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Two independent regulatory mechanisms synergistically contribute to P450-mediated insecticide resistance in a lepidopteran pest, *Spodoptera exigua*

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Abstract

Background Cytochrome P450 enzymes play a pivotal role in the detoxification of plant allelochemicals and insecticides. Overexpression of P450 genes has been proven to be involved in insecticide resistance in insects. However, the molecular mechanisms underlying the regulation of P450 genes in insects are poorly understood.

Results Here, we determine that upregulation of *CYP321B1* confers resistance to organophosphate (chlorpyrifos) and pyrethroid (cypermethrin and deltamethrin) insecticides in the resistant *Spodoptera exigua* strain. Enhanced expression of transcription factors *CncC/Maf* contributes to the increase in the expression of *CYP321B1* in the resistant strain. Reporter gene assays and site-directed mutagenesis analyses confirm that a specific binding site is crucial for binding *CncC/Maf* to activate the expression of *CYP321B1*. In addition, creation of a new binding site resulting from the *cis*-mutations in the promoter region of *CYP321B1* in the resistant strain facilitates the binding of the POU/homeodomain transcription factor *Nubbin*, and further enhances the expression of this P450 gene. Furthermore, we authenticate that changes in both *trans*- and *cis*-regulatory elements in the promoter region of *CYP321B1* act in combination to modulate the promoter activity in a synergistic manner.

Conclusions Collectively, these results demonstrate that two distinct but synergistic mechanisms coordinately result in the overexpression of *CYP321B1* involved in insecticide resistance in an agriculturally important insect pest, *S. exigua*. The information on mechanisms of metabolic resistance could help to understand the development of resistance to insecticides by other pests and contribute to designing effective integrated pest management strategies for the pest control.

Keywords Insecticide resistance, Cytochrome P450, *Spodoptera exigua*, *CncC/Maf*, Cis-regulatory element, Transcriptional regulation, Metabolic resistance

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Background

Cytochrome P450 enzymes play an important role in the adaptation to host plants and the detoxification of xenobiotics in insects [1–3]. Constitutive upregulation of P450 genes has shown to be associated with insecticide resistance in insects [4, 5]. Many genes belonging to the CYP4, CYP6, CYP9 and CYP12 families have been documented to be overexpressed and lead to insecticide resistance by enhancing the metabolic detoxification of them [2, 6]. For example, transcriptional upregulation of four P450 genes *CYP9M10*, *CYP9J34*, *CYP9J40* and *CYP6AA7* has been shown to confer resistance to permethrin in *Culex quinquefasciatus* [7, 8]. Similarly, overexpression of *CYP6FU1* gene leads to deltamethrin resistance in *Laodelphax striatellus* [9] and the enhanced expression of *CYP6BQ9* gene results in the majority of deltamethrin resistance in *Tribolium castaneum* [10]. However, the link between the genes belonging to the CYP321 subfamily and insecticide resistance remains largely unknown in insects.

The nuclear factor erythroid 2 related factor 2 (*Nrf2*) acts as a *trans*-regulatory factor to coordinate an evolutionarily conserved transcriptional activation pathway that mediates antioxidant and detoxification responses in laboratory mice [11–13]. Under basal (unstressed) conditions, Nrf2 is sequestered in the cytoplasm by Keap1, a Cul3-dependent ubiquitin ligase adaptor protein that targets Nrf2 for ubiquitination and proteasomal degradation in mammalian cells [14]. Oxidative stress and electrophiles disrupt the Nrf2-Keap1 interaction allowing Nrf2 to translocate to the nucleus where it dimerizes with the small Maf proteins; the heterodimers then bind to the antioxidant response elements (AREs) of detoxifying enzyme genes, including P450s, NAD(P)H: quinone oxidoreductase-1 (*NQO1*), GSTs, UDP-glycosyltransferase (UGT), and heme oxygenase 1 (*HO-1*) to initiate their transcription [15–18]. *CncC* (*Cap 'n' Collar isoform-C*), the insect ortholog of mammalian transcription factor *Nrf2*, as a heterodimer with *Maf-S* (*muscle aponeurosis fibromatosis*) is a central regulator of xenobiotic detoxification responses and contributes to the widespread overexpression of detoxification genes in insecticide-resistant strains of *Drosophila melanogaster* [17–19]. Transcription factors *CncC* and *Maf* also have been demonstrated as the key regulators for the expression of multiple P450 genes responsible for deltamethrin resistance in *T. castaneum* [20] and fenpropathrin resistance in *Tetranychus cinnabarinus* [21].

