation in the digestive system in different insect orders seems to be highly variable. dsRNase activity is relatively high in Lepidoptera and comparably low in Coleoptera (Shukla et al., 2016). Several putative genes for dsRNases were found in *H. armigera* (Zhang et al., 2017). We propose that high activity of dsRNases in the gut juice causes the degradation of dsRNAs and is chiefly responsible for the inefficient RNAi response in *H. armigera* (Figure 5).

Taken together, our results suggest that contrary to the case in Coleoptera, nuclear transformation and not plastid transformation may offer more promising avenues for crop protection against Lepidoptera. Our findings highlight striking differences in the susceptibility of different insects to environmental RNAi and underscore the importance of considering these species-specific factors in all attempts to design optimized RNAi strategies for pest control. Future efforts towards improving the RNAi effect in *H. armigera* by plant genetic engineering should be directed to approaches suitable to increase siRNAs production in plants and/or possibilities to block the degradation of dsRNA in the digestive system of the insect.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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