Engineered chloroplast dsRNA silences *cytochrome p450 monooxygenase*, *V-ATPase* and *chitin synthase* genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation

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Summary

In the past two decades, chloroplast genetic engineering has been advanced to achieve high-level protein accumulation but not for down-regulation of targeted genes. Therefore, in this report, *lepidopteran chitin synthase (Chi)*, *cytochrome P450 monooxygenase* (*P450*) and *V-ATPase* dsRNAs were expressed via the chloroplast genome to study RNA interference (RNAi) of target genes in intended hosts. PCR and Southern blot analysis confirmed homoplasmy and site-specific integration of transgene cassettes into the chloroplast genomes. Northern blots and real-time qRT-PCR confirmed abundant processed and unprocessed dsRNA transcripts (up to 3.45 million copies of P450 dsRNAs/μg total RNA); the abundance of cleaved dsRNA was greater than the endogenous *psbA* transcript. Feeding of leaves expressing *P450*, *Chi* and *V-ATPase* dsRNA decreased transcription of the targeted gene to almost undetectable levels in the insect midgut, likely after further processing of dsRNA in their gut. Consequently, the net weight of larvae, growth and pupation rates were significantly reduced by chloroplast-derived dsRNAs. Taken together, successful expression of dsRNAs *via* the chloroplast genome for the first time opens the door to study RNA interference/processing within plastids. Most importantly, dsRNA expressed in chloroplasts can be utilized for gene inactivation to confer desired agronomic traits or for various biomedical applications, including down-regulation of dysfunctional genes in cancer or autoimmune disorders, after oral delivery of dsRNA bioencapsulated within plant cells.

Introduction

In the past two decades, the concept of chloroplast genetic engineering has been advanced to achieve hyperexpression of foreign proteins, with recent advances in conferring novel agronomic traits (Clarke and Daniell, 2011; Jin *et al.*, 2012; Lee *et al.*, 2011), biomass/biofuel enhancement (Agrawal *et al.*, 2011; Jin *et al.*, 2011; Verma *et al.*, 2010a, 2013) or for various biomedical applications for oral delivery of vaccines (Arlen *et al.*, 2008; Davoodi-Semiromi *et al.*, 2010; Lakshmi *et al.*, 2013), autoantigens (Ruhlman *et al.*, 2007; Sherman *et al.*, 2014; Verma *et al.*, 2010b) or biopharmaceuticals (Boyhan and Daniell, 2010; Kohli *et al.*, 2014; Kwon *et al.*, 2013a,c; Ruhlman *et al.*, 2010; Shenoy *et al.*, 2014; Shil *et al.*, 2014). While application of RNAi technology through plant nuclear transformation has several limitations, delivery of small RNA prepared in more efficient systems as human therapeutics is also severely limited by their methods of delivery (Rothschild, 2014). Chloroplast transformation offers several advantages over nuclear transformation including expression of large amounts of foreign proteins, up to 70% of the total leaf protein (De Cosa *et al.*, 2001; Oey *et al.*, 2009; Ruhlman *et al.*, 2010). This is possible due to the polyploidy nature of the plastid genetic system with up to 10 000 copies of the chloroplast genome in each plant cell, resulting in the ability

to sustain a very high number of functional gene copies (Ruiz *et al.*, 2011). In addition, transgene containment due to maternal inheritance, lack of transgene silencing and position effect are unique advantages of chloroplast genetic engineering (Daniell, 2007; Verma and Daniell, 2007; Verma *et al.*, 2008).

However, chloroplast has the exceptional ability to produce abundant transcripts (up to 200 000 copies per ng total RNA), but this genome has never been exploited to produce and deliver siRNA or miRNA or dsRNA. As described below, there is a great need to down-regulate harmful genes to confer protection against various plant pests. In addition, down-regulation of dysfunctional genes causing cancer or autoimmune diseases or immune disorders in human health is highly desired. Due to the high level of chloroplast transcription, a large amount of the dsRNA could be synthesized and orally delivered *via* bioencapsulation in plant cells to target harmful genes (Kwon *et al.*, 2013c). To the best of our knowledge, there is no report on the expression the dsRNAs *via* the chloroplast genome modification.

Introduction of transgenic technology in crop plants has generated insect-resistant plants to reduce yield loss sed pesticide utilization (Bale *et al.*, 2008; Kos *et al.*, 2009). For example, expression of *Bacillus thuringiensis* (*Bt*) Cry toxin in crop plants has resulted in great success both economically and ecologically (Qaim and Zilberman, 2003; Wu *et al.*, 2008). However, there is

emerging population of insect resistance to biopesticides in transgenic crops and outbreak of nontarget pests (Bravo and Soberon, 2008; Gahan et al., 2001; Lu et al., 2010). Thus, new approaches, which are more effective and environmental friendly, are needed. Since the discovery of dsRNA which could silence genes (Fire *et al.*, 1998), RNA interference (RNAi) has been developed as an efficient and powerful tool in plants and animals to silence expression of harmful genes (Aravin *et al.*, 2001; Wesley *et al.*, 2001). Several insect genes have been down-regulated by injection of dsRNA (Bettencourt *et al.*, 2002; Ohnishi *et al.*, 2006). Oral delivery of high concentrations of dsRNA in the artificial diet is required (Turner *et al.*, 2006) because they are degraded in the digestive system. Bioencapsulation within plant cells should protect dsRNA and increase their efficacy. Therefore, it is important to develop an efficient method of delivery, which could be used for large-scale pest control in the field. Several dsRNAs specific to target genes of insects have shown protection from these herbivores (Baum *et al.*, 2007; Mao *et al.*, 2007). But, advances in commercial development are limited by low abundance of siRNA transcripts.

DsRNA transgenic plant-mediated RNAi was used for silencing the *hemipteran* insect midgut genes and the results demonstrated the potential of dsRNA-mediated RNAi for field-level control of planthoppers (Zha *et al.*, 2011). Viral vectors have been used to produce dsRNA in *Nicotiana attenuata* to provide a transient and rapid silencing of midgut genes of the plant's *lepidopteran* herbivore, *Manduca sexta* (Kumar *et al.*, 2012). The insect resistance is significantly improved in transgenic tobacco plants expressing dsRNA from the cotton bollworm (Zhu *et al.*, 2012). Wuriyanghan and Falk (2013) used tobacco mosaic virus (TMV) as vector to produce dsRNA in tobacco, tomato and tomatillo plants. Tomatillo plants infected with recombinant TMV containing *V-ATPase* or *B. cockerelli actin* sequences decreased *B. cockerelli* progeny production. Recently, Xiong *et al.* (2013) developed transgenic tobacco plants expressing dsRNA for a moult-regulating transcription factor gene (*HaHR3*) and showed increase in cotton bollworm resistance. El-Shesheny *et al.* (2013) reported that silencing the abnormal wing disc gene of Asian citrus psyllid by direct application of dsRNA disrupted the adult wing development and increased the nymph mortality. It has also been shown that silencing of the *HaHMG-CoA* reductase gene by RNAi inhibited oviposition of the cotton bollworm (Wang *et al.*, 2013). Despite a decade of these studies (mostly in model systems), commercial development is limited by use of viruses or prohibitively expensive application of dsRNA in the field.

