Gossypol-enhanced P450 gene pool contributes to cotton bollworm tolerance to a pyrethroid insecticide

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Abstract
Cotton plants accumulate phytotoxins, including gossypol and related sesquiterpene aldehydes, to resist insect herbivores and pathogens. To counteract these defensive plant secondary metabolites, cotton bollworms (Helicoverpa armigera) elevate their production of detoxification enzymes, including cytochrome P450 monooxygenases (P450s). Besides their tolerance to phytotoxin, cotton bollworms have quickly developed resistance to deltamethrin, a widely used pyrethroid insecticide in cotton field. However, the relationship between host plant secondary metabolites and bollworm insecticide resistance is poorly understood. Here, we show that exogenously expressed CYP6AE14, a gossypol-inducible P450 of cotton bollworm, has epoxidation activity towards aldrin, an organochlorine insecticide, indicating that gossypol-induced P450s participate in insecticide metabolism. Gossypol-ingested cotton bollworm larvae showed higher midgut P450 enzyme activities and exhibited enhanced tolerance to deltamethrin. The midgut transcripts of bollworm larvae administrated with different phytochemicals and deltamethrin were then compared by microarray analysis, which showed that gossypol and deltamethrin induced the most similar P450 expression profiles. Gossypol-induced P450s exhibited high divergence and at least five of them (CYP321A1, CYP9A12, CYP9A14, CYP6AE11 and CYP6B7) contributed to cotton bollworm tolerance to deltamethrin. Knocking down one of them, CYP9A14, by plant-mediated RNA interference (RNAi) rendered the larvae more sensitive to the insecticide. These data demonstrate that generalist insects can take advantage of secondary metabolites from their major host plants to elaborate defence systems against other toxic chemicals, and impairing this defence pathway by RNAi holds a potential for reducing the required dosages of agrochemicals in pest control.

Keywords: Helicoverpa armigera, insecticide tolerance, midgut, P450, plant secondary metabolite, transcriptome

Received 30 July 2011; revised received 7 February 2012; accepted 16 February 2012

Introduction
Plants biosynthesize diverse classes of secondary metabolites, and many of them function as defence chemicals against herbivores and pathogens (Grayer & Kokubun 2001; Howe & Jander 2008). In plants of the Cruciferae family, including Arabidopsis thaliana, glucosinolates are synthesized to enhance their immunity to herbivores (Halkier & Gershenzon 2006). Plants of the Umbelliferae and Rutaceae families are rich in furanocoumarins which are toxic to insects (Curini et al. 2006). In cotton plants, gossypol and related sesquiterpene aldehydes are the major secondary metabolites, which have been shown to possess fungistatic properties and insecticidal activities (Stipanovic et al. 2006; Wang et al. 2009).

To detoxify or tolerate various toxic compounds from plants or other sources, insects have developed...
sophisticated defence systems, including detoxification enzymes, particularly the cytochrome P450 monoxygenases (P450s) (Feyereisen 2005; Li et al. 2007; Schuler 2011). As a huge superfamily, P450s are able to catalyse various modification reactions, such as oxidation, epoxidation, O- and N-dealkylation, dehydrogenation and others (Meunier et al. 2004). These biochemical characters revealed that insect P450s have activities of metabolizing a broad spectrum of chemical compounds. Up to now, a large number of detoxification-related P450s from insects have been isolated and identified, of which many belong to the CYP6 family (Schuler 2011). For instance, CYP6B1 from black swallowtail (Papilio polyxenes) metabolized xanthotoxin, a major phytotoxin in its host plant parsnip (Pastinaca sativa) (Wen et al. 2003); CYP6A1 from house fly (Musca domestica) was shown to epoxidize a variety of plant terpenoids (Andersen et al. 1997); and CYP6AS from honey bee (Apis mellifera) could metabolize the flavonoid quercetin (Mao et al. 2009).

It has been demonstrated that, in response to toxic plant secondary metabolites, insects upregulate P450 genes so as to modulate their defence states (Schuler 2011). The P450 activities involved in alkaloid metabolism were enhanced in Drosophila larvae and adults after ingesting the cactus alkaloids-supplemented diets (Frank & Fogleman 1992; Danielson et al. 1994). In Papilio species such as black swallowtail, xanthotoxin induced the expression of P450s, which were responsible for the metabolism of this phytotoxin (Cohen et al. 1989). There are also P450 genes, such as Helicoverpa zea CYP6B8 and CYP321A1, that could be induced by and metabolize a broad range of compounds (xanthotoxin, angelicin, α-naphthoflavone), as well as chemical insecticides (cypermethrin, diazinon and aldrin) (Sasabe et al. 2004; Rupasinghe et al. 2007). Accordingly, H. zea showed cross-resistance to cypermethrin after xanthotoxin ingestion (Li et al. 2000a). Thus, insects respond to host plant secondary metabolites and modulate their defence states to survive in the toxic living environment.

Specialist herbivorous insects encounter relatively limited toxic secondary metabolites in their life cycle because they feed primarily on one or several closely related plant species. In contrast, generalist insects might encounter a wider range of toxins depending on host plant choice. Despite the adverse effect of gossypol, cotton bollworms, a generalist species, accommodate themselves to live on gossypol-supplemented cotton plants (Wu & Guo 2005), and populations of this species can be found also in other plants, including crop species of the Brassicaceae, Fabaceae and Solanaceae families. Accordingly, cotton bollworms ingest diverse plant secondary metabolites from their host plants (Hanny 1980; Li et al. 2002a).

Pyrethroids are effective insecticides in pests control and have played an important role in reducing crop loss by insect herbivores. However, high frequency usage of insecticides leads to environmental pollution and lowered insecticides efficiency due to the development of insect resistance. Cotton bollworms were found to have developed resistance to pyrethroids (Kranthi et al. 2002; Torres-Vila et al. 2002). Comparison of gene expression levels in pyrethroid sensitive and resistant strains showed that pyrethroid resistance was always accompanied with overexpression of multiple P450 genes (Yang et al. 2006; Heckel et al. 2008; Brun-Barale et al. 2010). Moreover, it was also found that insecticide resistance was different in populations fed on different host plant species (Xie et al. 2011), suggesting host plants and their second metabolites could influence insect susceptibilities to insecticides. As the main phytotoxin in cotton, gossypol may play a major role in shaping the defence states of cotton bollworms.

