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ORIGINAL PAPER

Cotton plants expressing *CYP6AE14* double-stranded RNA show enhanced resistance to bollworms

Ying-Bo Mao · Xiao-Yuan Tao · Xue-Yi Xue · Ling-Jian Wang · Xiao-Ya Chen

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Abstract RNA interference (RNAi) plays an important role in regulating gene expression in eukaryotes. Previously, we generated *Arabidopsis* and tobacco plants expressing double-stranded RNA (dsRNA) targeting a cotton bollworm (*Helicoverpa armigera*) P450 gene, *CYP6AE14*. Bollworms fed on transgenic *dsCYP6AE14* plants showed suppressed *CYP6AE14* expression and reduced growth on gossypol-containing diet (Mao et al., in Nat Biotechnol 25: 1307–1313, 2007). Here we report generation and analysis of dsRNA-expressing cotton (*Gossypium hirsutum*) plants. Bollworm larvae reared on T2 plants of the *ds6-3* line exhibited drastically retarded growth, and the transgenic plants were less damaged by bollworms than the control. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) showed that the *CYP6AE14* expression level was reduced in the larvae as early as 4 h after feeding on the transgenic plants; accordingly, the CYP6AE14 protein level dropped. These results demonstrated that transgenic cotton plants expressing *dsCYP6AE14* acquired enhanced resistance to cotton bollworms, and that RNAi technology can be used for engineering insect-proof cotton cultivar.

Keywords RNA interference (RNAi) · *Helicoverpa armigera* · Transgenic · *Gossypium hirsutum* · Insect-proof

Introduction

Crop plants are widely cultivated and suffer from attacks by insect herbivores (Gassmann et al. 2009; Wittstock et al. 2004; Christou et al. 2006; Ferry et al. 2006). Cotton bollworm (*Helicoverpa armigera*) is one of the most destructive agricultural pests, causing severe yield loss in crop production. Host plants of this generalist lepidopteran include not only cotton but also other crop species. In the past three decades, transgenic technology has been developed to generate insect-proof plants to reduce yield loss and pesticide utilization (Bale et al. 2008; Kos et al. 2009). Engineering crop plants to express genes coding for insecticidal crystalline proteins from *Bacillus thuringiensis* (*Bt*), so-called Cry toxins, has achieved great success both economically and ecologically (Qaim and Zilberman 2003; Wu et al. 2008). However, with lasting cultivation of *Bt* crops, increasing insect resistance to transgenic crops and

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Y.-B. Mao (✉) · X.-Y. Tao · X.-Y. Xue · L.-J. Wang · X.-Y. Chen
National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, People's Republic of China
e-mail: ybmao@sibs.ac.cn

outbreaks of nontarget pests were reported (Bravo and Soberon 2008; Gahan et al. 2001; Tabashnik et al. 2008; Lu et al. 2010), which calls for new approaches.

Since the discovery that ingested dsRNA could trigger RNAi in the nematode (*Caenorhabditis elegans*) (Fire et al. 1998), this gene-silencing approach has become a valuable tool in functional genomics (Cronin et al. 2009; Wesley et al. 2001). Injection of siCoo2-RNA into pea aphids led to *Coo2* suppression and caused significant mortality of insects fed on host plant (Mutti et al. 2008). When the diamondback moth, *Plutella xylostella*, was fed synthetic dsRNA against *CYP6BG1*, expression of the P450 gene was suppressed, resulting in reduced larval resistance to the insecticide permethrin (Bautista et al. 2009). It has been reported that plants could be armed with dsRNA to fend off insect pests, as transgenic plants producing dsRNAs against selected insect genes showed suppressive effects on cotton bollworm (Mao et al. 2007) and western corn rootworm (Baum et al. 2007) gene expression. Because the introduced dsRNA can be highly specific to target insects, this approach limits the adverse effects on nontarget organisms. RNAi-based gene regulation has been reported in different insect orders, including Lepidoptera, Hemiptera, Coleoptera, Diptera, and Hymenoptera (Huvenne and Smagghe 2010), which makes it possible to develop RNAi technology for control of various insect pests (Gordon and Waterhouse 2007).