Cotton bollworm cytochrome *P450 monooxygenase* gene located in the midgut of insects plays a central role in adaptation of the cotton metabolite, gossypol. The RNAi target to *P450* gene (CYP6AE14) has been expressed in tobacco and cotton (Hodgson *et al.*, 1995; Mao *et al.*, 2007, 2011). The feeding of plant cells expressing RNAi specific to cytochrome *P450 monooxygenase* gene of cotton bollworm decreased transcript in the midgut, retarded larval growth and impaired cotton bollworm tolerance to the gossypol (Mao *et al.*, 2007). Feeding of transgenic corn expressing dsRNAs target to *V-ATPase* gene showed a significant larval stunting and mortality (Baum *et al.*, 2007). Pest chitin synthases (CHS) are key enzymes for trachea, cuticle and midgut development (Merzendorfer, 2006). They are encoded by *CHSA* and *CHSB* genes. CHSA genes are specifically expressed in ectodermal cells, including tracheal and epidermal cells, while *CHSB* genes are expressed specifically in gut epithelial cells that produce the peritrophic matrix of the midgut (Merzendorfer and

Zimoch, 2003). It is well known that chitin is the main component of fungi and arthropod's body skeleton, which is absent in vertebrates and plants (Zimoch *et al.*, 2005). So, chitin synthases could be used as an ideal insect growth regulatory target for RNAi technology (Chen *et al.*, 2008; Tian *et al.*, 2009).

For most nuclear transformation of biopesticide genes, including those currently under commercial cultivation, the expression level is less than 1% of the total soluble protein (Ripoll *et al.*, 2003; Saha *et al.*, 2006). However, chloroplast transformation has resulted in much higher levels of expression and greater protection against resistant insects (De Cosa *et al.*, 2001; Dufourmantel *et al.*, 2007; Jin *et al.*, 2011, 2012; Kota *et al.*, 1999). In this report, three dsRNAs targeted to *Chi, P450* (CYP6AE14) and *V-ATPase* genes are expressed in the tobacco chloroplasts. This resulted in high transcript abundance (up to 3.5 million dsRNA copies per μg total RNA) that significantly reduced transcription of target genes in the insect midgut and stunted larval growth. This study opens the door for down-regulation of genes in plant pathogens, insects or for various biomedical applications of this technology.

Results

Construction of chloroplast transformation dsRNA vectors

Three target genes form *Helicoverpa armigera* were used for RNA interference (RNAi) induced through dsRNA *P450* gene (*P450*, GenBank: DQ986461.1, CYP6AE14), Vac *ATPase A* gene (*ATPase*, GenBank: HM629434.1) and *chitin synthase B* gene (*Chi*, EST cloned from midgut RNA of *H. armigera*). The candidate target sites of these genes were obtained by the GenScript siRNA Target Finder (http://www.genscript.com/siRNA_target_finder.html#). Sequences were filtered to eliminate candidates with unfavourable thermodynamic properties. Length of siRNA target site was set as 21mers. Low GC content (<60) was maintained to enhance siRNA functionality. For each target, free energy of sense and antisense was calculated and sequences with strong internal structures were eliminated. Likewise, tandem repeats were removed. Candidates were ranked based on algorithm using ΔE.

The dsRNA structure for the target genes is the typical 'sense-loop-antisense' structure. The loop structure for these dsRNA is 'TTCAAGAGA'. The sequences of stem-loops for the *P450, ATPase, Chi* genes are shown below and the loop sequence is indicated by underline: 5'-GCAACGAGGTCGAAATGAGTTCAAGAGACTCATTTCGACCTCGTTGC-3'; 5'-GTCACTGACGTAGTGCTGGTTCAAGAGACCAGCACTACGTCAGTGAC-3'; 5'-GGTGAGGACCGATGGCTCTTTCAAGAGAAGAGCCATCGGTCCTCACC-3'. More details on dsRNA primers and vector construction are shown in Table 1.

The primers Pa and Pb were used to amplify the *pbs*A promoter and part of dsRNA sequence by PCR. Similarly, primers Pc and Pd were used to amplify part of dsRNA and the *psb*A terminator. Because the 3' of PCR product of Pa and Pb primers have a 23~25 bp complementary sequence with the 5' of PCR product from Pc and Pd primers, they should anneal into a dsRNA expression cassette (PpsbA:: ds RNA::TpsbA) (Table 1). Formation of such dsRNA stem-loop structures is very common in chloroplast 5' UTR and 3' UTR transcripts (Merhige *et al.*, 2005; Ruhlman *et al.*, 2010; Zou *et al.*, 2003). Two restriction enzyme sites (*Sal*I and *Pst*I) were also introduced into this dsRNA expression cassettes by PCR (Table 1). The dsRNA expression cassettes were

Table 1 DsRNA structure for RNAi cassettes and primers for dsRNA synthesis

Target genes	dsRNA structure for the target genes (sense-loop-antisense)
Cytochrome P450	GCAACGAGGTCGAAATGAGTTCAAGAGACTCATTTCGACCTCGTTGC
ATPase A	GTCACTGACGTAGTGCTGGTTCAAGAGACCAGCACTACGTCAGTGAC
Chitin synthase B	GGTGAGGACCGATGGCTCTTTCAAGAGAAGAGCCATCGGTCCTCACC

Primers for dsRNA vectors construction	
Primer	Sequence (5′-3′)
Upstream primer: Pa (psbA promoter)	TTCCGTCGACGTAGAGAAGTCCGTATTTTTC SalI
Downstream primer: Pb-1 (P450 dsRNA for psbA poromoter)	GTCGAAATGAGTCTCTTGAACTCATTTCGACCTCGTTGCCAACAGTATAACATGACTTATATACTCGTGTCA
Downstream Pb-2 (ATPase dsRNA for psbA poromoter)	GACGTAGTGCTGGTCTCTTGAACCAGCACTACGTCAGTGACCAACAGTATAACATGACTTATATACTCGTGTCA
Downstream Pb-3 (chitin synthase dsRNA for psbA poromoter)	CCGATGGCTCTTCTCTTGAAAGAGCCATCGGTCCTCACCCAACAGTATAACATGACTTATATACTCGTGTCA
Downstream Pc (psbA terminator)	CAGTTGACCTGCAGCCCAAACAAATACAAAATCA PstI
Upstream Pd-1 (P450 dsRNA for psbA terminator)	TGAGTTCAAGAGACTCATTTCGACCTCGTTGCTTTTTTTCTAGAGATCCTGGCCTAGT
Upstream Pd-2 (ATPase dsRNA for psbA terminator)	TGGTTCAAGAGACCAGCACTACGTCAGTGACTTTTTTTCTAGAGATCCTGGCCTAGT
Upstream Pd-3 (Chitin synthase dsRNA for psbA terminator)	TCTTTCAAGAGAAGAGCCATCGGTCCTCACCTTTTTTTCTAGAGATCCTGGCCTAGT

Note: The loop of dsRNA "Sense-loop-antisense" structure or the restriction enzyme digestion site is indicated by underline.

double digested by *Sal*I and *Pst*I and then subcloned into the tobacco chloroplast transformation vector—pLD vector (Jin *et al.*, 2011, 2012; Verma and Daniell, 2007).

The chloroplast transformation vectors were constructed based on the pLD vector (Daniell *et al.*, 1998, 2001). In pLD-Chi: RNAi, pLD-P450: RNAi and pLD-ATPase: RNAi chloroplast vectors, the dsRNAs are regulated by the *psb*A promoter and its 5′ and 3′ untranslated regions (Figure 1a). Spectinomycin-resistance gene (*aad*A) was used as the selectable marker for plant regeneration and is driven by the *Prrn* constitutive *rRNA* operon promoter with GGAG ribosome-binding site. The inverted repeat region *trn*I/*trn*A was used as flanking sequences for homologous recombination into the chloroplast genome (Figure 1a).