In this investigation, we first performed the toxicological experiments to compare the deltamethrin tolerance rates between the control and the gossypol-pretreated larvae. Second, we measured the midgut detoxification enzyme activities towards specific substrates to illustrate the biochemical properties of the gossypol stimulation of insecticide tolerance. Third, we analysed expression levels of 6,490 genes in midgut of cotton bollworm in response to different plant secondary metabolites and deltamethrin by using cDNA microarray, followed by qRT–PCR confirmation which was focused on specific P450 genes involved in deltamethrin tolerance. Last, plant-mediated RNAi was applied to downregulate CYP9A14, which reduced the larval tolerance to deltamethrin.

Materials and methods

Plants and insect culture

Plants of cotton (Gossypium hirsutum L. cv. CCRI 12), including a pair of glanded and glandless cotton isogenic lines, namely CCRI 12-glanded and CCRI 12-glandless, were grown under 28–30 °C and 60–80% relative humidity. The second and third leaves from top (young leaves) were used for insect feeding.

Plants of Arabidopsis thaliana (ecotype Col-0) were grown in presterilized soil under at 22 °C on a 16-h day/8-h night cycle, and rosette leaves of the 1-month-old plants were used for feeding assay.

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, and the larvae
were fed on a modified artificial diet, as previously described (Mao et al. 2007).

Chemicals

Gossypol, xanthotoxin, tannic acid, quercetin, ethoxy-coumarin, 7-hydroxycoumarin, NADPH, p-nitroanisole, p-nitrophenol and Fast Blue RR salt were purchased from Sigma, α-naphthyl acetate and naphthol from Ruibio, deltamethrin from AccuStandard, aldrin and diel- drin from Riedel-dehaën, and β-pinene from Fluka.

Preparation of chemical-supplemented diets

To prepare the chemical-supplemented diets, the comp-ound to be tested was first dissolved in dimethyl sulfo-oxide (DMSO), and then added to the artificial diet before the solidification of agar (40–45 °C), mixed gently and decanted into a new dish. The control diet was prepared by adding the same volume of DMSO to artificial diet.

Analysis of deltamethrin tolerance of the larvae

The late 2nd instar larvae (weighted 5–7 mg each) were fed first on artificial diet supplemented with (Gos) or without (CK) 1.0 mg/g gossypol for 1 day, followed by recording the larval weight and transferring to diet contain-ting 5 μg/g deltamethrin (a sublethal concentration) for another day, the net weight increase was recorded. Alternatively, late 3rd instar larvae and leaves of gland-ed/glandless cotton cultivars were used to replace the late 2nd instar larvae and CK/Gos diet, respectively. Statistics was performed with Student’s t-test (α = 0.05), and results are shown as mean ± SD.

For toxicological analysis, the gossypol-pretreated early 3rd instar larvae (10–15 mg) were divided into five groups, each group contained 90 larvae (triplicates of 30 larvae) for five concentrations of deltamethrin-supplemented diets (20, 40, 80, 120, 140 μg/g), respec-tively. After 1 day, the dead larva was judged using a toothpick, which would show no response under touch- ing, and the number of deaths was recorded. Mortality rate is the percentage of death in each group. Statistic analysis of mortality data was performed with SPSS program, Mann–Whiney U test (α = 0.05). Regression analysis of the relationship between mortality and concentra-tions/doses of deltamethrin was established with EXCEL. LC₅₀ indicates the deltamethrin concentration lethal to half members of the group after 1 day.

Alternatively, LD₅₀ was measured as previously described (Brun-Barale et al. 2010). The 3rd instar lar-vae, which had been placed on glanded and glandless cotton leaves, respectively, were divided into five groups for topical application with five doses (6.25, 12.5, 18.75, 25, 31.25 ng per larva) of deltamethrin, respectively. After applying the deltamethrin solution (in acetone) to the thorax of the larva, control artificial diet was then added to keep feeding, the mortality was recorded after 1 day. LD₅₀ here indicates the deltameth- rin dose (ng per larva) lethal to half members of the group after 1 day.

Enzyme activity assay

To assay detoxification enzyme activities, total midgut proteins from gossypol- or deltamethrin-treated larvae were used. The early 4th instar larvae (20–25 mg each, 18–24 larvae) were reared on diet containing 1.0 mg/g gossypol or no gossypol (control) for 1 day, then the midgut was taken for further analysis. Alternatively, early 3rd instar larvae (7–12 mg each, 100–120 larvae) were reared on diet containing 5 μg/g deltamethrin for 7 day, then all the survival individuals were sorted into three groups in descending order of weight. The same stage instar larvae grown on artificial diet were chosen as control, and the midgut was taken for further analy-sis.

To prepare the midgut total proteins, two or three midguts were placed in a 1.5-mL eppendorf tube and homogenized in 500 μL of ice-cold homogenization buffer (0.1 M sodium phosphate buffer, pH 7.8, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF). The homogenate was centrifuged at 4 °C, 2400 × g for 10 min. The supernatant of each treatment was used immediately for enzyme assays. Protein content was determined by a Bradford dye reagent (Sigma) using bovine albumin as a standard.

Assays of O-demethylation of p-nitroanisol (PNOD) (Hansen & Hodgson 1971), O-deethylation of 7-ethoxy- coumarin (ECOD) (Ullrich & Weber 1972) and aldrin epoxidation (AE) (Lee & Scott 1989) activities by P450 monoxygenases were performed according to previ-ously published procedures with slight modifications.

For assay of P450 PNOD activities, 2 μL of 15 mg/mL p-nitroanisole was added to 200 μL total proteins (~200 μg), the mixture was incubated for 3 min at 30 °C, and the reaction was initiated by the addition of 30 μL of 10 mM NADPH. After 30 min, ethanol (200 μL) was added to precipitate the protein and stop the reaction. For blank control, proteins were added after etha-nol to account for the absorbance value of each sample. The tubes were centrifuged at 2400 × g for 10 min, and the supernatant was read in a spectrophotometer at 405 nm. The change in absorbance was calculated from the difference between the sample absorbance and the absorbance of each blank control. A p-nitrophenol stan-dard curve was used to obtain the molar extinction
coefficients. The extinction coefficient for p-nitrophenol in this assay was 15.91 ms/cm. The activity is expressed as nmole p-nitrophenol per min per mg protein.