Many plant secondary metabolites are toxic to or repel insects, enabling host plants to escape from insect herbivores (Gatehouse 2002). To counteract plant defenses, insects have developed adaptive mechanisms, which often involve a set of genes whose products metabolize the chemicals from plants (Wittstock et al. 2004). Most cotton cultivars accumulate gossypol and related sesquiterpene aldehydes in both aerial tissues and roots, and these phytoalexins form a chemical arsenal against herbivorous (Meng et al. 1999; Tan et al. 2000; Du et al. 2004; Stipanovic et al. 2006). Previously, we isolated a P450 monooxygenase gene, *CYP6AE14*, from *Helicoverpa armigera*; expression of *CYP6AE14* was induced by gossypol, and its expression level was correlated with larval growth when gossypol was present in the diet. When bollworms were fed on transgenic *Arabidopsis* plants producing dsRNA against *CYP6AE14* (*dsCYP6AE14*), expression of *CYP6AE14* was suppressed; after transferring to a gossypol-containing diet, the larvae showed decreased tolerance to gossypol (Mao et al. 2007). Therefore, if cotton plants are engineered to express *dsCYP6AE14*, they may be better protected from bollworms due to the presence of gossypol in cotton plants. Here we report the generation of transgenic *dsCYP6AE14* cotton plants, which indeed acquired enhanced resistance to cotton bollworms.

Materials and methods

Plant and insect culture

Cotton plants (*Gossypium hirsutum* cv. R15) were grown in greenhouse under 28–30°C, 60–80% relative humidity. Young leaves with the same condition and developing bolls at about 9 days post anthesis (9 DPA) were used for insect feeding tests.

Cotton bollworm (*Helicoverpa armigera*) eggs were obtained from Nanjing Agricultural University and reared as previously described (Peng et al. 2005). 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. Statistical data analysis was performed using Student′s *t*-test in Excel software.

Construction of vectors and cotton plant transformation

The dsRNA construct pBI121-*dsCYP6AE14* as described by Mao et al. (2007) contained a 35S promoter, a sense fragment of *CYP6AE14* complementary DNA (cDNA) from +472 to +940, a 120-nucleotide intron of *Arabidopsis RTM1* gene (Johansen and Carrington 2001), the *CYP6AE14* fragment in antisense orientation, and a NOS terminator (Fig. 1a). The *CYP6AE14* fragment (469 bp) was obtained by PCR amplification of *H. armigera* cDNA clones with primers GIPF (5′-GAAGATTTTCTCGATAAGG AAG-3′) and GIPR (5′-ATATAAAGCACTGTGC CACTAAG-3′). Binary vectors harboring the desired construct were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation, followed by transformation of cotyledon and hypocotyl explants

