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Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol

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We identify a cytochrome P450 gene (*CYP6AE14*) from cotton bollworm (*Helicoverpa armigera*), which permits this herbivore to tolerate otherwise inhibitory concentrations of the cotton metabolite, gossypol. *CYP6AE14* is highly expressed in the midgut and its expression correlates with larval growth when gossypol is included in the diet. When larvae are fed plant material expressing double-stranded RNA (dsRNA) specific to *CYP6AE14*, levels of this transcript in the midgut decrease and larval growth is retarded. Both effects are more dramatic in the presence of gossypol. As a glutathione-*S*-transferase gene (*GST1*) is silenced in *GST1* dsRNA–expressing plants, feeding insects plant material expressing dsRNA may be a general strategy to trigger RNA interference and could find applications in entomological research and field control of insect pests.

Many insects can metabolize toxic phytochemicals accumulated by plants to resist or evade herbivores[1–3]. *H. armigera* is an important lepidopteran pest responsible for severe yield loss in cotton and other crops. However, as the efficacy of insecticides, resistant crop varieties and transgenic *Bacillus thuringiensis* crops[4,5] is threatened by the emergence of resistance[6], new approaches to control this and other insect pests are needed.

Since the discovery that dsRNA can silence genes[7], RNA interference (RNAi) has been developed as an effective tool in plants and animals[8–10]. Gene silencing inhibits virus replication[11] and plants engineered to produce artificial microRNAs targeting virus genes can resist viral infection[12]. Although insect genes can be downregulated by injection of dsRNA[13–15] or by oral administration of high concentrations of exogenously supplied dsRNA as part of an artificial diet[16], an efficient method of delivering dsRNA is needed if RNAi technology is to control pests in the field.

An interest in understanding how *H. armigera* resists gossypol and related sesquiterpene aldehydes[17–19], which are toxic to many organisms[20,21], led us to demonstrate a key role for the P450 monooxygenase CYPAE14 in catabolism of gossypol. We demonstrate that plants expressing hairpin RNA directed against *CYP6AE14* provide sufficient levels of intact dsRNA to suppress gene expression in the insect midgut and that this has potential for managing insect pests of agricultural importance.

RESULTS

Analysis of larval tolerance to gossypol

When we fed third-instar larvae of *H. armigera* for 5 d with an artificial diet supplemented with different concentrations of gossypol, the larvae treated with a high concentration of gossypol (3 mg g^{-1} diet) exhibited obvious growth retardation (twofold weight increase), whereas control larvae increased their weight by more than tenfold. At lower concentrations (1–2 mg g^{-1}), gossypol-mediated inhibition of larval growth was milder (**Fig. 1a**). Thus, cotton bollworms can tolerate a relatively low concentration of dietary gossypol. Most cotton cultivars store gossypol in pigmented glands, but the leaves and seeds of glandless cultivars do not normally accumulate this toxin[17,18]. When third-instar larvae were reared on leaves of glanded or glandless cotton cultivars for 2 d and 3 d, respectively, larvae on the glanded cultivar grew slower than those on the glandless cultivar (**Fig. 1b**).

Insect P450 monooxygenases play a central role in adaptation to plant defense compounds and in developing insecticide resistance[22–24]. Adding 1 mg g^{-1} of either gossypol or piperonyl butoxide (PBO; an inhibitor of P450 monooxygenases) to an artificial diet had only mild effects on larval growth. However, when the two chemicals were combined, a drastic reduction in larval growth was observed (**Fig. 1c**). Development of the larvae was also affected. When both PBO and gossypol (2 mg g^{-1}) were administered, about half of the larvae failed to pupate (data not shown). Thus, tolerance of *H. armigera* to gossypol is likely to involve P450 activity.

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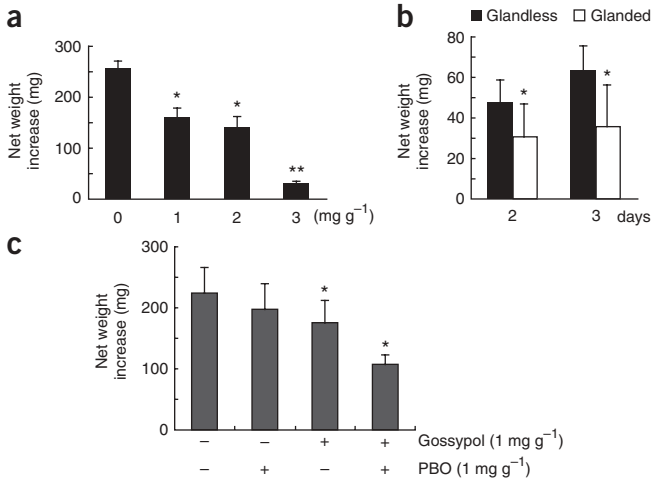

Figure 1 Net weight increases of larvae reared on a gossypol-supplemented diet. (**a**,**b**) Third-instar larvae were fed an artificial diet containing different concentrations of gossypol for 5 d (**a**), or with leaves of a glandless or a glanded cotton cultivar (**b**). (**c**) Fifth-instar larvae were fed an artificial diet with or without piperonyl butoxide (PBO), gossypol, or both, for 2 d. *, $P < 0.05$; **, $P < 0.01$.