A few studies have shown that *cis*-acting mutations in the promoter region of P450 genes regulate the expression of these P450 involved in insecticide resistance in insects. For example, the mutations alternating TF binding site in the 5'-promoter core region lead to the constitutive overexpression of *CYP6A2* gene and a TE insertion in the promoter region of *CYP6G1* upregulate the expression of this P450 gene in *D. melanogaster* [19, 22, 23]. Similarly, the expansion of a dinucleotide microsatellite in the promoter region of *CYP6CY3* enhances the transcriptional level of this P450 gene and results in the resistance to neonicotinoid insecticides in *Myzus persicae* [24]. However, whether *cis*- and *trans*-acting factors act in combination to regulate detoxification genes and if so, how these mechanisms interact remains poorly understood.

The beet armyworm, *Spodoptera exigua*, is a widely distributed polyphagous pest that seriously damages many cultivated crops including cabbage, soybean, cotton, and others [25]. The use of chemical insecticides remains currently the major method for the control of this pest. It has developed resistance to various groups of chemical insecticide classes, including chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids, IGRs and others [26], understanding the molecular mechanisms of the regulation of resistance genes is very important. Our recent analyses have shown that enhanced CYP450 enzyme activity is involved in resistance to chlorpyrifos and pyrethroids insecticides in *S. exigua*, and many CYP450 genes are upregulated in the resistant strain. One of these, *CYP321B1* (GenBank Accession No. KX443444.1) has 41-fold higher expression in the resistant strain and might confer resistance to chlorpyrifos and pyrethroid insecticides [27]. Here we report that constitutive overexpression of the *CYP321B1* gene indeed results in resistance to chlorpyrifos and pyrethroid insecticides. Upregulation of *CncC*/ *Maf* and a *cis*-acting mutation in the promoter region jointly control the constitutive overexpression of *CYP321B1* gene in a synergistic manner. Finding the molecular mechanisms underlying the regulation of detoxification genes will help us identify new target genes for pest control.

Results

Insecticide metabolism in vitro by recombinant CYP321B1

To investigate the functional role of CYP321B1 in insecticide metabolism in vitro, the recombinant protein was expressed in the Sf9 cells. Reduced CO-difference spectrum analysis revealed that recombinant CYP321B1 protein was successfully expressed as a good-quality functional enzyme (Fig. 1A). Furthermore, catalytic activity test with the ECOD substrate indicated that the recombinant protein efficiently catalyzed 7-ethoxy coumarin with a specific activity of 0.19 pmol min^{-1} $pmol^{-1}$ protein (Fig. 1B). High-performance liquid chromatography (HPLC) was utilized to evaluate the capacity of recombinant CYP321B1 for