Generation and molecular characterization of dsRNA transplastomic lines

Transplastomic plants were created as described previously (Jin *et al.*, 2011; Verma *et al.*, 2008). Several shoots emerged after bombardment of chloroplast vectors with gold particles coated with each pLD-RNAi plasmid in the first round of selection. The second round of selection advanced shoots towards homoplasmy, and the third round of selection in root induction medium established independent transplastomic lines. After third round selection and confirmation by the PCR and Southern analysis, the transplastomic lines were moved to the greenhouse for increasing biomass and further characterization. Two sets of primers, 5P/2M and 3P/3M, were used for PCR analysis to confirm site-specific integration of transgenes into the chloroplast genome. The 3P/3M primer pair annealed to the native chloroplast genome upstream of the site of integration and the *aad*A gene, resulting in a 1.65 kb PCR product. The 5P and 2M primers were used to confirm integration of dsRNA expression cassettes, which produced a 1.72 kb PCR product. PCR results confirmed the expected size

products in all of the transplastomic lines which were absent in the wild-type control plants (Figure 1a,c).

Southern blot analysis was used to determine homoplasmy and to confirm site-specific integration of transgenes into the chloroplast genome. Total plant DNA digestion with *Sma*I generated 5.6-kb fragments from transplastomic lines after hybridization with the [³²P]-labelled *trn*I/*trn*A flanking sequence probe. This confirmed site-specific integration of the transgenes into the spacer region between the *trn*I and *trn*A genes (Figure 1b). Furthermore, the absence of the 4.0-kb fragments in pLD-Chi: RNAi, pLD-p450: RNAi transplastomic lines confirmed that homoplasmy has been achieved (Figure 1d,e). However, in pLD-ATPase: RNA lines, A2 and A4 showed 4.0-kb fragment similar to wild type suggesting these two lines are negative lines, while other three lines A5, B3 and B5 showed two bands, suggesting heteroplasmy (Figure 1f). All other transplastomic lines generated a single 5.6-kb hybridizing fragment suggesting they are homoplasmic.

Higher level transcription of dsRNA *via* the chloroplast genome

To investigate transcription and processing of the dsRNA via the tobacco chloroplast genome, northern hybridization analysis was carried out in the Southern blot-positive transplastomic lines, using 3′ psbA UTR as the probe. Expected transcript sizes were detected in P450: RNAi (2.3, 2.4, 2.6, 2.11 and 10.4), ATPase: RNAi (A1, A3, A6, B2, B4, B5, B14) and Chi: RNAi (1–5) transplastomic lines, while no dsRNA product was observed in WT (Figure 2a–c). Both processed and unprocessed dsRNA transcripts were observed in all three dsRNA transplastomic lines. It is interesting to note that dsRNA transcripts (cleaved and unprocessed) were several fold higher than the highly expressed endogenous *psb*A gene. This may be due to double the copy number of transgenes (because of transgene insertion within the
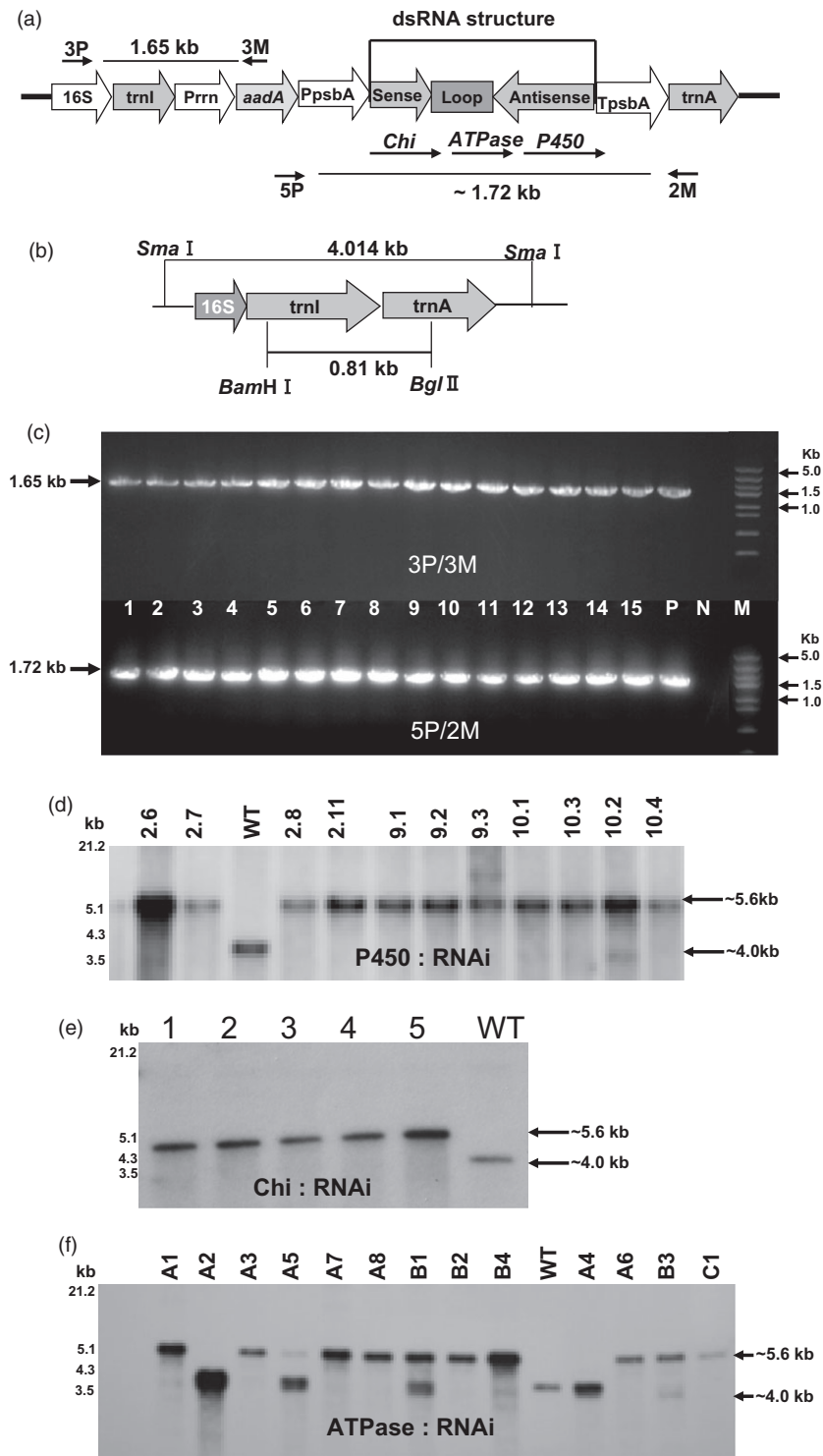
Figure 1 Chloroplast dsRNA vectors, evaluation of transgene integration and homoplasmy. (a) The pLD-dsRNA chloroplast transformation vector map. The dsRNA structure for the target genes is the typical 'sense-loop-antisense' structure. For the *P450 gene*, the sequence of stem-loops is shown as below: 'GCAACGAGGTCGAAATGAGTTCAAGAGACTCATTTCGACCTCGTTGC'. For the *Chi* and *ATPase* genes, the RNAi vectors also have the similar 'sense-loop-antisense' structures. The sizes of these 'sense-loop-antisense' structures are ~70 nt. (b) Schematic representations of the chloroplast flanking sequences used for homologous recombination, probe DNA sequence (0.81 kb), when digested with *Sma*I. (c) PCR analysis of the wild-type and transplastomic lines using the primer pair 3P/3M (Upper) and 5P/2M (Lower). Primer annealing sites are shown in (a). Lanes 1–5: P450:RNAi; 5–10: Chi: RNAi; 10–15 ATPase: RNAi transplastomic lines. P, positive control; N, negative control; M, DNA marker. (d) Southern blot analysis of P450: RNAi transplastomic lines hybridized with chloroplast flanking sequence probe (WT, wild-type and 11 transplastomic lines). (e) Southern blot analysis of Chi: RNAi transplastomic lines (WT, wild-type; 1–5, transplastomic lines). (f) Southern blot analysis of ATPase: RNAi lines (WT, wild-type; A2, A4 are negative lines; A5, B1 and B3 are heteroplasmic lines; A1, A3, A7, A8, B2, B4, A6 and C1 are homoplasmic lines.
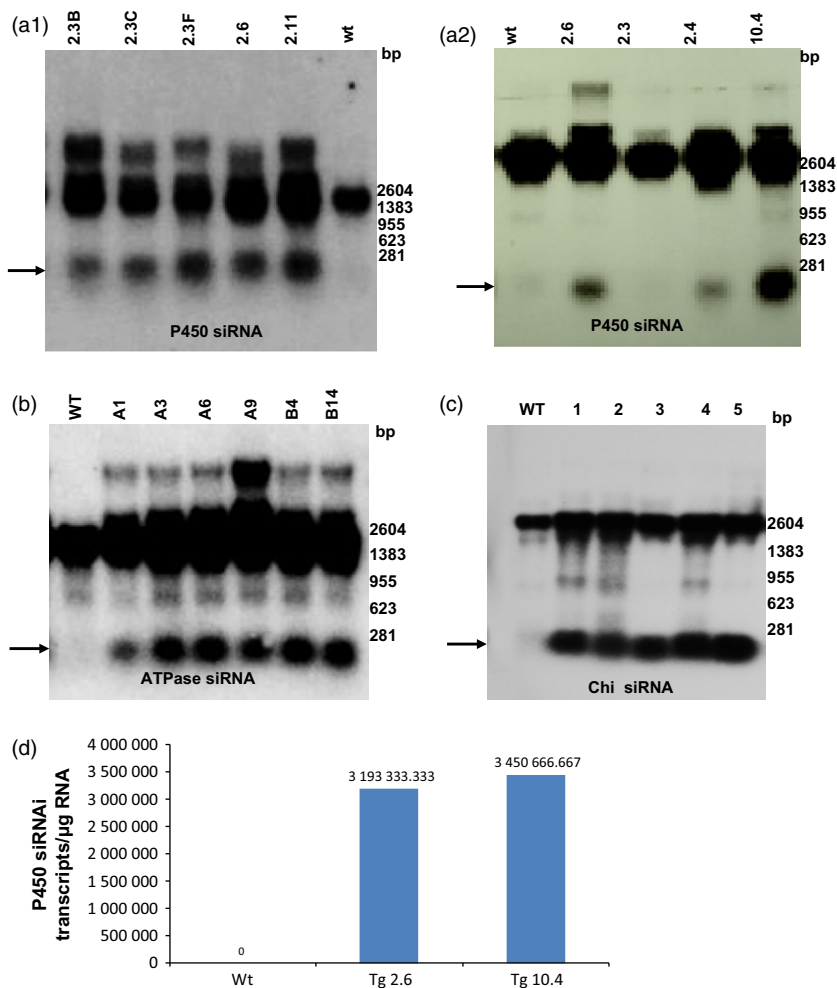
Figure 2 Northern blot and real-time qRT-PCR analysis of dsRNA in transplastomic plants: For the northern blot, equal amount of 2 μg total RNA of every sample was loaded and separated on 1.0% denaturing agarose gel. The PCR-amplified product from 3′ psbA UTR was used as probe for northern blot (a) Hybridization signals of dsRNAs (unprocessed and cleaved) were detected in P450: RNAi transplastomicclines (2.3, 2.4, 2.6, 2.11 and 10.4), whereas the cleaved dsRNA product was absent in wild-type plants. (b) Hybridization signals of dsRNAs (unprocessed and cleaved) were detected in A1, A3, A6, B2, B4, B5 and B14 ATPase: RNAi transplastomic lines whereas the cleaved dsRNA product was absent in wild type. (c) Hybridization signals of dsRNAs were detected in 1–5 RNAi: Chi transplastomic lines whereas the cleaved dsRNA product was absent in wild type plants. Note: the expected dsRNAs are indicated by the arrow in (a–c). (d) Transcript abundance of dsRNA in the wild type and two independent transplastomic lines-2.6 and 10.4.