Isolation of gossypol-inducible midgut genes

To enrich for genes potentially involved in the tolerance of gossypol, we constructed a cDNA library from RNAs expressed in the midguts of fifth-instar larvae exposed to gossypol (1 mg g^{-1}) for 1 d. Analysis of ~1,000 unique, expressed sequence tags (>300 bp) identified several gossypol-inducible genes (data not shown), one of which contains a 1,581-bp open reading frame encoding a putative P450 monooxygenase, CYP6AE14. This gossypol-inducible P450 is most similar to members of the CYP6 family, with 67% protein sequence similarity to CYP6AE12 of *H. armigera* and 58% similarity to CYP6AE1 of parsnip webworm (*Depressaria pastinacella*). Insect CYP6 enzymes often confer tolerance to or detoxify xanthotoxins[25], and *CYP6AE1* is furanocoumarin inducible[26]. As induced metabolism of xenochemicals is a common feature of animal detoxification systems[27,28], this gossypol-inducible P450 is likely to participate in *H. armigera* defenses against gossypol.

Correlation of *CYP6AE14* expression with larval growth

H. armigera CYP6AE14 is encoded by a single-copy gene (**Supplementary Fig. 1** online). Induction of *CYP6AE14* expression by gossypol is dose dependent in the range of 0.1 to 1 mg g^{-1} (**Fig. 2a**). *CYP6AE14* transcript level was high in the midgut, low in malpighian tubes and fatty bodies, and undetectable in the ovary and brain (**Fig. 2b**). Immunohistochemistry revealed that CYP6AE14 proteins are densely localized on the inner surface of the midgut (**Fig. 2c**). This spatial pattern of gene expression and protein distribution is consistent with the putative role of CYP6AE14 in defenses against gossypol. To determine a further link between *CYP6AE14* expression and cotton bollworm tolerance to gossypol, we weighed each larva and then measured *CYP6AE14* transcript levels in the midgut using quantitative RT-PCR (qRT-PCR). Larval weight increase correlated with *CYP6AE14* expression levels when gossypol was included in the diet ($r \leq 0.8$; 2 mg g^{-1} gossypol). However, this correlation was not observed in the absence of gossypol (**Fig. 2d**).