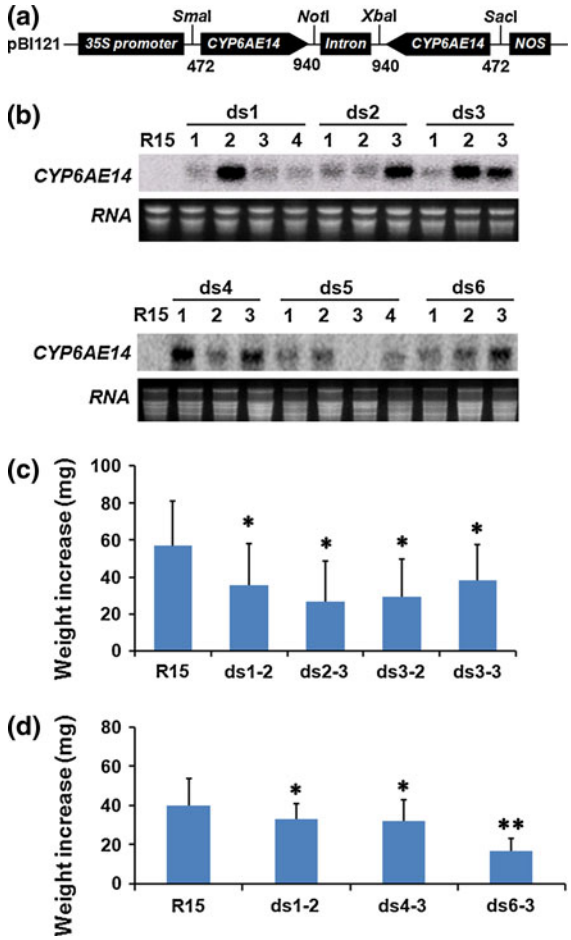

Fig. 1 Effect of T1 transgenic cotton on larvae growth. **a** The dsRNA construct pBI121-*dsCYP6AE14* contained a 35S promoter, a sense fragment of *CYP6AE14* cDNA from +472 to +940, a 120-nucleotide intron of *Arabidopsis RTM1* gene (Johansen and Carrington 2001), the *CYP6AE14* fragment in antisense orientation, and a NOS terminator. **b** northern blot detection of dsRNA homologues to *CYP6AE14* in the leaves of transgenic (*ds1-ds6*) and nontransgenic control (R15) plants. **c**, **d** Net weight increase of larvae reared on leaves of T1 transgenic cotton plants. Third-instar larvae previously grown on artificial diet were transferred to nontransgenic (R15) or T1 transgenic cotton plant leaves for 4 days, respectively. Values are means ± standard deviation (SD). $*P < 0.05$; $**P < 0.01$

from *Gossypium hirsutum* cv. R15 (Li et al. 2002). After the stages of callus induction, proliferation, embryogenic callus induction, embryo differentiation, and finally plantlet regeneration, the plantlets were transferred to pots in greenhouse for further growth. Transgenic plants were screened by kanamycin selection and further confirmed by PCR for presence of the neomycin phosphotransferase II (*NptII*) gene and RNA gel blot for presence of the *dsCYP6AE14* transcripts.

T1 seeds were germinated and rescreened to determine transgenic lines using PCR assay (*NptII* gene detection), and RNA and DNA gel blot assays. Transgenic plants were denoted using the format *ds6-3*, where "ds" represents a positive transgenic line, "6" is an independent primary transformant, and "3" is the number of the T1 progeny of that plant.

RNA analysis

Total RNAs were isolated from *H. armigera* by Trizol reagent (Invitrogen, Carlsbad, CA) or from cotton as described (Wu et al. 2002). The RNAs were separated on 1.0% denaturing agarose gel and transferred to Hybond-N^+ filter membrane (Amersham Pharmacia Biotech, Uppsala). 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 ultraviolet (UV) cross-linked and hybridized with ExpressHyb solutions (Clontech, Palo Alto, CA). 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, Madison, WI). For RT-PCR, first-strand cDNA was prepared using the ReverTra Ace kit (TOYOBO, Osaka). 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.

DNA analysis

Genomic DNA was isolated as described (Benbouza et al. 2006). Transgenic lines were determined by PCR detection of presence of *NptII*. The *NptII* gene was detected using primers kanF: 5′-GGCGATACCG TAAAGCACGAGGAA-3′ and kanR: 5′-GCTATGA CTGGGCACAACAGACAAT-3′, respectively, generating a 680-bp fragment.

Genomic DNA (20 μg) was digested with indicated enzyme for 16 h, separated on 0.8% agarose gel, and transferred onto Hybond N1 membrane (Amersham Pharmacia Biotech, Uppsala). DNA gel blot analysis of *G. hirsutum* cv. R15 and transgenic

cottons was carried out using probes that were obtained by PCR using primers as described for vector construction.

Protein analysis

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, St. Louis, MO), 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.