inverted repeat region) and transcription driven by the psbA promoter as well as the 16S rRNA promoter driving the entire ribosomal operon. Also, the cleaved dsRNA transcript was more abundant than the endogenous *psb*A transcript. The northern blot-positive transplastomic plants were used for insect feeding bioassays.

Real-time PCR (qRT-PCR: real-time quantitative reverse transcription PCR) analysis was performed to quantify the transcription level of dsRNAs. In this report, dsRNA of *p450* gene was chosen for qRT-PCR analysis. These results showed that there were more than three million (3.19-3.45 millions) copies of P450 dsRNA in each microgram total RNA from transplastomic leaves, further confirming results observed in northern blots. However, cleaved dsRNA was not detected in the wild-type tobacco leaves (Figure 2d), confirming probe specificity.

P450 monooxygenase suppression via chloroplast dsRNA feeding reduced the *Helicoverpa armigera* tolerance to gossypol

Gossypol and related sesquiterpene biosynthesized in cotton are toxic to many organisms. However, most *Helicoverpa* insects resist gossypol because the P450 monooxygenases in the midgut of these insects permit them to tolerate gossypol. Therefore, to suppress *P450* gene, two-step insect bioassay was conducted. In the first step, third-instar larvae of *H. armigera* were fed on P450: RNAi-expressed leaves for four days. Then,

all these larvae were transferred to the following diet or leaves: artificial diet with 3 mg/g gossypol or without gossypol; cotton leaves with glands/glandless; and wild-type tobacco leaves. Addition of gossypol in the artificial diet or in leaves affects the growth and development of the larvae (Figure 3). For the gossypol tolerance test, the third-instar larvae of *H. armigera* were fed on artificial diet supplemented with 0–4 mg/g of gossypol. These results showed that gossypol-mediated toxicity to larvae was moderate. The weight increases (163–190 mg) on low concentration gossypol diet (1–2 mg/g) were comparable to respective controls (246 mg). However, the larval growth was dramatically suppressed at higher concentrations (3–4 mg/g) of gossypol diet with net weight increase of 15.1–55.4 mg (Figure 4a). Thus, *H. armigera* insects can tolerate relatively low concentration of gossypol. Therefore, 3 mg/g of gossypol concentration was used for subsequent insect bioassays.