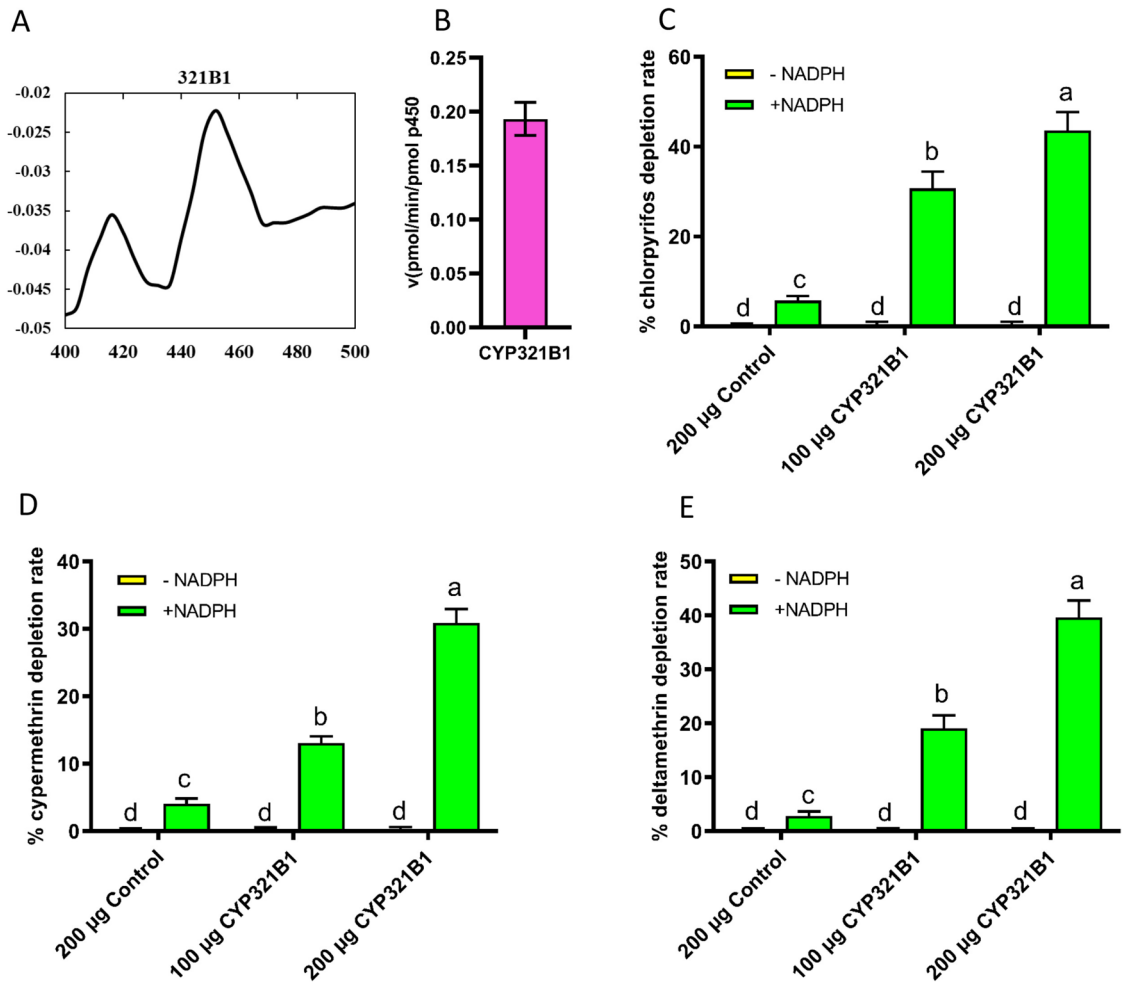

Fig. 1 Upregulation of *CYP321B1* gene involved in resistance to chlorpyrifos, cypermethrin and deltamethrin. **A** Carbon monoxide difference spectra were used to assess the integrity of the recombinant CYP321B1 protein. **B** Enzyme activity of recombinant P450 protein was evaluated by using a model fluorescent substrate (ECOD). Error bars display SD. Chlorpyrifos (**C**), cypermethrin (**D**) and deltamethrin (**E**) metabolism by recombinant CYP321B1 protein. Error bars represent the mean ± SD values ($n = 3$). Different letters on the right of error bars indicate significant differences based on ANOVA with Tukey's HSD multiple comparison test ($p < 0.05$)

degrading insecticides including chlorpyrifos, cypermethrin and deltamethrin. After incubating insecticides with the microsome for 1.5 h, 30.7 ± 3.7% and 43.6 ± 4.1% of chlorpyrifos, 13.1 ± 1.0% and 30.9 ± 2.2% of cypermethrin, and 19.1 ± 2.3% and 39.7 ± 3.1% of deltamethrin were degraded by 100 and 200 μg recombinant CYP321B1 in the presence of NADPH, respectively (Fig. 1C, D and E). No depletion in insecticide was found when recombinant CYP321B1 was incubated with these insecticides without NADPH. Only trace levels of degradation of these insecticides when the proteins of non-transfected Sf9 cells were used. These results suggest that overexpression of *CYP321B1* is associated with resistance to chlorpyrifos, cypermethrin and deltamethrin in *S. exigua*.

Overexpression of *CYP321B1* enhanced the tolerance of *D. melanogaster* to insecticides

To identify whether the overexpression of *CYP321B1* was involved in insecticide resistance in vivo, the GAL4/UAS system was utilized to generate the transgenic *D. melanogaster* flies expressing CYP321B1 (actin5C-CYP321B1). The expression level of *CYP321B1* in F1 progeny was confirmed by RT-PCR (Fig. 2A and Additional file 1: Fig. S2) and RT-qPCR (Fig. 2B). When the transgenic flies expressing CYP321B1 (actin5C > CYP321B1) were exposed to 0.05 mg/L chlorpyrifos for 3 days, the