Total proteins of the midgut of *H. armigera* were extracted and loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel (20 μg proteins per lane). After electrophoresis, the proteins were electrotransferred to a Hybond-C membrane (Amersham, Buckinghamshire, UK). Blots were incubated with the primary antibody for 1 h, than incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antiserum as the secondary antibody for 45 min. The blot was developed using enhanced chemiluminescence (ECL) detection solution (Tiangen, China) and exposed to X-ray films (Kodak, Japan).

Analysis of sesquiterpene aldehydes

Total sesquiterpene aldehydes were quantitated with a phloroglucinol/HCl assay (Liu et al. 1999). A total of 500 mg sample was immersed in liquid nitrogen, ground to a fine powder, and extracted with 70% acetone for 30 min. After centrifugation, an equal volume of reagent (1% phloroglucinol, 2 N HCl in 95% ethanol) was added to the acetone extract, followed by incubation at 55°C for 5 min. Absorbance at 555 nm was measured immediately. Standard curve was prepared with gossypol (Sigma).

Results

Generation of transgenic cotton plants producing *dsCYP6AE14*

To examine if RNAi technology could be applied directly to cotton plants for enhanced insect resistance, we transferred the *dsCYP6AE14* construct into cotton plants (*Gossypium hirsutum* cv. R15). Twenty-eight primary (T0) transgenic cotton lines were obtained. Transcripts of *dsCYP6AE14* in T1 generation plants were examined by northern blotting. Of the 20 individual plants from five T1 lines, at least 6 (*ds1-2*, *ds2-3*, *ds3-3*, *ds4-1*, *ds4-3*, and *ds6-3*) showed relatively high expression level of the dsRNA (Fig. 1a). When placed on the control or the transgenic *dsCYP6AE14* cotton leaves for 4 days, second-instar larvae exhibited retarded growth on *dsCYP6AE14* leaves. Among these lines, *ds6-3* exhibited the most obvious adverse effect on larval growth (Fig. 1b, c). T2 generation plants of the *ds6-3* line were then generated. In a PCR assay, all of the T2 plants showed positive signals of *NptII*, a marker gene used for screening transgenic plants (Supplementary Fig. 1). RNA blot showed that the *dsCYP6AE14* transcripts were present in all of the T2 plants examined (Fig. 2a). Southern blot indicated that there were two copies of *dsCYP6AE14* in *ds6-3* and the T2 plants (Fig. 2b). The *ds6-3* line was then used for further investigation.

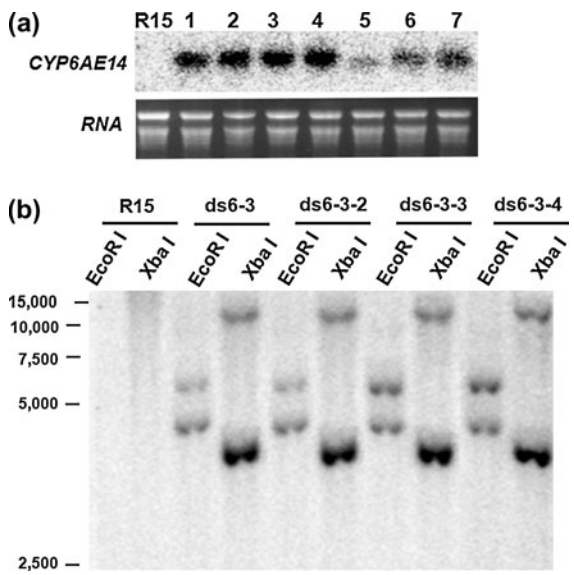

Fig. 2 northern and Southern blots of *dsCYP6AE14* in T1 and T2 lines of *ds6-3*. **a** northern blot to detect dsRNA of *CYP6AE14* in leaves of T2 plants of *ds6-3*. **b** Southern blot assay to determine copies of *CYP6AE14* in T1 and T2 plants of *ds6-3*. Genomic DNA of *ds6-3* and three individuals of T2 generation (ds6-3-2, ds6-3-3, and ds6-3-4) were digested by enzymes as indicated