For P450 ECOD, 2 µL of 5 mg/mL ethoxycoumarin was added to the reaction mixture. After reaction, the product was measured with a luminescence spectrometer (PerkinElmer LS50B) at an excitation wavelength of 380 nm (slit 5 nm), and an emission wavelength of 460 nm (slit 2.5 nm). The 7-hydroxycoumarin standard was used to convert absorption into concentration. The activity is expressed as pmole 7-hydroxycoumarin per min per mg protein.

For P450 AE, 2 µL of 10 mg/mL aldrin was added to the reaction mixture. After reaction, the product of dieldrin was extracted twice by 500 µL of redistilled hexane and detected in a gas chromatography/mass spectrometry system as described (Lee & Scott 1989). A dieldrin standard curve was used to convert peak area into mass of product. The activity is expressed as pmole dieldrin per min per mg protein.

For glutathione S-transferase (GST) towards 3,4-dichloronitrobenzene (DCNB), a GSH-ST detection kit (Nanjing Jiancheng Bioengineering) was used, in which GST catalyses the conjugation of γ-glutathione (GSH) to DCNB through the thiol group of the glutathione. After reaction at 37 °C for 10 min, the remaining GSH was then detected by reaction with the general thiol reagent (5-5-dithiobis[2-nitrobenzoic acid], DTNB) to form the 412 nm chromophore, 5-thio-nitrobenzoic acid (TNB), as described (Teitze 1969). The activity is expressed as nmole GSH decreased min/mg/protein.

For choline esterase (ChE) towards acetylcholine, a ChE detection kit (Nanjing Jiancheng Bioengineering) was used. After incubation at 37 °C for 10 min, the degradation product of acetylcholine by ChE, choline, was detected by reaction with DTNB for 6 min, and the absorbance was measured at 412 nm. The activity is expressed as nmole choline per min per mg protein.

For esterase activity towards a-naphthyl acetate (α-NA), the assay was conducted as previously described (Asperen 1962; Yang et al. 2004). Twenty microlitre of total proteins (~80 µg) was added to 300 µL of sodium phosphate buffer (0.1 M pH 7.6) in silica cuvette, then 200-µL substrate solution (5 mL 0.1 M pH 7.6 phosphate buffer, 10 mg Fast Blue RR salt and 0.1 mL 100 mM α-NA) was added, mixed gently, and the absorbance was immediately measured once every 15 s at 450 nm and the recording lasted for 2 min. The mixture without protein was used as blank control. A naphthol standard curve was used to convert absorption into concentration. The activity is expressed as nmole naphthol per min per mg protein.

In vitro assay of deltamethrin metabolism

For the assay of deltamethrin metabolism, the early 4th instar larvae (20–25 mg each, 18–24 larvae) were reared on galled or glandless cotton for 1 day; then, the midguts of larvae were taken.

Three microlitre of 5 mg/mL deltamethrin (dissolved in methanol) and 30 µL of 10 mM NADPH were added to 300 µL protein solution (~800 µg), the mixture was incubated at 30 °C for 30 min and stopped by adding 125 µL 2 N HCl. Permethrin was added after terminating the reactions and used as an internal standard for normalizing the slight variations in extraction. The remaining deltamethrin was then extracted and detected by reverse phase HPLC as described (Sasabe et al. 2004). Deltamethrin disappearance was calculated by subtracting the remaining substrate from zero-time controls. The activity is expressed as nmole deltamethrin disappearance per min per mg protein.

Microarray analysis

Six different compounds or galled and glandless cotton leaves were used for treatments. Late 3rd instar larvae (15–20 mg, 18–24 larvae) fed on artificial diets containing 1.0 mg/g gossypol, 0.5 mg/g xanthotoxin, 1.0 mg/g tannic acid, 1.0 mg/g quercetin, 1.0 mg/g β-pinene, 5 µg/g deltamethrin and 1.0 mg/g DMSO as control, respectively. Alternatively, early 4th instar larvae were reared on leaves of the galled and the glandless cotton cultivars. Each treatment had three biological replicates. One day later, the midguts were obtained for RNA extraction and gene expression analysis.

Total RNAs were extracted by Trizol reagent (Invitrogen). RNA samples prepared from cotton bollworm midguts were sent to Shanghai Biochip for 454 sequencing. From the sequencing results, 6490 contigs were chosen for generating microarray. cDNA and cRNA preparations from total RNA, labelling and fragmentation of the cRNA, and hybridization were carried out by NimbleGen Systems as described (Peterson & Freeman 2009). The microarray hybridization was performed in triplicates for each experiment. Significance analysis of microarrays (SAM) was performed to screen differentially expressed genes, in which a false discovery rate (FDR) with q = 0.05 was employed (Tusher et al. 2001). Genes with a q-value ≤0.05 and a relative fold change in expression ≥2 or ≤0.5 were chosen for further analysis. Hierarchical clustering of differentially expressed genes was performed with Cluster 3.0 program using the correlation (uncentered) similarity metric and average linkage clustering method; the resulting tree-images were visualized using JAVA TREEVIEW.

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Quantitative real-time PCR

The first-strand cDNA was synthesized by a reverse transcription kit (Invitrogen). Primers used were shown in Table S3 (Supporting information), and 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 denaturing at 95 °C for 20 s and annealing/extension at 60 °C for 30 s. A housekeeping gene actA3b (GenBank No. X97615.1) was used to normalize the gene expression levels.