The primary objective of this research is to evaluate suppression of the *P450* gene by dsRNA expressed via the chloroplast genome. So, the transcription level of this targeted gene was detected by qRT-PCR. After feeding on the two independent transplastomic lines (L2.6 and L10.4) for 4 days, the midgut of tested larvae was isolated and used for qRT-PCR analysis. The transcription level of the *P450* gene located in the insect midgut was significantly decreased in both L2.6 ($P < 0.01$)- and L10.4-fed insects ($P < 0.05$) (Figure 5a). Similarly, the transcription level
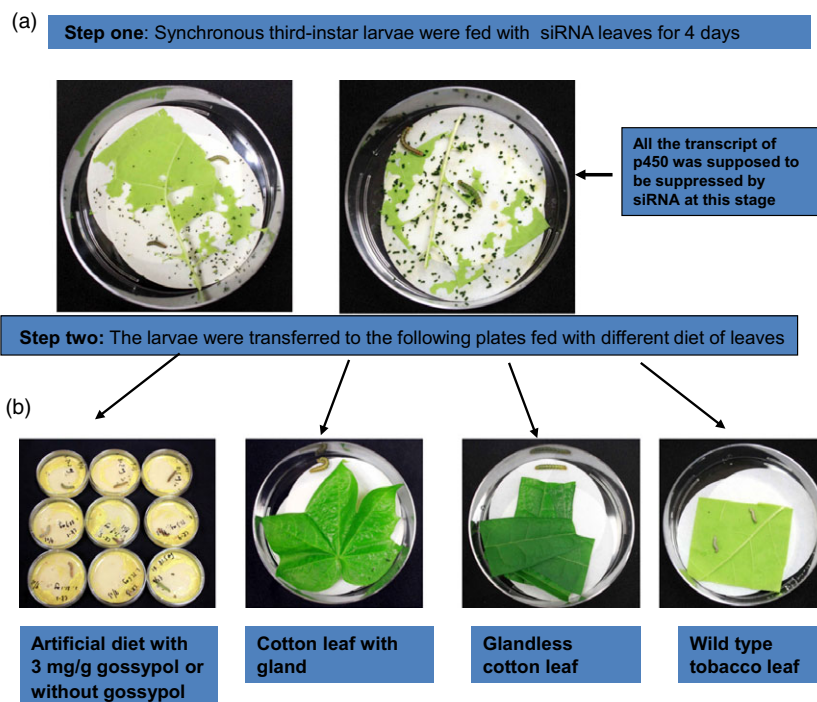
(a)

Step one: Synchronous third-instar larvae were fed with siRNA leaves for 4 days

All the transcript of p450 was supposed to be suppressed by siRNA at this stage

Step two: The larvae were transferred to the following plates fed with different diet of leaves

(b)

Artificial diet with 3 mg/g gossypol or without gossypol

Cotton leaf with gland

Glandless cotton leaf

Wild type tobacco leaf

Figure 3 Strategy for P450: RNAi transplastomic plant insect bioassay. Two steps are needed for this insect bioassay. In the first step, third-instar larvae of *Helicoverpa armigera* were fed on P450: RNAi leaves for 4 days. (a) In this step, most of the P450 transcripts should be degraded siRNA. (b) Then, these larvae were transferred to the following diet or leaves: artificial diet with 3 mg/g gossypol or without gossypol; cotton leaves with glands; glandless cotton leaf and wild-type tobacco leaf. In the second step, gossypol in the artificial diet or leaves should affect larval growth and development. The weight change of larvae was measured at this step.

in both groups continued to decrease, and the transcription of *P450* in the L2.6 was too low to be detected in some samples (Figure 5b).

In parallel experiments, other insects were subjected to the insect bioassay by feeding on different diet/leaves. The larval growth was significantly affected by the gossypol after four days of feeding on transplastomic leaves expressing dsRNA (Figure 4b). The net weight increase of larvae fed on the diet supplemented with 3 mg/g of gossypol was 45 mg, which was one-sixth of the net weigh increase when larvae fed on the diet without gossypol. Most *Gossypium* species store gossypol in the pigmented glands, but some glandless cultivars do not accumulate this toxin (Luo *et al.*, 2001). Larvae fed with glanded leaves grew slower than those on the glandless leaves (net weight increase 56.5 mg vs 214 mg).

Helicoverpa armigera showed stunted development after feeding on leaves expressing dsRNA-Chi and dsRNA-ATPase via the chloroplast genome

For the Chi and ATPase: RNAi feeding bioassay, third-instar larvae were fed with the transplastomic leaves expressing dsRNA for 4 days. The bioassay results showed that siRNA significantly reduced *H. armigera* growth. The net weight increase of larvae fed on dsRNA-ATPase leaves was 57.7 mg within 4 days, whereas it was 130.7 mg when fed on wild-type untransformed leaves (Figure 6a). Similarly, net weight increase of larvae fed with dsRNA-Chi-expressing leaves was significantly ($P < 0.05$) lower than those fed with wild-type untransformed leaves. The average net increase of larvae fed on dsRNA-Chi leaves was 47.6 mg, whereas it was 136.7 when larvae were fed with wild-type untransformed leaves (Figure 6b).

Silencing the *P450, ATPase* and *Chi1* genes leads to the higher larvae mortality and lower pupation rate

The transformation from larvae to pupae is a key step in the metamorphosis of *Helicoverpa* insects. In addition to the net

weight increase of insects after feeding on different diet or leaves, the larval mortality and pupation rate were also observed in all treatment groups. After feeding with different diet or leaves for 4 days, all larvae were transferred to the artificial diet until pupation. During this transfer, some larvae failed to form pupae and finally died (Figure 7d, 1–4 dead larvae). Only a few larvae developed into pupation stage (Figure 7d, number 5 live pupa). The larvae (with P450 monooxygenase silencing) fed on the gossypol diet or glanded leaves showed significantly lower pupation rate ($P < 0.05$) when compared to larvae fed on nongossypol or glandless or wild-type untransformed leaves (Figure 7a).

The larval population decreased when the larvae were fed with transplastomic leaves expressing dsRNA-ATP and dsRNA-Chi for 4 days. During this growth period, some larvae moved slowly and stopped feeding and finally failed to complete the pupation. Only 56.7% of the larvae were successfully transformed into pupa after feeding with dsRNA-ATPase leaves, whereas 83.3% of the larvae transformed to pupa when fed with wild-type untransformed leaves (Figure 7b). Similar bioassay results were obtained for insects fed with dsRNA-Chi-expressing leaves. The pupation rate in the control group (80%) was significantly higher ($P < 0.05$) than the dsRNA-Chi-fed group (46.7%, Figure 7c).

Discussion

Chloroplast genetic engineering has been in development for more than two decades (Clarke and Daniell, 2011; Daniell *et al.*, 2002), and potential advantages continue to be explored recently (Kwon *et al.*, 2013a,b; Daniell *et al.*, 2005; Jin *et al.*, 2011, 2012; Jin and Daniell, 2014; Kohli *et al.*, 2014; Shenoy *et al.*, 2014; Sherman *et al.*, 2014; Shil *et al.*, 2014; Verma *et al.*, 2013). These successful reports showed that a wide range of products can be produced in plastids. More than 100 transgenes have been stably expressed via the chloroplast genome, including genes coding for biopharmaceutical proteins, industrially valuable
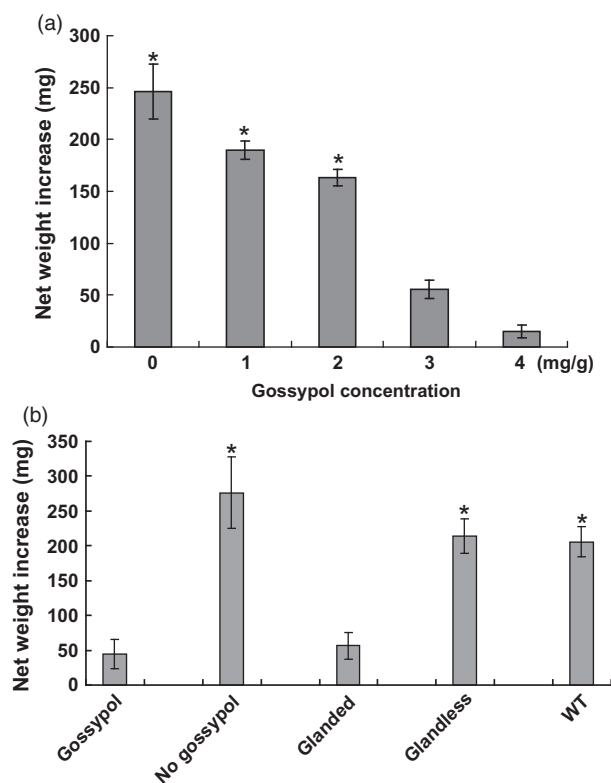
Figure 4 Effect of gossypol and siRNA on larval growth. The third-instar larvae were fed on P450: RNAi leaves for 4 days and were then transferred to the artificial diet or leaves (see the protocol in Figure 3). (a) Net weight increase of larvae reared on the artificial diet supplemented with gossypol (1–4 mg/g). The third-instar larvae were fed on the artificial diet for 5 days, and then, the weight increase was measured. (b) Net weight increase of larvae reared on artificial diet with (3 mg/g) or without gossypol, cotton leaves with or without glands and wild-type tobacco leaves. Net weight increase values consist of means ± standard deviation. Asterisk indicates *P* < 0.05 compared with wild-type control group.