CYP6AE14 expression in bollworms was suppressed by *dsCYP6AE14* cotton

To see if the RNAi signal could be transmitted into bollworm midgut cells during ingestion of the transgenic cotton tissues, second-instar larvae, previously reared on artificial diets, were transferred to leaves of the T2 *ds6-3* plants, and RNA gel blotting was performed to detect the small RNA (sRNA) fragments of *CYP6AE14*. The sRNAs of *CYP6AE14* were detected in midgut of the bollworms at day 3 post transferring to the transgenic leaf (Fig. 3a). *CYP6AE14* gene expression was then examined by both qRT-PCR and RNA blot at different intervals. The data show that *CYP6AE14* expression level was decreased as early as 4 h post transferring, and the suppression was evident at 8 and 16 h (Fig. 3b, c). Consistent with the gene expression level decrease, the CYP6AE14 protein level drastically dropped in larvae reared on T2 *ds6-3* leaves for 3 days (Fig. 3d). These data demonstrate that *CYP6AE14* expression in the cotton bollworm midgut could be suppressed by transgenic cotton leaves expressing *dsCYP6AE14*.

Transgenic *dsCYP6AE14* cotton plants showed enhanced protection from bollworms

To detect the effects of T2 plants on bollworm growth, second-instar larvae reared on leaves of the wild-type and T2 *ds6-3* plants were weighed, respectively. Larvae on the *ds6-3* plant leaves exhibited obvious growth retardation, with only 60% weight increase in comparison with those on the wild-type leaves, after assaying for 4 days; growth retardation became more evident when the assay was extended to 6 and 10 days (Fig. 4a, b). It was reported that high concentrations of gossypol in diets would inhibit bollworm growth (Mao et al. 2007). We then measured gossypol equivalents in the wild-type and the transgenic cotton leaves. Quantitative analysis of gossypol with a phloroglucinol/HCl assay (Liu et al. 1999) showed that gossypol equivalents was slightly lower in the *ds6-3* than in the wild-type leaves, but the difference was not significant (Fig. 4c). Therefore, different growth rates of the larvae on the control and transgenic *ds6-3* leaves were not due to variations of gossypol content. Because the P450