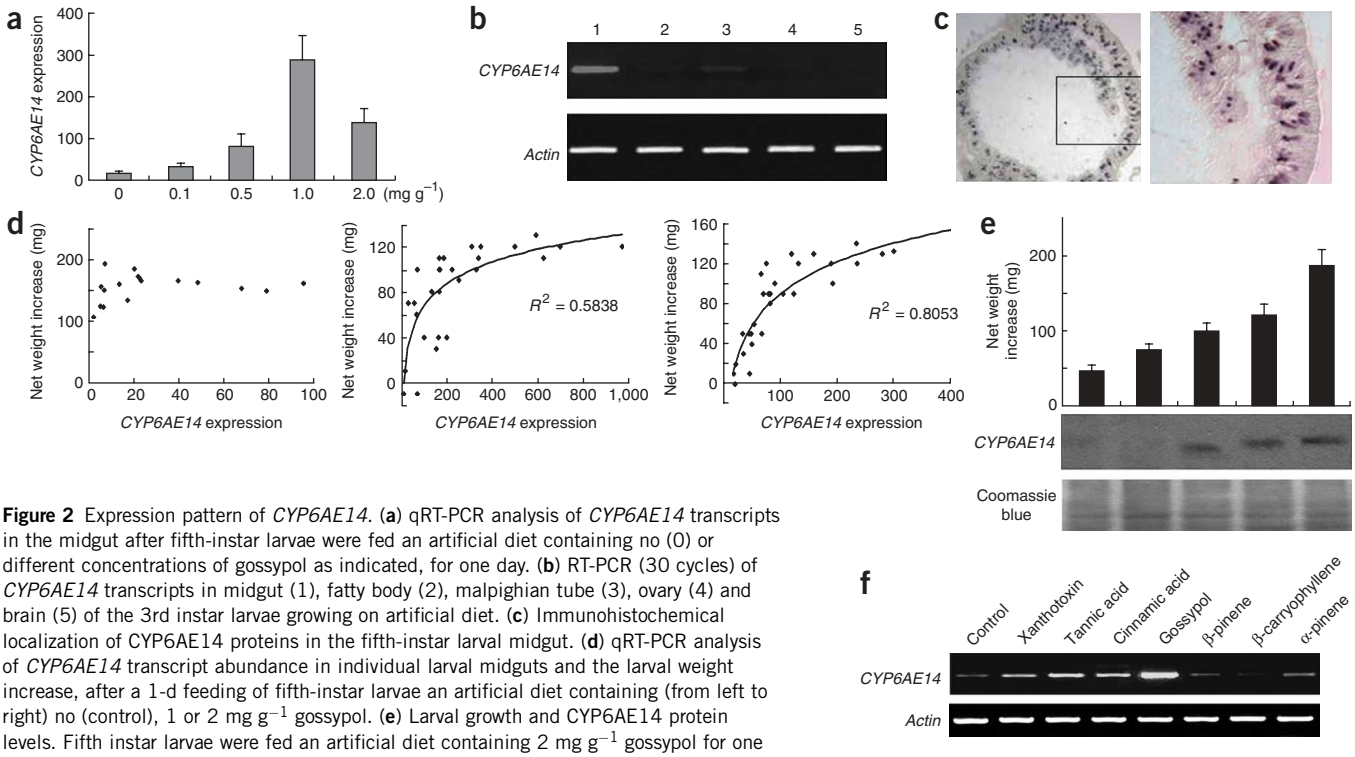

Figure 2 Expression pattern of *CYP6AE14*. (**a**) qRT-PCR analysis of *CYP6AE14* transcripts in the midgut after fifth-instar larvae were fed an artificial diet containing no (0) or different concentrations of gossypol as indicated, for one day. (**b**) RT-PCR (30 cycles) of *CYP6AE14* transcripts in midgut (1), fatty body (2), malpighian tube (3), ovary (4) and brain (5) of the 3rd instar larvae growing on artificial diet. (**c**) Immunohistochemical localization of CYP6AE14 proteins in the fifth-instar larval midgut. (**d**) qRT-PCR analysis of *CYP6AE14* transcript abundance in individual larval midguts and the larval weight increase, after a 1-d feeding of fifth-instar larvae an artificial diet containing (from left to right) no (control), 1 or 2 mg g^{-1} gossypol. (**e**) Larval growth and CYP6AE14 protein levels. Fifth instar larvae were fed an artificial diet containing 2 mg g^{-1} gossypol for one day; 60 larvae were divided into five groups according to their weights; CYP6AE14 proteins in the midgut of each group were detected by western blot. (**f**) RT-PCR (24 cycles) of *CYP6AE14* transcripts in midgut after fifth-instar larvae were fed an artificial diet containing no chemical (control) or 1 mg g^{-1} of the chemicals as indicated, for one day.

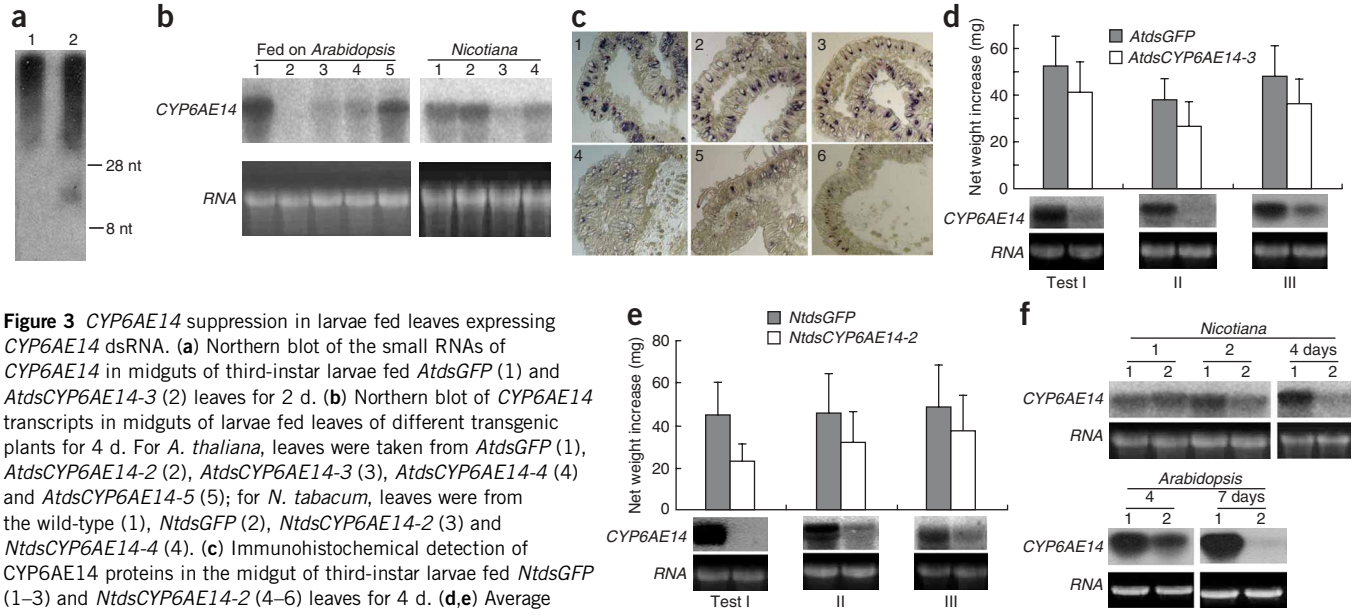

Figure 3 *CYP6AE14* suppression in larvae fed leaves expressing *CYP6AE14* dsRNA. (**a**) Northern blot of the small RNAs of *CYP6AE14* in midguts of third-instar larvae fed *AtdsGFP* (1) and *AtdsCYP6AE14-3* (2) leaves for 2 d. (**b**) Northern blot of *CYP6AE14* transcripts in midguts of larvae fed leaves of different transgenic plants for 4 d. For *A. thaliana*, leaves were taken from *AtdsGFP* (1), *AtdsCYP6AE14-2* (2), *AtdsCYP6AE14-3* (3), *AtdsCYP6AE14-4* (4) and *AtdsCYP6AE14-5* (5); for *N. tabacum*, leaves were from the wild-type (1), *NtdsGFP* (2), *NtdsCYP6AE14-2* (3) and *NtdsCYP6AE14-4* (4). (**c**) Immunohistochemical detection of CYP6AE14 proteins in the midgut of third-instar larvae fed *NtdsGFP* (1–3) and *NtdsCYP6AE14-2* (4–6) leaves for 4 d. (**d**,**e**) Average weight increase (upper) and the midgut *CYP6AE14* transcript level (down) of third-instar larvae fed *AtdsGFP* and *AtdsCYP6AE14-3*, or *NtdsGFP* and *NtdsCYP6AE14-2* leaves for 4 d. Statistical analysis of net weight increase was performed with student *t*-test ($P < 0.05$). (**f**) Northern blot of *CYP6AE14* transcripts in midguts of third-instar larvae fed *NtdsGFP* (1) and *NtdsCYP6AE14-2* (2) leaves for 1, 2 and 4 d (right), or *AtdsGFP* (1) and *AtdsCYP6AE14-3* (2) leaves for 4 d and 7 d, respectively.