Vectors, plant transformation and RNAi through dsRNA ingestion

Vectors for double-strand RNA (dsRNA) expression were constructed as previously described (Mao et al. 2007). The pBSK-intron vector, a pBluescript II SK vector (Strategene) containing a 120-nucleotide intron of A. thaliana RTM1 gene between the Not I and Xba I sites, was used as a backbone for dsCYP9A14 construct. Sense and antisense CYP9A14 fragments with restriction enzyme sites at both ends were obtained by PCR amplification of H. armigera cDNA clones with primer pairs (Table S3, Supporting information). The two PCR fragments were inserted at inverted repeats into the corresponding sites of pBSK-intron vector. The generated dsCYP9A14 construct was then used to replace GUS in pBI121 (Clontech) to generate the Pro35S:: dsCYP9A14 construct. Sense and antisense CYP9A14 fragments with restriction enzyme sites at both ends were obtained by PCR amplification of H. armigera cDNA clones with primer pairs (Table S3, Supporting information). The two PCR fragments were inserted at inverted repeats into the corresponding sites of pBSK-intron vector. The generated dsCYP9A14 construct was then used to replace GUS in pBI121 (Clontech) to generate the Pro35S:: dsCYP9A14 construct. The final RNAi vector was introduced into Agrobacterium tumefaciens strain GV3101. Transgenic plants of Arabidopsis were generated by a floral dip method (Clough & Bent 1998), screened on half-strength MS agar medium containing 30 μg/mL kanamycin.

Analysis of dsRNA expression levels was performed on T1 plants. For dsRNA ingestion, late 2nd instar larvae (4–6 mg each, 180–200 larvae) were placed in six-well plate and fed with leaves of the wild type (WT) or the T2 plants of dsCYP9A14 transgenic line (ds-8) of Arabidopsis for 4–5 days, followed by assay of deltamethrin tolerance as described previously.

Results

Gossypol ingestion enhances cotton bollworm tolerance to deltamethrin

To find out the effect of plant secondary metabolites on insecticide tolerance, we examined the involvement of gossypol in cotton bollworm tolerance to deltamethrin, a widely used pyrethroid insecticide in cotton field. We first determined the effect of gossypol treatment prior to deltamethrin exposure. The late 2nd instar larvae of H. armigera were divided into two groups, one was fed on gossypol-free (control) diet and the other on diet supplemented with gossypol (1.0 mg/g) for 1 day, respectively, before transferring to a sublethal concentration of deltamethrin (5 μg/g) for another day. We found that all the gossypol-pretreated larvae survived, whereas 9% of the control died. Furthermore, gossypol-pretreated larvae exhibited faster weight increase than the control after transferring to deltamethrin-added diet (Fig. 1A), as well as the reduced mortality under a series of deltamethrin-supplemented diets (Fig. 1B). We then used gossypol-containing and glandless (gossypol-free) cotton cultivars for comparison, which showed similar consequences (Fig. 1C), and the deltamethrin dose lethal to 50% (LD50) of the gossypol-containing 3rd instars was ~28.6 ng per larva, about 1.5-fold increase from that of the larvae from the glandless cotton (Fig. 1D). In addition, we used the 4th instar larvae for assay of deltamethrin metabolism activities of midgut proteins, which showed that the larvae fed on gossypol-contaminated cotton leaves had a deltamethrin diminishing rate twice as high as that of the larvae on glandless cotton leaves (Fig. 1E). These results indicate that gossypol present in diet elevated the level of bollworm tolerance to deltamethrin.

Gossypol induces cotton bollworm midgut P450 activities

Cytochrome P450 monooxygenases, glutathione S-transferases and esterases constitute the three major detoxification enzymes of insects. To analyse biochemical properties of the gossypol induction of deltamethrin tolerance, activities of these detoxification enzymes in midgut were analysed. In the assay of GST towards DCNB, no obvious differences of midgut activities were observed between the gossypol-treated and the control larvae (Table 1). For the total esterases towards α-NA, the activities in the gossypol-treated group were increased by about 1.3-fold from the control (Table 1). For P450 monooxygenase activities, the PNOD and the AE activities in the gossypol-treated group were increased by 1.6- and 1.7-fold, respectively (Table 1). Previously, we isolated a gossypol-inducible P450 gene, CYP6AE14 (Mao et al. 2007). Baculovirus-mediated expression demonstrated that the CYP6AE14 protein metabolized aldrin, an organochlorine, into dieldrin (Fig. S2, Supporting information). ChE towards acetylcholine, which was not considered a detoxification reaction, was also examined. We found that the gossypol treatment did not show a clear effect on the ChE activity (Table 1). Together, these data demonstrate that gossypol ingestion elicited a clear elevation of midgut esterase, and predominantly, the P450 monooxygenase activities.
Fig. 1 The effect of gossypol on cotton bollworm tolerance to deltamethrin. (A) Net weight increase in gossypol-pretreated larvae on deltamethrin-supplemented diet. The late 2nd instar larvae had previously fed on control (CK) or 1.0 mg/g gossypol-supplemented (Gos) diet for 1 day; after recording the initial weight, two independent groups of each treatments were transferred to 5 μg/g deltamethrin-supplemented (Del) and CK dietary, respectively, weight increases were recorded 1 day later. Error bars represent standard deviation. Different letters indicate significant differences (Student’s t-test, \( P < 0.05 \)). (B) Mortality of gossypol-pretreated larvae under different concentrations of dietary deltamethrin. The early 3rd instar larvae grown on CK or Gos diet were randomly divided into five groups and fed with artificial diet containing 20, 40, 80, 120, 140 μg/g deltamethrin, respectively. Mortality was recorded after 1 day. For clarity, only the positive (CK) or negative (Gos) SD is shown. Statistical significance of mortality was determined with Mann–Whiney U test: \( P = 0.346, P = 0.106, P = 0.049, P = 0.049 \) and \( P = 0.037 \) for Gos vs. the corresponding CK under 20, 40, 80, 120, 140 μg/g deltamethrin, respectively. LC_{50} indicates the deltamethrin concentration lethal to half members of the group after 1 day. The LC_{50} (μg/g) was \( \sim 120 \) for ‘Gos’ and \( \sim 100 \) for ‘CK’. (C) Net weight increase in glanded and glandless cotton-pretreated larvae on deltamethrin-supplemented diet. Experiments were performed as described in A except that the late 3rd instar larvae and glanded/glandless cotton cultivars were used to replace the late 2nd instar larvae and CK/Gos diet, respectively. Error bars represent standard deviation. Different letters indicate significant differences (Student’s t-test, \( P < 0.05 \)). (D) Mortality of the larvae pretreated with glanded and glandless cotton leaves under topical application of different doses of deltamethrin. The glanded and glandless cotton cultivar leaves were used to replace CK/Gos diet as described in B, and instead of dietary deltamethrin, a series doses of deltamethrin (6.25, 12.5, 18.75, 25, 31.25 ng) were topically applied to the thorax of the larva. Statistical significance of mortality was determined with Mann–Whiney U test: \( P = 0.031, P = 0.049, P = 0.048, P = 0.127 \) and \( P = 0.048 \) for glanded vs. the corresponding glandless under 6.25, 12.5, 18.75, 25, 31.25 ng deltamethrin/ larva, respectively. LD_{50} indicates the deltamethrin dose lethal to half members of the group after 1 day. The LD_{50} (ng per larva) was \( \sim 28.6 \) for ‘Glanded’ and \( \sim 19.4 \) for ‘Glandless’. (E) Deltamethrin metabolism activities of midgut proteins. Total proteins were isolated from 4th instar larvae fed on glanded or glandless cotton leaves for 1 day. The activity is expressed as nmole deltamethrin diminished per min per mg protein. Mann–Whiney U test: \*\( P < 0.05 \).
We then tried to identify which detoxification enzyme system took the major responsibility for deltamethrin tolerance. The 3rd instar larvae were first placed on deltamethrin-supplemented diet (5 \( \mu \)g/g); 7 days later, the survival individuals were divided into three groups based on larval weights (Fig. 2A), and the enzyme activities of each group were then determined. In comparison with the control group (CK), the group with the best growth conditions (GI) showed different degrees of elevation in midgut enzyme activities. While the esterase \( \alpha \)-NA activity in GI was \( \sim \)1.5-fold higher (Fig. 2C), the P450s exhibited a more drastic increase in activities: the ECOD activity was increased by \( \sim \)1.9-fold, the PNOD and the AE activities by as much as 3.2-fold in comparison with the control (Fig. 2D). Furthermore, the larval growth of the deltamethrin-treated groups showed a high degree of correlation with the level of P450 activities (Fig. 2A,D). Four groups of larvae with ranged weights from control diet were also tested in parallel, which exhibited low correlation between the enzyme activity and the larval growth (Fig. S3, Supporting information). These results suggest that midgut