Figure 5 Quantity of P450 transcript in the midgut of tested larvae after feeding on the wild-type and transplastomic leaves. The third-instar larvae were fed on transplastomic P450: RNAi or wild-type tobacco leaves for 4 days or 7 days, and then, the midgut of these insects were isolated under stereomicroscope and then washed with ddH₂O to remove all the debris. The cleaned midgut tissues were stored in 70% ethanol at −20 °C. RNA was isolated from these midgut tissues and used for real-time qRT-PCR analysis. (a) P450 transcription level of the tested larvae fed with wild-type and two transplastomic line L2.6 and L10.4 for 4 days. (b) P450 transcription level of the tested larvae fed with wild-type and two transplastomic lines L2.6 and L10.4 for 7 days. The primers used for quantification of dsRNAs of P450 in the transplastomic plants leaves were as follows: upstream primer (5′-AGAGACTCATTTCGACCTCGTTGCT-3) and downstream primer (5′-GGAGCAATAGCACCCTCTTGATAGAA-3′). The upstream primer anneals with P450 and the downstream primer anneals with TpsbA. All the results were obtained from at least three independent biological replicates. Transcription values consist of means ± standard deviation. Asterisk indicates *P* < 0.05 when compared with wild-type control group. Two asterisks indicate *P* < 0.01 when compared with wild-type control group.

enzymes, antibiotics, vaccine antigens, biomaterials, and genes that confer important agronomic traits (Clarke and Daniell, 2011; Daniell et al., 2009; Verma and Daniell, 2007). Most of these publications focused on evaluation and quantization of proteins but not transcript abundance. Only a few papers focused on the quantification of transcription of foreign genes in chloroplasts by qRT-PCR and then evaluated transgene copy number, transcript abundance in transplastomic plants (Ruiz et al., 2011). In this report, the expression of dsRNA via the chloroplast genome was successfully achieved for the first time. Most importantly, the qRT-PCR data provided direct proof of higher transcription level of dsRNA in the transplastomic cells, up to 3.5 million copies of dsRNA in 1 μg (10⁶ pg) RNA. Cleaved dsRNA is more abundant than the highly expressed endogenous chloroplast *psbA* gene. Higher accumulation of dsRNAs in plant cells is a requirement for the effective silencing of targeted genes.

The data presented here demonstrate that RNAi is a powerful tool for gene silencing and mediated post-transcriptional down-regulation of chitin synthases, P450 monooxygenase and V-ATPase gene transcripts and impact on development of larvae fed with transplastomic leaves. In the P450 bioassays, we observed that the P450-targeted transcripts in the insect midgut decreased significantly over time. The P450 transcript level of transplastomic lines after 7 days of feeding declined up to 92% when compared
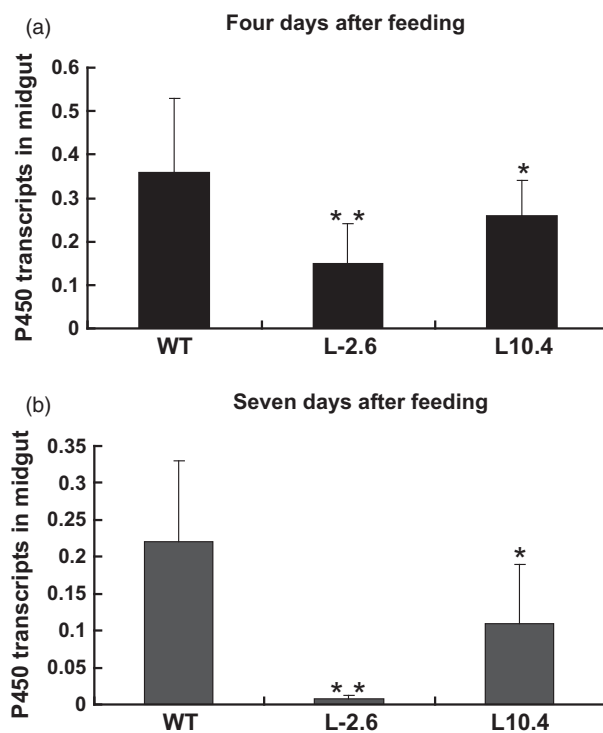
to respective controls. The correlation between dosage and effectiveness of RNAi has been shown in several siRNA injection studies (Boisson et al., 2006; El-Shesheny et al., 2013; Whyard et al., 2009). When compared to the in vitro spray/microinjection of siRNA solution or feeding the artificial diet supplemented with siRNA, the transplastomic plant-mediated RNAi offers several advantages: (i) higher doses of chloroplast-derived transcripts, (ii) stable expression throughout plant life cycle and (iii) higher RNAi activity free from degradation of siRNA in vitro or in the digestive system due to bioencapsulation within plant cells.

Most importantly, we observed high abundance of dsRNA transcripts in transplastomic lines and their processing. Several lines of evidence support processing of dsRNA within chloroplasts, although unprocessed RNA is more abundant than cleaved transcripts. When primers specific for dsRNA transcript (P450/
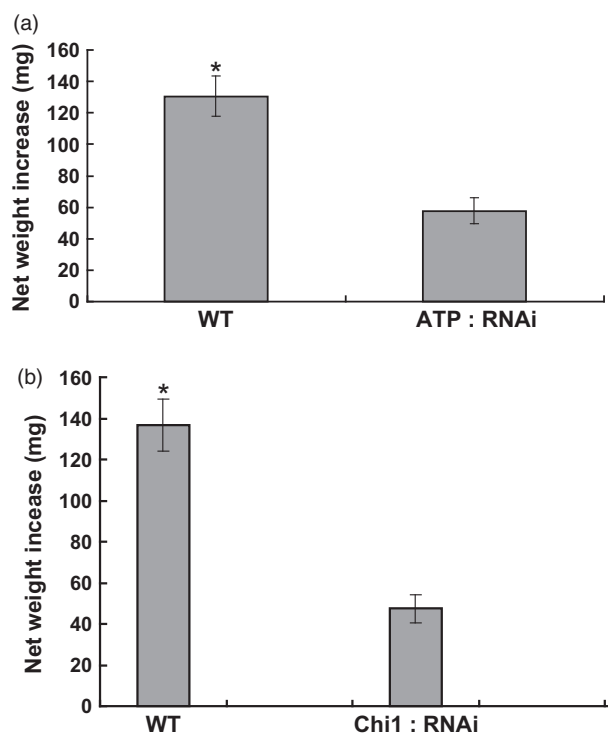
Figure 6 Net weight changes of *Helicoverpa armigera* feeding on transplastomic leaves expressing ATP and Chi dsRNAs. (a) Net weight increase of *H. armigera* fed with ATP: RNAi transplastomic tobacco leaves for 4 days. (b) Net weight increase of *H. armigera* fed with Chi: RNAi transplastomic tobacco leaves for 4 days; Net weight increase values consist of means ± standard deviation. Asterisk indicates *P* < 0.05 compared with wild-type control group.