Western blot analysis revealed a similar correlation between CYP6AE14 abundance and larval growth (**Fig. 2e**). Expression of *CYP337B1v2*, another *H. armigera* P450 gene, was high in malpighian tubes and ovaries, low in midguts and fatty bodies and not responsive to dietary gossypol (**Supplementary Fig. 2a**,**b** online). As expected, *CYP337B1v2* expression in the midgut was not correlated with larval growth in the presence of gossypol (**Supplementary Fig. 2c**). These data suggest that *CYP6AE14* is required for cotton bollworm tolerance of gossypol. We also tested the effects of several other plant metabolites on *CYP6AE14* expression. In comparison with a marked induction by gossypol, *CYP6AE14* was weakly induced by other phenolic compounds tested, and volatile terpenes exerted little or no effect (**Fig. 2f**). Therefore, *CYP6AE14* induction by gossypol is specific.

Suppression of *CYP6AE14* expression by ingestion of dsRNA-producing plant material

Because cotton bollworm requires an elevated level of *CYP6AE14* gene expression to grow on gossypol-containing food, downregulation of *CYP6AE14* might reduce larval tolerance of gossypol. As the RNAi effect is diffusible[29–32], we suspected that its mobility might enable suppression of insect genes if larvae are fed plant material that expresses dsRNA directed against a target of interest. To this end, we constructed *dsCYP6AE14*, an inverted repeat derived from the *CYP6AE14* coding sequence. A similar dsRNA construct of the green florescence protein gene, *dsGFP*, was generated as a control (**Supplementary Fig. 3a** online). Of several independent lines of *Arabidopsis thaliana* and *Nicotiana tobacum* plants producing the small interfering RNAs (siRNAs, ~21–24 nt), none displayed obvious phenotypic changes. When third-instar larvae, previously reared on artificial diets, were transferred to leaves of the transgenic plants, RNA blot analysis revealed small *CYP6AE14* RNA fragments in the midgut 2 d after transfer (**Fig. 3a**). Four days after transfer, larvae grown on *dsCYP6AE14* leaves had fewer *CYP6AE14* transcripts in their midguts compared to larvae on *dsGFP* or wild-type leaves (**Fig. 3b**). CYP6AE14 levels were also reduced (**Fig. 3c**). Among the larvae placed on leaves of *A. thaliana* lines, those from *AtdsCYP6AE14-2, 3* and *4* leaves had lower *CYP6AE14* expression levels than the group from *AtdsCYP6AE14-5*. Similarly, on tobacco leaves, the larvae from *NtdsCYP6AE14-2* leaves had a lower *CYP6AE14* expression level than those from *NtdsCYP6AE14-4* (**Fig. 3b**). Plants of *NtdsCYP6AE14-2* and *AtdsCYP6AE14-2, 3, 4* lines had higher levels of the siRNAs than the *NtdsCYP6AE14-4* and *AtdsCYP6AE14-5* lines, respectively, suggesting that the gene knockdown efficiency is related to *dsCYP6AE14* expression levels in transgenic plants (**Supplementary Fig. 3b**). The experiments were repeated with *AtdsCYP6AE14-3* and *NtdsCYP6AE14-2* leaves, and suppression of *CYP6AE14* expression in the midgut was observed consistently. Furthermore, in comparison with the wild-type plant leaves, larval growth was slightly affected by feeding on *dsCYP6AE14*, but not by *dsGFP* expression (**Fig. 3d**,**e** and **Supplementary Fig. 4** online).

We then analyzed the time course of *CYP6AE14* suppression by *dsCYP6AE14* plants. Third-instar larvae were reared on *NtdsCYP6AE14-2* and *NtdsGFP* leaves and after 1, 2 and 4 d, larvae were weighed and *CYP6AE14* transcripts in the midgut quantified. In larvae reared on *NtdsCYP6AE14-2* leaves, *CYP6AE14* transcript levels were largely unchanged at day 1, started to decrease at day 2 and were much reduced at day 4 (**Fig. 3f**); accordingly, growth retardation became evident at day 2 (**Supplementary Fig. 4**). Similarly, when *AtdsCYP6AE14-3* leaves were used for feeding, *CYP6AE14* transcripts decreased substantially at day 4 and were further reduced to undetectable levels by day 7 (**Fig. 3f**).