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<th>GST-DCNB (nmole per min per mg pro)</th>
<th>Esterase-( \alpha )-NA (nmole per min per mg pro)</th>
<th>P450 PNOD (nmole per min per mg pro)</th>
<th>P450 AE (nmole per min per mg pro)</th>
<th>ChE (nmole per min per mg pro)</th>
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<td>Gos</td>
<td>31.36 ± 3.05 a</td>
<td>193.96 ± 19.40 a</td>
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<td>CK</td>
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<td>153.24 ± 16.26 b</td>
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Proteins were isolated from midguts of the 4th instar larvae grown on 1.0 mg/g gossypol (Gos)-supplemented or control (CK) diets for 1 day. Each assay was repeated three times, the results are shown as mean ± SD, and the different letters followed indicate significantly different (\( P < 0.05 \), Mann–Whiney U test). Ratio = mean in Gos/mean in CK. The results were consistent in at least three independent experiments.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** Activities of major detoxification enzymes of deltamethrin-treated cotton bollworm larvae. (A) Groups of differently weighted larvae survived from deltamethrin treatment. The 3rd instar larvae grown on 5 \( \mu \)g/g deltamethrin-containing diet for 7 days; then, the survival individuals were divided into three groups (GI–GIII) based on the larval weight, and the same stage instar larvae grown on artificial diet were chosen as control (CK). Error bars represent standard deviation. Different letters indicate significant differences (Student’s t-test, \( P < 0.05 \)). (B) GST activity towards DCNB. Ratios of each group against control (CK) are given at the bottom. (C) Esterase activity towards \( \alpha \)-NA. (D) P450 activity towards different substrates. Left to right: P450 ECOD, P450 PNOD and P450 AE.

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P450s play a key role in the bollworm tolerance to deltamethrin.

Gossypol and deltamethrin elicit similar expression profiles of midgut P450s

To illustrate the gossypol-induced defence reactions at gene expression level, microarrays were used to analyse the changes in transcript abundance in midgut in response to five phytochemicals (gossypol, xanthotoxin, tannic acid, quercetin and β-pinene) and the insecticide deltamethrin. The differentially expressed midgut genes (Fold Change ≥2 or ≤0.5, q-value ≤0.05) were then picked up for further analysis (Table S1, Supporting information). Among the 6490 genes analysed, gossypol upregulated 98 and downregulated 55, whereas deltamethrin upregulated 120 and downregulated 80, respectively. Of these, 67 genes were co-regulated by deltamethrin and gossypol (Fig. 3A). In our experiments, xanthotoxin had a drastic effect on the regulation of midgut gene expression, with the number of up- and downregulated genes being 190 and 126, respectively, of which 126 were co-regulated by deltamethrin and xanthotoxin (Fig. 3A). The other three compounds exhibited a less effect on gene expression (Fig. 3A). In total, there were ~500 genes that responded to the six chemical treatments. Gene Ontology (GO) analysis of these 500 differentially expressed genes revealed that the majority took part in the metabolic processes and cellular processes (Table S1, Supporting information).

We then focused on the differentially regulated detoxification enzymes. The microarray analysis totally uncovered 37 differentially regulated putative P450 genes (Tables 2 and S2, Supporting information), which belong to CYP6A, CYP6B, CYP9A, CYP337B and CYP321A subfamilies. Deltamethrin, xanthotoxin and gossypol upregulated 22, 20 and 20 putative P450 genes, respectively, whereas the numbers of upregulated P450s by tannic acid, quercetin and β-pinene were only 6, 5 and 5, respectively (Tables 2 and S2, Supporting information). Among these P450 genes, some were previously reported: CYP321A1 metabolized a variety of phytochemicals and insecticides (xanthotoxin, angelicin, α-NF, cypermethrin, diazinon and aldrin) (Sasabe et al. 2004; Rupasinghe et al. 2007), CYP9A subfamily members and CYP6B7 were found to be overexpressed in pyrethroid resistance strains (Yang et al. 2006), and CYP6AE14 was shown to participate in gossypol metabo
tolerance (Mao et al. 2007). There were also multiple new putative P450s differentially expressed in midgut in response to chemical treatments (Table S2, Supporting information). These data clearly indicate that expression levels of multiple P450s in cotton bollworm midgut changed significantly in response to plant secondary metabolites of xanthotoxin and gossypol, as well as to the artificial pesticide deltamethrin.