TpsbA) were used in qRT-PCR, dsRNA transcript products were observed in transplastomic lines but not in untransformed wild-type plants. Processed, cleaved and stabilized dsRNA with the 3′psbA UTR should be 272 nucleotides. Correct size of this transcript was observed in all three dsRNA transplastomic lines but not in untransformed wild-type plants. Because no intercistronic processing element was engineered, in the absence of processing, only dicistron or polycistron transcripts should have been observed without any dsRNA cleavage and processing. According to our current understanding of processing of transgene transcripts (Martinez de Alba *et al.*, 2013), after generation of primary transcripts, RISC complex cleaves mRNA and cleaved products are stabilized by SGS3; RDR6 then generates dsRNA and DCL2/4 forms siRNA duplexes. However, none of these proteins are known to be present in plastids, and yet we observed cleaved, stabilized and protected dsRNA products. So, it is possible that dsRNA processing occurs *via* a different mechanism in chloroplasts. Formation of stem-loop structure by dsRNA could leave single-stranded regions for ribonucleolytic cleavage (Hotto *et al.*, 2010). In addition, plastid noncoding RNAs (pncRNA) biogenesis relies on the assembly of RNases that generate mRNAs and rRNAs and are protected by secondary structures as well as RNA-binding proteins (Hotto *et al.*, 2012). In this case, dsRNA is further protected by the psbA 3′UTR, stabilizing this partially processed dsRNA product. Future investigations will examine the presence or absence these processing proteins and further enhance processing by targeting them to chloroplasts or co-express them along with dsRNA genes. Therefore, there is great potential to

achieve very high levels of dsRNA expression in this ideal delivery system in which dsRNA is protected by bioencapsulation within plant cells.

Recent development in transcriptomics, specifically strand-specific RNA sequencing by next-generation sequence technology, has allowed high-throughput, comprehensive detection of low-abundance transcripts typical of the noncoding RNAs studied in eukaryotes and bacteria. Most recently, a few plastid pncRNAs had been identified (Germain *et al.*, 2011; Zhao *et al.*, 2007; Zhelyazkova *et al.*, 2012a,b) and even fewer had been investigated for functional role in gene regulation (Hegeman *et al.*, 2005; Hotto *et al.*, 2010; Zghidi-Abouzid *et al.*, 2011). Another important source of pncRNA identification includes profiling 21- to 24-nt miRNAs and siRNAs (Hotto *et al.*, 2012; Shah *et al.*, 2010). Using RNA-Seq technology, pncRNAs of <40 nt were identified from *Arabidopsis* leaves, tomato fruit and flower barley leaf plastids and rice (*Oryza sativa*) leaves, respectively (Mohorianu *et al.*, 2011; Ruwe and Schmitz-Linneweber, 2012; Zhelyazkova *et al.*, 2012a,b). Although there has been more pncRNA identification and pncRNA biogenesis, their functionality is still not clear. Till now, there is no evidence for an RNA interference pathway in plastids. However, bacteria could generate a size class of 20- to 50-nt ncRNAs termed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs), which are involved in silencing foreign genes. So, in the future, based on this report, chloroplast transformation technology should enable us to study RNA interference pathway(s) in plastids.

Plant-mediated RNAi has been an important crop protection strategy targeting insect pests with greater specificity than currently available pesticides or Bt toxin. Especially in the case of *lepidopteran* insect control, this technology has exhibited great potential. CYPs (e.g. P450) were first successfully used to targeted genes to control *lepidopteran* insects, which could detoxify synthetic insecticide compounds such as gossypol in *Gossypium* plants. Several past reports showed that silencing these CYPs would suppress transcript levels of the targeted genes, attenuate their function and finally decrease larval growth or survival (Kumar *et al.*, 2012; Mao *et al.*, 2007, 2011; Zha *et al.*, 2011). As CYPs belong to a multigene family, they have been proposed to be ideal targets for RNAi strategy (Kumar *et al.*, 2012). Therefore, in this first attempt of gene silencing *via* the chloroplast genome, three dsRNA targets were selected as suitable candidate genes. Several previous reports demonstrated successful targeted insect gene silencing with transiently expressed or *Agrobacterium*-transformed stable plant-mediated RNAi (Kumar *et al.*, 2012; Mao *et al.*, 2011; Pitino *et al.*, 2011; Wuriyanghan and Falk, 2013; Xiong *et al.*, 2013). In this report, successful silencing of three midgut *lepidopteran* insect genes with chloroplasts-derived dsRNA suggests that the plant-mediated RNAi approach is feasible *via* the chloroplast genome.

As described above, there is a great need to down-regulate harmful genes to confer protection against various plant pathogens. In addition, down-regulation of out of control genes causing cancer or autoimmune diseases or immune disorders is highly desired in human health (Bagasra and Prilliman, 2004). Due to the high level of chloroplast transcription, a large amount of the dsRNA could be synthesized and orally delivered *via* bioencapsulation in plant cells to target disruption of harmful genes. To the best of our knowledge, there is no report on the expression the dsRNAs via the chloroplast genome. Taken together, results reported here demonstrate that the approach of chloroplast-
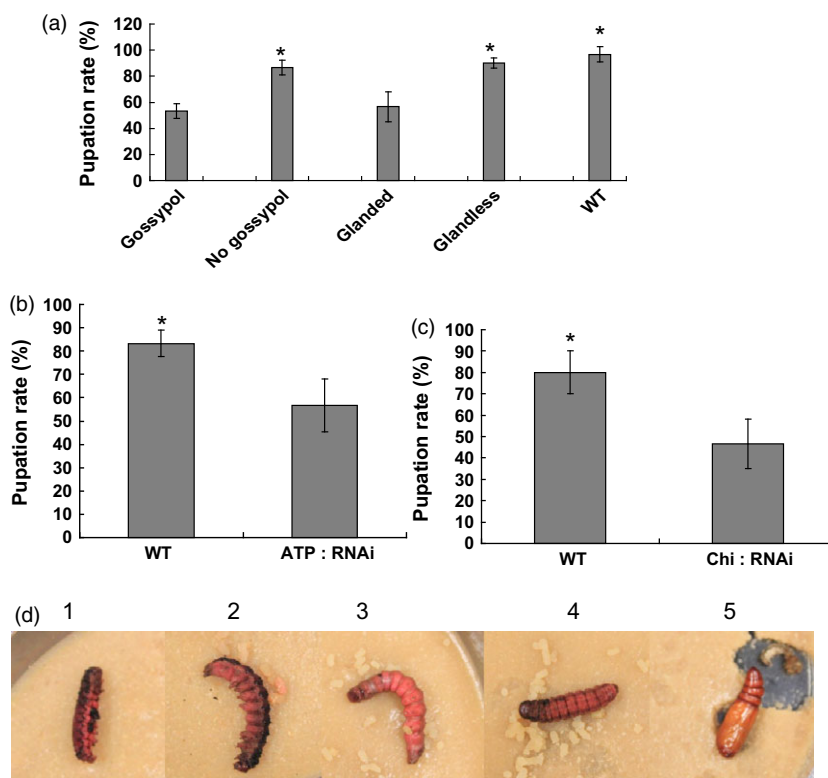
Figure 7 Larval development and insect mortality bioassay. (a) Pupation rate of larvae fed with P450: RNAi tobacco leaves transferred to different diets or leaves. Gossypol: artificial diet containing 3 mg/g gossypol; no gossypol: artificial diet without gossypol; glanded: glanded cotton leaves; glandless: cotton leaves without glands; WT: wild type tobacco leaves. (b–c) Pupation rate of larvae after feeding on ATP: RNAi and Chi: RNAi tobacco leaves. (d) Larval mortality and pupal phenotype. 1: Pupation failure led to mortality after feeding artificial diet containing 3 mg/g gossypol; 2: dead larva after feeding glanded cotton leaves; 3: dead larva after feeding with ATP: RNAi tobacco leaves; 4: dead larva after feeding with Chi: RNAi tobacco leaves; 5: successful pupation of insect fed on wild type tobacco leaves all the time during the bioassay. Pupation values consist of means ± standard deviation. Asterisk indicates $P < 0.05$ compared with wild-type control group.