Catalase (CAT) activity, which is fundamental to insect antioxidant defenses, can be inhibited by superoxide anions that are mainly produced by P450s in microsomes[33]. CAT activity was higher in the midguts of larvae fed a PBO-supplemented diet; by contrast, CAT activity was lower when the diet was supplemented with gossypol, or when larvae were fed leaves of a glanded cultivar (**Supplementary Fig. 5a**,**b** online). These results imply that inhibition of P450s could elevate CAT activities. Because *CYP6AE14* is highly expressed in the

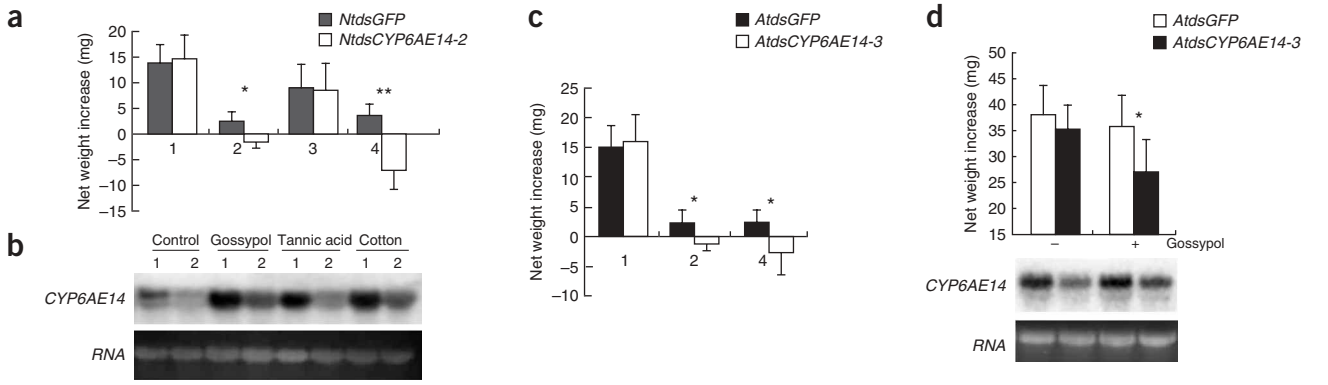

Figure 4 Enhanced effect of gossypol on larval growth fed on dsRNA leaves. (**a**,**b**) Third-instar larvae were fed on *NtdsGFP* or *NtdsCYP6AE14-2* leaves for 4 d and then transferred to artificial diet supplemented with no chemical (1), 1 mg g^{-1} of gossypol (2) and tannic acid (3), and to glanded (gossypol-containing) cotton leaves (4), respectively. After 2 d, weight increases (**a**) were recorded and *CYP6AE14* transcripts in midguts (**b**) were detected by northern analysis. (**c**) Growth of larvae after transfer to an artificial diet, as described for **a**, for 2 d; larvae were previously grown on *AtdsGFP* and *AtdsCYP6AE14-3* leaves, respectively, for 4 d. (**d**) Larval growth (upper) and northern blot of *CYP6AE14* transcripts (down) of third-instar larvae were fed *AtdsGFP* and *AtdsCYP6AE14-3* for 3 d on leaves sprayed with mock solution (–) or gossypol solution (+). The final concentration of gossypol in the leaf was ~0.5–1 mg g^{-1} dry weight. *, $P < 0.05$; **, $P < 0.01$.

midgut, suppression of *CYP6AE14* expression was expected to provoke CAT activity. Midgut CAT activity of the larvae reared on *dsCYP6AE14* leaves was indeed higher than that from larvae reared on *dsGFP* leaves (**Supplementary Fig. 5c**,**d**).

CYP6AE14 suppression reduced the larval tolerance to gossypol

As growth of larvae reared on a gossypol-containing diet correlates with *CYP6AE14* expression (**Fig. 2d**,**e**), we anticipated that the negative effect of gossypol on larval growth would be magnified if *CYP6AE14* was downregulated by RNAi. To test this, we transferred larvae reared on tobacco (*NtdsCYP6AE14-2* and *NtdsGFP*) leaves for 4 d to artificial diets containing different supplements. After 2 d, rapid growth on the control diet was evident for both groups. However, when gossypol was included, larvae fed *NtdsCYP6AE14-2* leaves did not grow at all. Their average weight even decreased, whereas growth of *NtdsGFP* larvae was retarded (**Fig. 4a**). If gossypol was replaced by tannic acid, a mild but insignificant ($P = 0.82$) growth delay was observed (**Fig. 4a**). Thus, the adverse effect of *dsCYP6AE14* leaves on larval growth became significant ($P = 0.02$) only when gossypol was included in the diet. To examine the effect of gossypol in cotton tissues after *CYP6AE14* suppression, we fed larvae leaves of a glanded cotton cultivar; larval growth of the *NtdsCYP6AE14-2* group was severely inhibited (**Fig. 4a**). Northern blot analysis indicated that 2 d after removal from tobacco leaves, the *NtdsCYP6AE14-2* group still showed much less *CYP6AE14* expression in the midgut than the *NtdsGFP* group, even after induction by gossypol (**Fig. 4b**). We also tested the sensitivity of larvae from the *AtdsCYP6AE14-3* leaves to gossypol, and the results were similar (**Fig. 4c**). To imitate exposure of larvae to *dsCYP6AE14*-expressing cotton plants, we sprayed *A. thaliana* leaves with a gossypol solution (50 μg ml^{-1}) so that the leaves contained both gossypol and the dsRNA. Growth of larvae fed gossypol-painted *AtdsCYP6AE14-3* leaves was clearly retarded (**Fig. 4d**). Together, these data demonstrate that plant-mediated RNAi of *CYP6AE14* gene expression greatly enhances the toxicity of gossypol to *H. armigera*.