Cluster analysis of differentially expressed genes revealed that the P450s expression profile induced by deltamethrin was most similar to that of gossypol (Fig. 3B). Xanthotoxin also significantly induced multiple P450s (Fig. 3B and 4A, Table S2, Supporting information), which explains the higher deltamethrin tolerance of the bollworm after xanthotoxin pretreatment (Fig. S1C, Supporting information). However, xanthotoxin ingestion resulted in more severely retarded larval growth than the other phytochemicals tested (Fig. S1C, Supporting information), indicating that this furanocoumarin is highly toxic to cotton bollworm. There were totally 21 differentially regulated putative esterase genes (Table S2, Supporting information), and their expression profile elicited by deltamethrin was most similar to that by xanthotoxin (Fig. S4, Supporting information). For GSTs, substantially fewer genes were differentially regulated (Tables 2 and S2, Supporting information).

As glanded cotton leaves had a similar or better effect on enhancing deltamethrin tolerance of cotton bollworms than diet-added gossypol (Fig. 1), subsequent analysis used leaves of the glanded and the glandless cotton cultivars for feeding. Compared with those on glandless cotton, the larvae grew on glanded cotton leaves had 51 up- and 12 downregulated genes (Table S1, Supporting information). Of these differentially expressed genes, most were annotated to participate in metabolism: 12 P450s, 10 dehydrogenases, nine esterases and two GSTs were upregulated, and one P450, one dehydrogenase and five proteases were downregulated (Table 3). Similar to the results in the gossypol-supplemented artificial diet treatment, the differential expressed P450 genes, which were corroborated by qRT–PCR (Fig. 3C and 4B), were classified into CYP321A, CYP337B, CYP6AE, CYP6B and CYP9A subfamilies, five of them, including CYP337B1,

| Table 2 Detoxification enzyme genes differentially expressed in cotton bollworm midguts in response to deltamethrin and five phytochemicals |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Treatment | Del | Gos | Xan | Tac | Que | bpi | Total |
| Up | P450 | 22 | 20 | 20 | 6 | 5 | 20 |
| Esterase | 5 | 5 | 6 | 4 | 2 | 8 | 16 |
| GST | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Down | P450 | 3 | 0 | 1 | 2 | 3 | 1 |
| Esterase | 4 | 0 | 4 | 3 | 0 | 0 | 5 |
| GST | 2 | 0 | 1 | 0 | 0 | 0 | 3 |

Microarray results showing cotton bollworms response to deltamethrin (Del), xanthotoxin (Xan), gossypol (Gos), tannic acid (Tac), quercetin (Que) and β-pinene (bpi). In total, 37 putative P450s, 21 esterases and four GSTs genes were found differentially expressed after treatments. Up: genes ≥2-fold upregulated; Down: genes ≥2-fold downregulated. For details, see Table S2.

| Table 3 Functional groups of differentially expressed genes in midguts of the larvae fed on glanded and glandless cotton leaves |
|-----------------|-----------------|-----------------|-----------------|
| Gene Description | GO-terms | Number | (Function) |
| Biosynthesis & Metabolism | | | |
| Cytochrome P450 | Monoxygenase activity | 13 | (1) |
| Dehydrogenase | Oxidoreductase activity | 11 | (1) |
| Esterase | Hydrolase activity | 9 | (0) |
| Trypsin-like protease | Endopeptidase activity | 5 | (5) |
| Glutathione S-transferase | Transferase activity | 2 | (0) |
| UDP-glucuronosyltransferase | Transferase activity | 2 | (0) |
| Cysteine dioxygenase | Oxidoreductase activity | 1 | (1) |
| Glucose dehydrogenase | Oxidoreductase activity | 1 | (1) |
| 3-oxyacyl-(acyl-carrier-protein) reductase | Oxidoreductase activity | 1 | (0) |
| Juvenile hormone epoxide hydrolase | Ether hydrolase activity | 1 | (0) |
| Glutamate cysteine ligase | Ligase activity | 1 | (0) |
| Stress response protein | | | |
| Oxidative stress protein | Zinc ion binding activity | 1 | (0) |
| Heat shock protein | Stress response | 1 | (0) |
| Transporter | | | |
| Sugar transporter | Transporter activity | 1 | (1) |
| ABC transporter | ATPase/transporter activity | 2 | (0) |
| Organic cation transporter | Transporter activity | 1 | (0) |
| Storage protein | | | |
| Arylphorin precursor | Nutrient reservoir activity | 2 | (0) |
| mRNA processing | Pre-mRNA splicing regulation | 1 | (0) |
| WW domain-binding protein | | | |
| Others | | | |
| Total | | 63 | (12) |

Differentially expressed genes are ≥2-fold upregulated or ≥2-fold downregulated. The numbers in brackets indicate the downregulated genes.
CYP6AE11, CYP6B7, CYP9A12 and CYP9A14, were previously reported to be overexpressed in some cotton bollworm field strains with different levels of pyrethroid resistance (Yang et al. 2006; Heckel et al. 2008; Brun-Barale et al. 2010). Moreover, the analysis identified five new putative P450 genes (JN176568, JN176569, JN176570, JN176571, JN176573), which were differentially expressed (Fig. 3C, Table S2, Supporting information). All these data suggest that gossypol exerts a prominent effect on changing oxidation-reduction states mainly through induction of P450s and dehydrogenases.