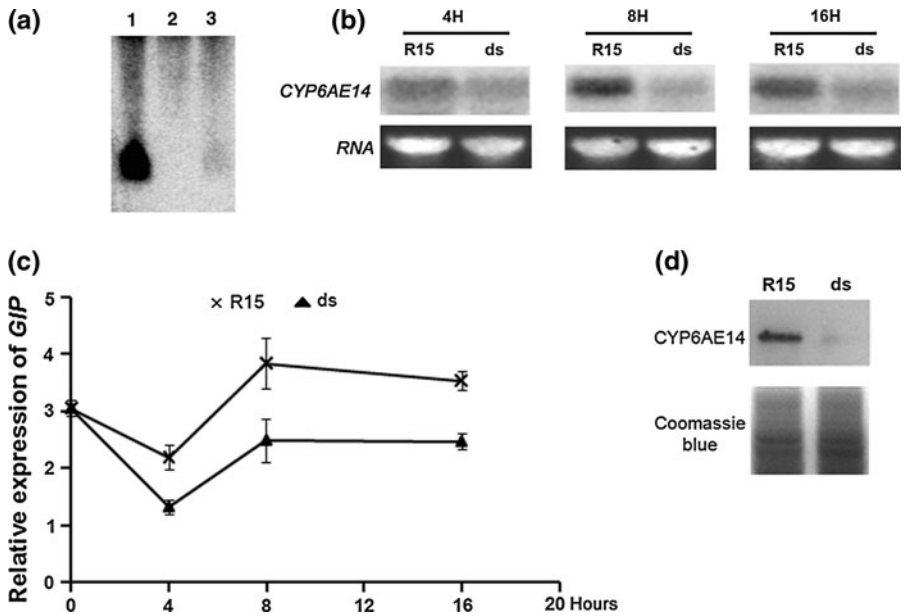

Fig. 3 Suppression of *CYP6AE14* expression in larvae fed on *ds6-3* T2 plants. **a** northern blot of the small RNAs of *CYP6AE14* in midgut of third-instar larvae fed on nontransgenic control R15 (lane 2) and *ds6-3* T2 plants (lane 3) for 3 days. *Lane 1*: total RNAs from *ds6-3* T2 plants as positive control. **b** and **c** northern blot (**b**) and qRT-PCR (**c**) analysis of *CYP6AE14* transcripts in midgut of second-instar larvae fed on control (R15) or *ds6-3* T2 (ds) plants for indicated time. **d** western blot detection of CYP6AE14 proteins in midgut of second-instar larvae fed on nontransgenic control R15 or *ds6-3* T2 plants for 3 days. Values are means ± SD

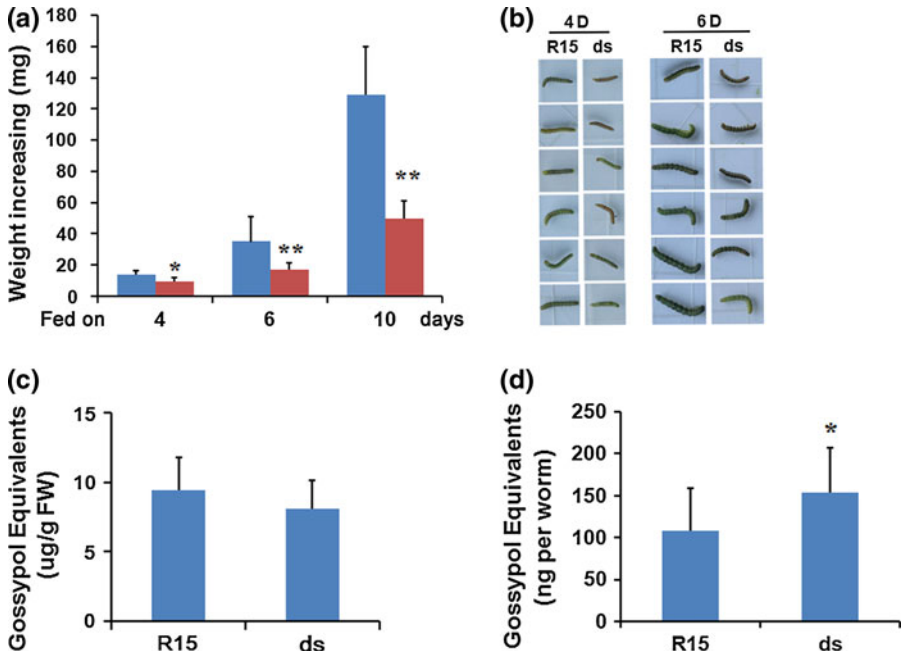

Fig. 4 Effect of *ds6-3* T2 plants on larvae growth. **a** Net weight increase of larvae fed on leaves of nontransgenic control R15 (*blue*) or *ds6-3* T2 (ds) plants (*red*) for indicated days. **b** Images of larvae that were fed on leaves of nontransgenic control R15 or *ds6-3* T2 plants for 4 and 6 days, respectively. **c** Gossypol equivalents in leaves of nontransgenic control R15 or *ds6-3* T2 plants. **d** Gossypol equivalents in midgut of the larvae fed on leaves of nontransgenic control R15 and *ds6-3* T2 plants, respectively, for 6 days. Values are means ± SD. $*P < 0.05$; $**P < 0.01$

enzyme of CYP6AE14 plays a key role in the bollworm response to gossypol-containing diet (Mao et al. 2007), we assumed that downregulation of *CYP6AE14* would impair gossypol detoxification in bollworms, thus resulting in accumulation of higher concentrations of gossypol in the larvae midgut. We found that gossypol equivalent was indeed higher (about 1.4-fold) in larvae fed on the *ds6-3* leaves compared with those fed on the wild-type leaves (Fig. 4d).