Plant-mediated insect RNAi as a functional tool in *H. armigera* gene suppression

To determine whether the plant-mediated insect RNAi, exemplified here with *CYP6AE14*, was applicable to other midgut-expressed genes, we tested another *H. armigera* gene, *GST1* (GenBank accession no. EF033109), which encodes a glutathione-*S*-transferase. Expression of *GST1* was not altered by gossypol treatment (**Fig. 5a**). Four days after third-instar larvae were transferred to leaves of the *A. thaliana* line *AtdsGST1-5*, which expresses an inverted repeat of *GST1* (*dsGST1*, see **Supplementary Fig. 3a**), levels of *GST1* transcripts in the midgut were reduced. These experiments were repeated several times (**Fig. 5b**). Accordingly, total GST activity in the midgut also decreased (**Fig. 5c**). These results demonstrate that, at least in the midgut, expression of *CYP6AE14* and other genes of this herbivorous insect can be suppressed by feeding on plants engineered to produce the respective dsRNA.

Finally, we investigated the effective forms of plant RNAs in mediating insect gene silencing. *A. thaliana* has four Dicer-like (DCL) nucleases. DCL1 is a sophisticated enzyme capable of producing both microRNAs and siRNAs, and the three other DCLs are mainly involved in siRNA production[34]. To generate plants that predominately produce the long dsRNA of the transgene, we used a *dcl2 dcl3 dcl4* triple mutant[35]. After introducing the *dsCYP6AE14* gene into the triple mutant, we selected four lines (*dcl AtdsCYP6AE14-11* through *dcl AtdsCYP6AE14-14*). For comparison, we chose two more transgenic wild-type lines (*AtdsCYP6AE14-7* and *AtdsCYP6AE14-8*)

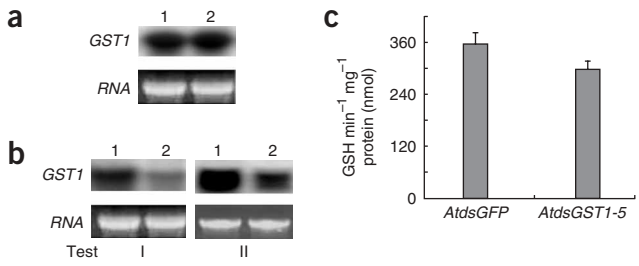

Figure 5 Suppression of *GST1* by dsRNA-producing plants. (**a**) Northern blot of *GST1* transcripts in midgut of fifth-instar larvae fed an artificial diet containing no (1) or 1 mg g^{-1} gossypol (2) for one day. (**b**) Northern blot of *GST1* transcripts in midguts of larvae fed *AtdsGFP* (1) or *AtdsGST1-5* (2) leaves for 4 d. (**c**) Total GST activity in larval midguts as described for **b**. $P < 0.001$.

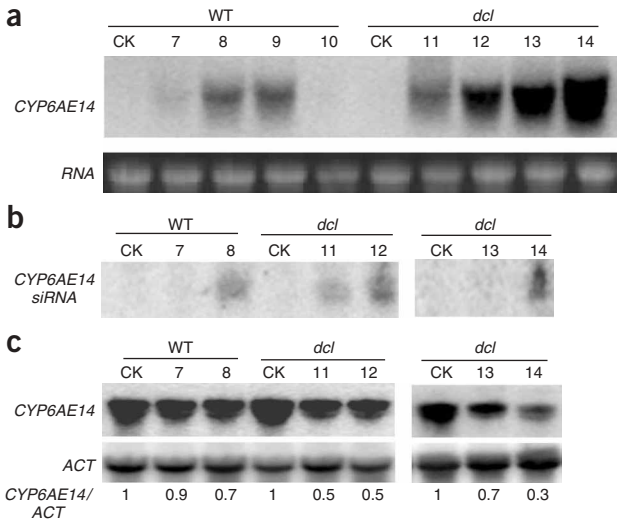

Figure 6 Suppression of *CYP6AE14* by *dcl2 dcl3 dcl4* triple mutant plants expressing *dsCYP6AE14*. (**a**,**b**) Northern blot detection of *CYP6AE14* dsRNA (**a**) and siRNA (**b**) in transgenic plants of the wild-type (WT) and the *dcl2 dcl3 dcl4* triple mutant (*dcl*). (**c**) *CYP6AE14* transcript levels in midgut of the third-instar larvae fed leaves of different transgenic plant lines for 3 d; RNA hybridization signals were quantified using a Fuji phosphorimager, and normalized using *ACT* (*ACTA3b*, X97615) expression as loading control. CK indicates nontransgenic plants and 7–14 refer to independent transgenic lines. The levels of *CYP6AE14* mRNA in midguts of the larvae fed nontransgenic plants (CK) was arbitrarily set to 1.

with different levels of *dsCYP6AE14* expression (**Fig. 6a**). Northern blot analysis showed that the triple mutant accumulated substantially higher levels of the long dsRNA of *CYP6AE14* than the wild-type plants (**Fig. 6a**). In one of the mutant lines, *dcl AtdsCYP6AE14-13*, siRNAs of *CYP6AE14* were largely absent but the long dsRNA was abundant; production of the siRNAs in the other three lines was not abolished (**Fig. 6b**), probably owing to DCL1 activity[35]. When third-instar larvae were fed these transgenic leaves for 3 d, we found that in general, the triple *dcl* mutant lines exhibited a more profound effect in silencing *CYP6AE14* expression in the midgut (**Fig. 6c**). Because the most striking feature of the triple mutant plants in this experiment is their accumulation of more abundant long dsRNA of *CYP6AE14* and siRNAs are scarce in *dcl AtdsCYP6AE14-13* leaves, the long dsRNA may also suppress insect gene expression after entering the midgut.