Multiple gossypol-inducible P450s participate in deltamethrin tolerance

To determine a further link between the expression of detoxification genes and cotton bollworm deltamethrin tolerance, transcript levels of 22 differentially expressed P450s and eight esterase genes in each larva were compared with the larval growth in the deltamethrin-supplemented diet. The transcript levels of eight of 22 P450s had close correlations with larval growth, including four CYP9A and two CYP6B subfamily members, CYP6AE11 and CYP321A1 (Fig. 5). Interestingly, five of them (CYP321A1, CYP9A12, CYP9A14, CYP6AE11 and CYP6B7) were upregulated by glanded cotton (Fig. 3C and 4B). The transcript levels of the other 14 P450s had relatively lower correlations with larval growth (Fig. S5, Supporting information). For esterase genes, three of eight had a relatively higher regression coefficient than the other five but still much lower than that of the eight P450s mentioned earlier (Fig. S5, Supporting information). These data suggest that the cotton bollworm resistance to pyrethroid insecticide tolerance involves multiple P450s and not a single detoxification gene.

RNA interference to reduce the bollworm deltamethrin tolerance

Previously, we showed that silencing CYP6AE14 gene of cotton bollworm by plant-mediated RNAi impaired larval tolerance of gossypol, providing a novel strategy for field control of insect pests (Mao et al. 2007, 2011). As gossypol-induced genes are also related to deltamethrin tolerance, we anticipated that downregulation of P450 genes responsive to both gossypol and deltamethrin by RNAi might create hypersensitivity of the insect to deltamethrin. To test this hypothesis, we generated transgenic Arabidopsis plants expressing dsRNA against CYP9A14 (dsCYP9A14). CYP9A14 was chosen because it was overexpressed in the pyrethroid resistance strains (Yang et al. 2006) and its expression was inducible by both gossypol and deltamethrin (Fig. 4A).
The expression of *dsCYP9A14* in transgenic *Arabidopsis* was detected by RT–PCR (Fig. S6A, Supporting information), and a high expression line (*ds-8*) was selected for subsequent feeding assay. The 3rd instar larvae grown on *ds-8* or wild-type plant (WT) leaves for 3 days were then transferred to deltamethrin-supplemented diet (5 μg/g) for another day. The larvae taken from *ds-8* leaves had a lower CYP9A14 transcript level than those from WT leaves (Fig. 6A), and, consequently, a much reduced larval growth on deltamethrin-supplemented diet (Fig. 6B) and a 35% decrease in LD₅₀ (Fig. 6C).

**Discussion**

Plants have a suite of biochemical pathways that synthesize phytotoxins for defence against herbivores or pathogens (Grayer & Kokubun 2001; Howe & Jander 2008). On the other hand, the inducibility of detoxification genes in response to host plant secondary metabolites has helped insect herbivores protect themselves against these toxins for an optimal survival (Schuler 2011). In this investigation, gossypol treatment induced multiple P450 genes, and many of them were shown to contribute to the tolerance to or metabolism of phytotoxins and insecticides (Table S2, Supporting information). Of particular interests, we found that five of the gossypol-inducible P450s were related to deltamethrin tolerance (Fig. 5). Commonly, a single substrate could be metabolized at alternative positions by a group of P450s or a single P450 could metabolize multiple substrates (Schuler 2011). Phytochemical-inducible P450s with the character of ‘broad-range’ substrates are of great value to generalist insect herbivores. By ingesting one or a few toxic compounds from the host plant, they accomplish much wider range of resistance to other toxic secondary metabolites, or even environmental chemicals, such as insecticides. This would cost less material/energy of biosynthesis and render them easier to obtain food and adapt to environmental changes.

It is well established that insect P450s have evolved under selective pressure of toxic compounds. A detailed investigation showed the evolutionary relationship between CYP6B subfamily genes, which are responsible for furanocoumarins metabolism in parsnip webworm (*depressaria pastinacella*) (Li et al. 2004) and Papilio species (Hung et al. 1996; Li et al. 2001, 2002b; Wen et al. 2006). CYP9A12 and CYP9A17 of cotton bollworm shared 94% amino acid identity with each other (Zhou et al. 2010). We found that both of them were induced by gossypol and related to deltamethrin tolerance (Fig. 3A and 5). Another pair of P450 genes, CYP6AE11 and CYP6AE14, sharing 92% amino acid sequence identity with each other, was upregulated in the larvae fed on glanded cotton. Elevated CYP6AE14 expression was found necessary for cotton bollworm tolerance of gossypol (Mao et al. 2007), and CYP6AE11 was recently found to be overexpressed in pyrethroid resistance strain (Brun-Barale et al. 2010); together with our correlation analysis (Fig. 5), these data suggest that the two closely related gossypol-inducible P450s have functional emphases on toxic compounds. This pair of P450s may
serve as an example representing the evolutionary relationship between phytotoxin and pesticide detoxification: in response to selective agrochemicals, particular P450s have evolved the ability to metabolize artificial insecticide in addition to their original activity towards phytotoxin.

Insecticide resistance could occur due to detoxification activity of a single P450, such as CYP6D1 in house fly (Wheelock & Scott 1992; Scott & Lin 2011), or multiple P450s, such as CYP9A12, CYP9A14, CYP6B7 in cotton bollworm (Yang et al. 2006). Data presented in this article attributed cotton bollworm tolerance of deltamethrin to multiple P450s. It is interesting to understand the factors that have selected this mechanism. We argue that gossypol-enhanced genes constitute a pool for the development of cotton bollworm tolerance of pyrethroid insecticides. Under the selective pressure of cotton secondary metabolites, in particular gossypol and related sesquiterpene aldehydes, cotton bollworms have developed detoxification systems to adapt to these polyphenol aldehydes. The activation of P450s by gossypol potentiates the defence state of bollworms, and this phenotypically plastic adaptation (Agrawal 2001) has provided them with higher survival rates when encountering synthetic insecticides. Although xanthotoxin significantly stimulated cotton bollworm tolerance to deltamethrin (Fig. S1C, Supporting information), it is highly toxic (Fig. S1C, Supporting information) possibly because cotton bollworms have encountered this phytotoxin only rarely (Li et al. 2000b) and not yet acclimated themselves to the dose (0.5 mg/g) we applied. On the other hand, to cotton bollworms, gossypol is the major secondary metabolite of their major host plant; in response to deltamethrin, cotton bollworms developed the expression profile of differentially expressed P450 genes that was most similar to that triggered by gossypol. Thus, the presence of highly active phytotoxins in host plant provides a selective pressure for the herbivorous insects to develop a rich pool of defence genes, which is at least one of the reasons why cotton bollworms have quickly acquired pyrethroid resistance.