We have demonstrated that bollworms from the *dsCYP6AE14* cotton had lower growth rates and higher gossypol equivalents in midgut. Retarded growth could be caused by antifeedant effect of gossypol, resulting in less consumption of cotton tissues by the larvae. Alternatively, the consumption was not changed but the higher gossypol absorption inhibited bollworm growth. Leaf consumption assay was thus performed. Second-instar larvae were transferred to *ds6-3* leaves; during the first 3 days of the assay, ingestion of the *ds6-3* leaves was reduced by 21% (Fig. 5a). When measurement of larval leaf intake was extended to another 3-day period (days 4–6), a greater difference (44%) was observed (Fig. 5b).

Damage of bolls by bollworms causes severe quality and production losses of cotton. To test the protection of bolls by the RNAi transgenic cotton, larvae that previously lived on leaves from the wild-type and *ds6-3* leaves for 10 days were then transferred to bolls (about 9 days post anthesis, DPA). We found that, after 1 day, the larvae from the wild-type leaves burrowed into bolls and consumed the contents, whereas the larvae from the *ds6-3* leaves chewed only shallow gouges in the boll surface, whereas the boll contents were almost intact (Fig. 5c). Together, these data indicate that the transgenic *dsCYP6AE14* cotton plants were less damaged by cotton bollworms in comparison with the untransformed control.

Discussion

RNAi-triggered gene suppression through uptake of dsRNAs has been reported for many insect species, including those of Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, and Orthoptera (Huvenne and Smagghe 2010). It was shown that bacteria could be used to express dsRNA; after feeding a lepidopteran pest (*Spodoptera exigua*) with a bacteria-mixed diet, the target gene was silenced (Tian et al. 2009). To counteract plant nematodes, viruses were modified for dsRNA production in plant. RNAi was then observed in nematodes reared on the virus-infected plants (Valentine et al. 2007; Dubreuil