DISCUSSION

Expression of some of at least 16 full-length *H. zea* and *H. armigera* P450 monooxygenases for which cDNAs are available is induced by pyrethroid insecticides (CYP6B2, 6, 7, 8)[36–38], by plant allelochemicals (CYP6B8, 27, 28 and CYP321A1)[39,40] or by plant signaling molecules (CYP6B8, 28, 9, 27)[41]. CYP6AE14 is the first gossypol-inducible P450 gene from bollworms. Although a firm conclusion awaits elucidation of biochemical function, three lines of evidence suggest that CYP6AE14 is directly involved in the ability of cotton bollworm to tolerate gossypol. First, *CYP6AE14* is highly expressed in the midgut, the main digestion and detoxification organ of the larva. Second, larval growth correlates with *CYP6AE14* expression levels when gossypol is present in the diet. And third, when *CYP6AE14* expression is suppressed, as achieved here by plant-mediated RNAi, larval tolerance of gossypol is greatly reduced. Importantly, only in the presence of gossypol does a high level of *CYP6AE14* expression become necessary for normal growth of the cotton bollworm. As all of the widely planted cotton cultivars contain a substantial amount of gossypol, CYP6AE14 is an attractive target for controlling cotton bollworm.

RNAi occurs widely in eukaryotic organisms, and the silencing signal is capable of intercellular movement[7,42–45]. Mobility of RNAi effects is observed in *Caenorhabditis elegans*[7]; a robust RNAi response occurs not only in nematodes fed *Escherichia coli* that produce dsRNA, but also in their progeny[30].

The ability to downregulate *CYP6AE14* and *GST1* expression in the midgut by feeding cotton bollworms dsRNA-producing leaves suggests that plant-mediated RNAi may be a general approach for gene-silencing in herbivorous insects. As the potential to silence the insect gene is retained in a *dcl2 dcl3 dcl4* triple mutant, the long dsRNA produced in plants could suppress insect gene expression. Further research should establish whether dicer activities are present in the midgut and whether the dsRNAs are diced into siRNAs before spreading into midgut cells.

Using dsRNA to knock-down specific genes has been well documented in recent years. Injection remains the most used method to deliver dsRNA molecules into animal tissues, but may injure the tissues under study, depends largely on the operator's skill and is unsuitable for controlling field pests. The plant-mediated herbivorous insect RNAi reported here lays the basis for a new strategy: suppressing a critical insect gene(s) by feeding insects with plant tissues engineered to produce a specific dsRNA. This method could help to dissect gene functions of herbivorous insects in a convenient and repeatable way. Notably, when cotton bollworms were grown on plants producing *dsGFP*, no adverse effect on larval growth was observed. The insect RNAi effect triggered by ingestion of transgenic dsRNA-producing plants is therefore gene-specific and could be used to protect crop plants against insect damage.

A. thaliana expresses nearly 1.5 million small RNA species present in inflorescences and seedlings[46], for which only a small fraction have identified target genes. Why do plants produce so many small RNAs? Our demonstration that dsRNA, or its effect, can be transmitted from plants to insects invites investigation as to whether the various plants small RNAs or their double-stranded precursors have participated in the co-evolution of plants and insects.

METHODS

Plant and insect culture. Plants of *Arabidopsis thaliana* (ecotype Col-0), tobacco (*Nicotiana tabacum*) and cotton (*Gossypium hirsutum* cv. Xu-142) were grown in presterilized soil at 22 °C (for *A. thaliana*) or 26 °C (for tobacco and cotton), on a 16-h-day/8-h-night cycle. Allele-specific PCR was carried out to confirm the homozygous state of the triple mutant T-DNA insertions.

Cotton bollworm (*Helicoverpa armigera*) eggs were obtained from Nanjing Agricultural University and reared in the laboratory at 25 °C and 70% relative humidity on a 14-h-day/10-h-night cycle. The larvae were fed on a modified artificial diet as described[47]. Plants (4 ~ 5-weeks-old) and leaves of tobacco plants (~1 month after transferring to soil) were also used for feeding experiments. If needed, the leaves were sprayed with a gossypol solution, which was prepared by adding the stock solution (1 mg gossypol in 1 ml ethanol) to water to a final concentration of 50 μg ml^{-1}. For each feeding experiment, synchronous larvae were selected, weighed individually and divided into groups; each group contained 20–30 individuals. After feeding on different diets for indicated days, larvae were weighed and midguts were taken for further analysis. Statistics of data was performed with student *t*-test in the Excel program.