RNAi has been developed into a powerful tool of gene regulation for both plants and animals. Gene suppression in insects by ingested dsRNA makes it possible to generate insect-proof plants by RNAi technology (Mao et al. 2007, 2011). Increasing evidence (Kong et al. 2010), including data of this report, has shown that bollworms growing on glanded cotton accumulate a high concentration of gossypol and have enhanced insecticide tolerance. To reduce crop damages caused by this pest, extra

Fig. 6 Effects of transgenic dsCYP9A14 Arabidopsis plants on deltamethrin tolerance of cotton bollworm. (A) qRT–PCR analysis of CYP9A14 transcript levels in midgut of 3rd instar larvae fed on leaves of the wild type (WT) or the dsCYP9A14 transgenic line (ds-8) for 3 days. The dsCYP9A14 plants were engineered to express the double-stranded RNA against the bollworm CYP9A14. (B) Net weight increase of the larvae prefed with WT and ds-8 leaves after transferring into artificial diet containing 5 µg/g deltamethrin for 1 day. Plant feeding assay was performed as described in A. Student’s t-test: *P < 0.05. (C) Mortality rates of the larvae fed on WT and ds-8 leaves under topical application of different doses of deltamethrin. The 4th instar larvae (20–30 mg each) from each treatment were divided into 3 groups, each group contained 30 larvae (triplicates of 10 larvae) for three doses (0.625, 1.25, 2.5 ng/mg larva) of deltamethrin, respectively. The mortality was recorded after 1 day. For clarity, only the positive (ds-8) or negative (WT) SD is shown. Statistical significance of mortality was determined with Mann–Whiney U test: P = 0.067, P = 0.043, P = 0.456 for ds-8 vs. the corresponding WT under deltamethrin doses of 0.625, 1.25, 2.5 ng/mg larva, respectively.
insecticides are often required. Data presented herein demonstrate the bollworm tolerance of deltamethrin can be reduced by engineering plant-mediated RNAi against gossypol-inducible P450s. Thus, besides directly impairing the insect development and growth, RNAi technology holds an additional potential to cripple the pest tolerance to insecticides, reducing the dosage of agrochemicals needed for pest control in field.

Acknowledgements

We are grateful to Jian-Hao Jiang for assistance in Sf9 cell culture and exogenous protein expression, and Wen-Li Hu for GC-M5 analysis. This research was supported by The National Science Foundation of China (30870380), The National High-tech Research Program of China (2009AA10Z112) and The Ministry of Agriculture of China (2008ZX08009-001, 2011ZX08005-001), and the CAS/SafeA International Partnership Program for Creative Research Teams.

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This study was conducted by X.Y.T during her PhD thesis. X.Y.T, X.Y.X, Y.C and Y.B.M have a common research program with the focus on plant-insect interactions and the RNAi technology for insect control. Y.X.C’s lab also works on the bio-synthesis of sesquiterpenoids (such as gossypol) as well as trichome and cotton fiber development. Y.P.H’s interest is on the functional genome of insects.

Data accessibility

6490 contigs from 454 sequencing used for microarray analysis are shown in the supporting information (Table S1, Supporting information).

Microarray results in response to six chemicals, and gossypol or glandless cotton leaves are uploaded as online supplementary material (see also Table S1, Supporting information).

The mortality data in toxicological analysis, results of enzyme assays and qRT–PCR curves are provided (Table S4, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of differentially expressed genes in cotton bollworm midguts based on microarray.
Table S2 The differentially expressed detoxification genes in cotton bollworm midguts in response to six compounds.

Table S3 Primers used in this investigation.

Table S4 The mortality data in toxicological analysis, results of enzyme assays and qRT-PCR curves.

Fig. S1 Cotton bollworm larvae weight. (A and B) Initial weight of the larvae shown in Fig. 1A,B, respectively. (C) Initial weight of late 2nd larvae after treatments with 1 mg/g gossypol, 0.5 mg/g xanthotoxin and 1 mg/g tannic acid, respectively, for 1 day (upper) and the net weight increase after transferring to 5 μg/g deltamethrin for another day (down).

Fig. S2 Aldrin epoxidation activity of CYP6AE14. Recombinant baculoviruses were generated in Bac-to-Bac baculovirus expression system (Invitrogen Life Technology). CYP6AE14 and house fly NADPH P450 reductase were co-expressed in SF9 cells. (A) GC-MS detection of the aldrin epoxidation product dieldrin. (B) Western blot with an anti-His antibody to detect the expressed protein.

Fig. S3 Activities of midgut detoxification enzymes of differently weighted 3rd-5th instar larvae. The individuals (3rd-5th instars) grown on artificial diet were divided into four groups (GI to GIV) depending on their weights (A) and GST-DCNB (B), Esterase-αNA (C) and P450-PNOD, P450-ECOD and P450-AE (D) activities were tested.

Fig. S4 Cluster diagram of expression of esterase and GST genes in response to six compounds. For detail see Fig. 3B. This cluster analysis included 21 putative esterase genes and 37 total contigs of putative GST, detailed information of the genes is provided (Table S2, Supporting information).

Fig. S5 Larval growth increase in relation to the expression level of three P450, three esterase and two UDP-GT genes under deltamethrin treatment. The experiment was performed as described in Fig. 5. Among the 22 P450, eight esterase and two UDP-GT genes being studied, results with regression coefficient ($r^2$) > 0.5 are shown here besides in Fig. 5. For gene sequences and expression profiles, see Tables S1 and S2 (Supporting information).

Fig. S6 RT–PCR detection of dsCYP9A14 transcripts in different lines of dsCYP9A14 transgenic Arabidopsis (A) and Mortality of the larvae fed with leaves of dsCYP9A14 or WT plants of Arabidopsis for 4–5 days (B). Tub: β-tubulin.

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