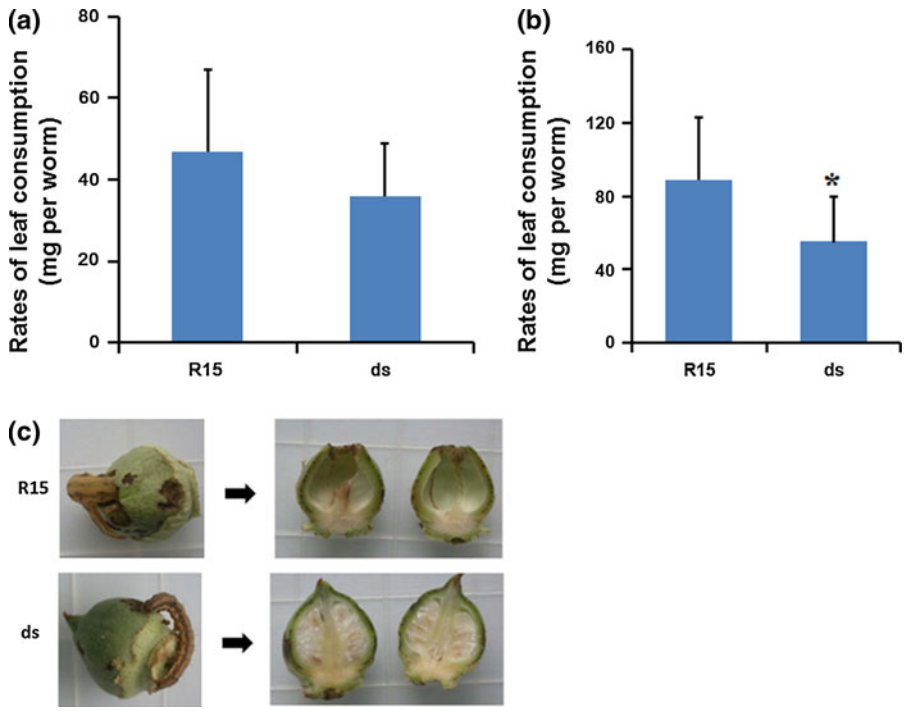

Fig. 5 Transgenic cotton plants were less damaged by bollworms than the control. Second-instar larvae were divided into 2 groups. Each group contained 40 individual larvae and were fed on control (R15) and *ds6-3* T2 (ds) leaves with similar conditions. **a** Consumption of leaves of control and ds6-3 T2 plants by second-instar larvae for the first 3 days. **b** Leaf consumption from the 4th to 6th day was recorded. Values are means ± SD. $*P < 0.05$. **c** Image of larvae on cotton boll. Larvae previously reared on leaves of nontransgenic control R15 or *ds6-3* T2 plants for 10 days were transferred to cotton boll for another day

et al. 2009). For sucking insects, RNA interference was successfully achieved by feeding insects with sugar solutions containing dsRNA synthesized in vitro (Bautista et al. 2009; Walshe et al. 2009; Zhou et al. 2008). Each of these approaches, however, suffers from certain limitations, such as low uptake efficiency, high cost, and inapplicability for field control of pests. Bacteria-based RNAi can be a convenient platform for functional genomics of some insect species, but this technology cannot produce plant with improved insect resistance. Viruses are highly efficient in triggering dsRNA production in plant, but the dsRNA production is not stable in offspring, and the heterogeneity in RNAi efficiency between virus-infected plants may limit its utilization. In this investigation, the introduced dsRNA of *CYP6AE14* was stably expressed not only in T1 but also in T2 generation, and enhanced resistance to bollworms was observed in both generations, suggesting that transgenic plant-based RNAi may be a feasible strategy for generating insect-proof plants.

Bt crops have been grown worldwide and offer a high degree of protection (Qaim and Zilberman 2003; Wu et al. 2008). At present, *Bt*-based strategies seem more effective than RNAi-based technology for agricultural pest control; however, recent reports of resistance to *Bt* toxins existing in field populations of insects (Bravo and Soberon 2008; Gahan et al. 2001; Tabashnik et al. 2008) call for new approaches. Utilization of RNAi in pest control offers an alternative. On the other hand, *Bt* insecticide proteins have no or little effects on sap-sucking homopteran pests such as aphids, leafhoppers, and whitefly (Price and Gatehouse 2008). Since gene suppression by ingested dsRNA has been reported in aphids (Whyard et al. 2009), and RNAi knockdown of a salivary transcript led to lethality in the pea aphid (Mutti et al. 2006, 2008), RNAi-based technology also may be a promising approach for protection of plants from sucking pests.

Growth inhibition of cotton bollworms due to ingestion of *dsCYP6AE14*-expressing plants occurred

only when gossypol was present in diet, demonstrating that genes involved in detoxification or defense reactions against plant secondary metabolites can serve as targets of pest control. This has the advantage of controlling specific pests that feed on a crop species producing a defined group of defensive compounds, minimizing effects on nontarget insects.

Insect P450 monooxygenases play a central role in adaptation to plant defense products (Scott et al. 1998; Zeng et al. 2009). The catalytic activities of most eukaryotic P450 monooxygenases rely on cytochrome P450 reductase, cytochrome B5, and cytochrome B5 reductase as partners (Feyereisen 1999; Yang et al. 2010). We noticed that the *dsCYP6AE14* cotton plant did have deleterious effects on bollworms, but was not lethal. If multiple genes involved in the P450 complex were targeted by RNAi, the deleterious effects would be magnified. As a recently developed genetic tool, further studies are needed to optimize RNAi-based strategies for crop protection.

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