derived dsRNA will be useful not only in plant biotechnology but also in various biomedical applications.

Materials and methods

Generation and molecular characterization of transplastomic plants

Young tobacco leaves (4 weeks old) were bombarded with chloroplast dsRNA vectors described in the Results Section using the biolistic device PDS1000/He, and transplastomic lines were recovered as described previously (Jin *et al.*, 2011). Bombarded leaves were then subjected to three rounds of selection. First two rounds of selection were performed on the regeneration medium of plants (RMOP) and the third round of selection was made on hormone-free half MS medium (1/2 MSO) medium. Growth media were supplemented with 500 mg/L spectinomycin. After selection, putative transplastomic shoots were transferred to pots in the greenhouse to increase biomass.

Tobacco genomic DNA was isolated from the untransformed and spectinomycin-resistant transplastomic lines using DNeasy Plant Mini Kit(Qiagen, Valencia, CA, USA). PCR analysis was used to confirm transgene integration into the chloroplast genome using two sets of primers— 3P-3M and 5P-2M as described previously (Jin *et al.*, 2011, 2012). The 3P primer (5'-AAAACCCGTCCTCAGTTCGGATTGC-3') anneals with the native chloroplast genome, and 3M primer (5'-CCGCGTTGTTTCATC AAGCCTTACG-3') anneals with the *aad*A gene. Therefore, this pair of primers was used to check site-specific integration of the selectable marker gene into the chloroplast genome. The 5P primer (5'-CTGTAGAAGTCAC CATTGTTGTGC-3') anneals with the selectable marker gene *aad*A, and 2M primer (5'-TGACTGCCCACCTGAGAGCGGACA 3') anneals with the *trn*A gene, which were used to confirm integration of the transgene expression cassette.

Southern blot analysis of putative transplastomic lines was performed according to our previous protocol (Jin *et al.*, 2011, 2012; Singh *et al.*, 2009). In brief, total genomic DNA (~2 μg) isolated from third round of selection was digested with *Sma*I and separated on a 0.8% agarose gel and then transferred to a nylon membrane. The pUC-Ct vector DNA was digested with *Bgl*II and *Bam*HI to generate a 0.81-kb fragment of the flanking sequence. The digested DNA bands were labelled with ^{32}P α[dCTP], and the membrane was hybridized using Stratagene QUICK-HYB hybridization solution followed by manufacturer's protocol.

Northern hybridization, real-time PCR (qRT-PCR) analysis of small RNAs

Total RNA was isolated from fully expanded wild-type and transplastomic tobacco leaves by Qiagen RNeasy Mini Kit (Qiagen) and quantified by Nanodrop.

For the northern blot, equal amount of total RNA (2 μg) was separated on 1.0% denaturing agarose gel and transferred to the nylon membrane (Nytran SPC; Whatman, Buckinghamshire, UK). The PCR-amplified product from 3' psbA UTR was used as the probe for northern blot of P450:RNAi, Chi: RNAi and ATPase: RNAi transplastomic lines. Membrane was hybridized with PCR-amplified DNA fragment labelled with ^{32}P[dCTP] using the QUICK-HYB hybridization solution following manufacturer's protocol (Stratagene, La Jolla, CA).

For the real-time qRT-PCR, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). The third-instar larvae were fed on transplastomic P450: RNAi or wild-type tobacco leaves for 4 days or 7 days, and then, the midguts of these tested insects were isolated under stereomicroscope and then washed with ddH$_2$O to remove any debris. The cleaned midgut tissues were stored in 70% ethanol at −20 °C until use. After removing the genomic DNA using DNase I, 1.0–2.0 μg of total RNA from tobacco leaves or cotton bollworm midgut tissue was reverse-

transcribed to cDNA using a commercially available kit (Applied Biosystems). Quantitative real-time PCR was performed with My-IQiCycler (BioRad, Hercules, CA) using 2× SYBR Green master mixes. The primers used for quantification of dsRNAs of P450 in the transplastomic plants leaves were as follows: upstream primer (5′-AGAGACTCATTTCGACCTCGTTGCT-3) and downstream primer (5′-GGAGCAATAGCACCCTCTTGATAGAA-3′). The upstream primer anneals with P450, and the downstream primer anneals with TpsbA. Quantification was performed by the delta cycle time method with *Helicoverpa zea* β-*Actin* as an internal standard for normalization. All the results were obtained from three independent biological replicates.

Insect culture and feeding bioassays

Cotton bollworm (*H. armigera*) eggs were purchased from Benzon Research Laboratory (http://www.benzonresearch.com/insectlist.htm). They were hatched at 25 °C, and all the larvae were fed with the artificial diet (Southland Products Incorporated: http://www.tecinfo.com/~southland/). All dishes were kept at 25 °C on a 14-h-day/10-h-night cycle. For the gossypol tolerance experiments, third-instar larvae were transferred to the artificial diet supplemented with gossypol (0–4 mg) for 4 days and the increase in weight was recorded.

For the P450: RNAi bioassay, the larvae were reared on artificial diet until they developed to third-instar stage. These third-instar larvae were fed with tobacco leaves expressing P450 RNAi for 3 days. Then, the larvae were transferred to the following diets or leaves: (A) artificial diet; (B) artificial diet supplemented with 1–3 mg/g of gossypol; (C) glanded (gossypol containing) cotton leaves; (D) glandless cotton leaves, respectively, for 4 days. After feeding for 4 days, the increase in weight of the larvae from these four groups was recorded. For the Chi and ATPase RNAi feeding bioassay, third-instar larvae were fed on the transplastomic leaves expressing dsRNA targeted to *Chi* and *ATPase* genes for 4 days. The increase in weight of the larvae was recorded. For each treatment, 10 larvae were used and the experiment was repeated three times. After different feeding experiments, all larvae were put back on the artificial diet until pupae emerged. The mortality and pupation rate were recorded at the end of these experiments.

Statistical analysis

All statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC). Significance of variance was determined after the one-way ANOVA ($P > 0.05$) and is presented in all graphs as mean ± SE.

Acknowledgements

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