Vectors and plant transformation. The dsRNA construct (pBI121-*dsCYP6AE14* for wild-type and pCAMBIA1300-*dsCYP6AE14* for wild-type or the

dcl2 dcl3 dcl4 triple mutant plants) contained a 35S promoter, a sense fragment of *CYP6AE14* cDNA, a 120-nucleotide intron of *A. thaliana RTM1* gene[48], the *CYP6AE14* fragment in antisense orientation, and a NOS terminator. Vectors for *dsGFP* and *dsGST1* were similarly constructed (**Supplementary Fig. 3a**). The *CYP6AE14* and *GST1* fragments were obtained by PCR amplification of *H. armigera* cDNA clones with primers GIPF (5′-GAAGATTTTCTCGATAAG GAAG-3′) and GIPR (5′-ATATAAAGCACTGTGCCACTAAG-3′) for *CYP6AE14*; GSTF (5′-GACCTTGGCAGACCTCAG-3′) and GSTR (5′-CCAG CTCGAACCACTTTT-3′) for *GST1*. The *GFP* fragment was obtained by amplification of pCAMBIA 1302 plasmid (http://www.cambia.org/daisy/bios/585.html) with primers GFPF (5′-CGATTTCAAGGAGGACGG-3′) and GFPR (5′-CCATGCCATGTGTAATCCC-3′). Binary vectors harboring the desired construct were transferred into *Agrobacterium tumefaciens* strain GV3101 (for *A. thaliana*) and strain LBA4404 (for tobacco) by electroporation. Transgenic *A. thaliana* plants were generated by a floral dip method[49], and screened on solid plates containing 25 mg l^{-1} kanamycin. For tobacco, a leaf disc cocultivation method was used for generating transgenic plants, which were selected on medium containing 100 mg l^{-1} kanamycin. Transgenic plant lines were further analyzed by PCR and northern blot analysis.

RNA analysis. Total RNAs were isolated from *H. armigera* or plant tissues by Trizol reagent (Invitrogen). The RNAs were separated on 1.0% denaturing agarose gel and transferred to the Hybond-N^+ filter membrane (Amersham Pharmacia Biotech). For small RNAs, the RNA samples, 40 μg per lane, were loaded on a TBE-urea gel (15%), after electrophoresis, they were electroblotted onto the Hybond-N^+ membrane. The membranes were UV cross-linked and hybridized with ExpressHyb solutions (Clontech). Probes were obtained by PCR using primers as described for vector construction. The probes were randomly labeled with ^{32}P-dCTP using the Prime-a-Gene Labeling System (Promega). For RT-PCR, the first strand cDNA was prepared using the ReverTra Ace kit (TOYOBO). Real-time RT-PCR (qRT-PCR) was performed on a Bio-Rad iCycler with iQ SYBR Green Supermix (Bio-Rad), following a two-step protocol: 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 20 s.

Protein detection. The rabbit antiserum against a CYP6AE14 fragment (150-311 amino acid residues) was raised, and the antibody was purified by binding with Protein A-Sepharose CL6B (Sigma), followed by selective elution of IgG with 50 mM glycine, pH 3.0, 0.5 mM NaCl, neutralized with 1 M Tris/HCl to pH 7.0, and used at 1:500 dilution.

For western blot analysis, total proteins of the midgut of *H. armigera* were extracted and loaded onto a 12% SDS-PAGE gel (20 μg proteins per lane); after, by electrophoresis, the proteins were electrotransferred to a Hybond-C membrane (Amersham). Blots were incubated with the primary antibody for 1 h. Immune-detection was carried out with alkaline phosphatase (AP)-conjugated anti-rabbit antiserum as the secondary antibody for 45 min, and the western blue stabilized substrate for AP was used as a chromogen. For immunohistochemistry, midgut tissues were formalin-fixed and paraffin-embedded, followed by cutting into 8-μm thick sections. After deparaffinization through a series of xylene baths, and rehydration through a series of graded alcohol solutions, the protein signals were detected as described for western blot analysis.

Enzyme assay. Midgut was taken from each larva and washed several times with ice-cold physiological saline (0.7% NaCl). After removing the contents, the midgut was rinsed with physiological saline, homogenized in elution buffer (0.25 M sucrose, 0.05 M Tris-HCL, 1 mM EDTA, pH 7.4) and sonicated. The homogenates were centrifuged for 10 min at 5,000*g*, 4 °C. The amount of proteins was determined by Coomassie Blue G dye assay. CAT activity was determined by monitoring spectrophotometrically at 240 nm the degradation of a standard concentration of hydrogen-peroxide, and given as nmol H_2O_2 min^{-1} mg^{-1} protein. Glutathione *S*-transferase (GST) activity was determined by monitoring changes in absorbance at 412 nm in spectrophotometer, and given as nmol GSH min^{-1} mg^{-1} protein.

Accession numbers. GenBank: *H. armigera* ESTs, EE399397–EE399947; cDNA sequences of *CYP6AE14* (DQ986461) and *GST1* (EF033109).

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

X.-Y.C. and Y.-B.M. designed the research; Y.-B.M. performed most of the experiments; Y.-B.M. and J.-W.W. designed the dsRNA constructs; W.-J.C., L.-J.W., G.-J.H., X.-Y.T. did some of the RNA analysis experiments; Y.-B.M., X.-Y.C. and Y.-P.H. wrote the manuscript.

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