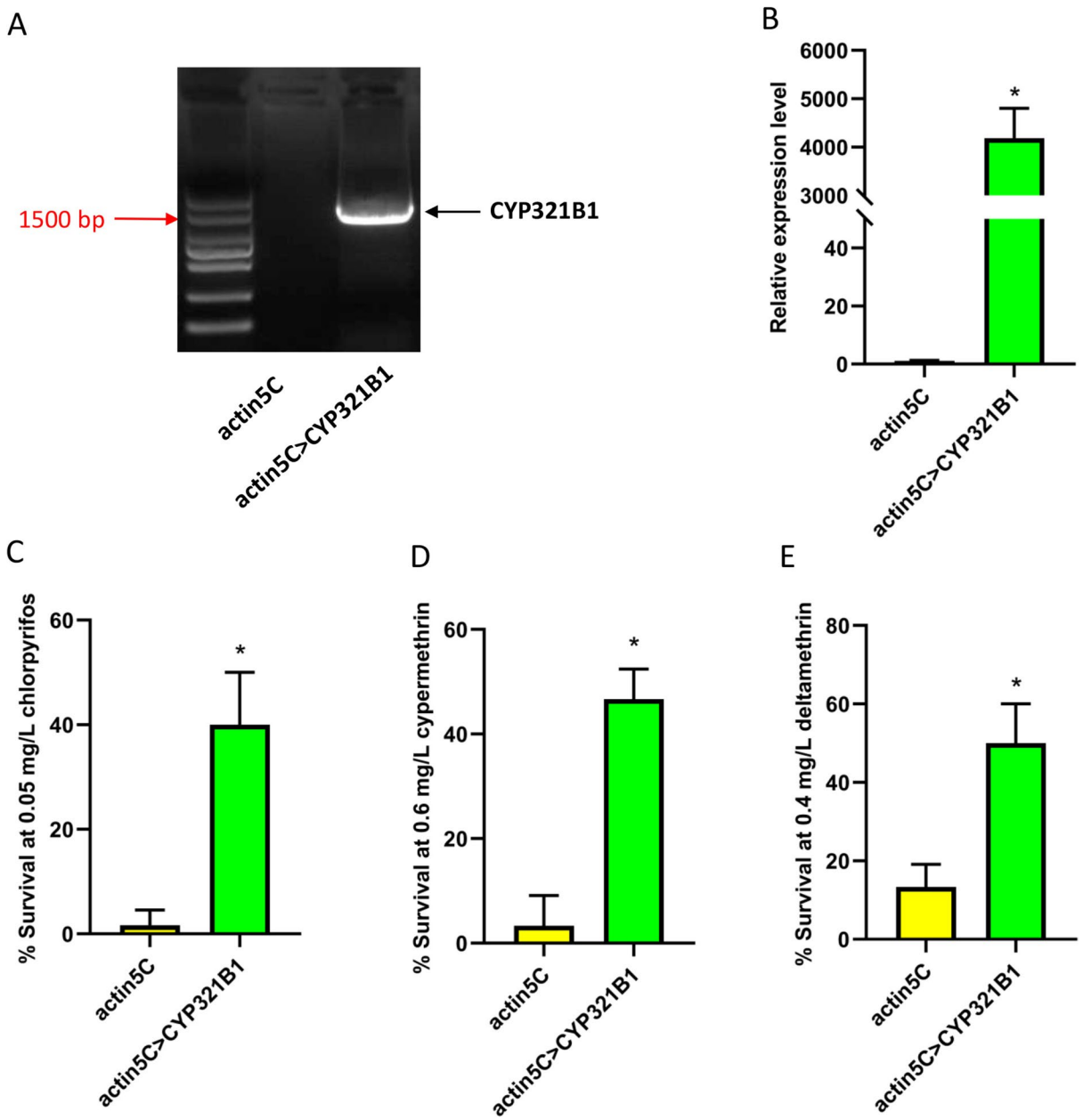

Fig. 2 Susceptibility of transgenic flies expressing CYP321B1 to chlorpyrifos, cypermethrin and deltamethrin. **A** PCR was used to measure the mRNA level of *CYP321B1*. **B** The transcriptional level of *CYP321B1* was analyzed by qRT-PCR. The $2.^{-\Delta\Delta CT}$ method was used for quantification of the gene expression level. Student's t-test was used for statistical analysis. Asterisks on the standard error bars indicate significant differences compared with the control. A value of $P < 0.05$ was considered statistically significant. Susceptibility of transgenic flies expressing CYP321B1 to chlorpyrifos (**C**), cypermethrin (**D**) and deltamethrin (**E**) was investigate. The progeny from the cross between the w1118 and Act5C-GAL4 lines was used as a control (actin-5C). Three independent biological replicates were carried out and the mean ± SD was displayed. A significant difference in survival between transgenic flies expressing CYP321B1 and controls is marked with asterisks on the right of error bars (Student's t-test, $p < 0.05$)

mortality was 60.00% compared to 98.50% mortality in the control flies (Fig. 2C). The mortalities of flies expressing CYP321B1 exposed to 0.6 mg/L cypermethrin were 53.33% compared to the control flies which exhibited mortality of 96.67% (Fig. 2D). A significant difference was also observed after exposure to 0.4 mg/L deltamethrin, with flies expressing CYP321B1 displaying 50.00% mortality compared with 86.67% for controls (Fig. 2E). These results demonstrate that overexpression of *CYP321B1* confers resistance to the three insecticides in *S. exigua*.

Homology modeling and docking

Homology modeling and molecular docking was utilized to determine the possible interactions of CYP321B1 protein with insecticides. These results revealed that this P450 protein were tightly bound to chlorpyrifos, cypermethrin and deltamethrin. The free binding energies of CYP321B1-chlorpyrifos, CYP321B1-cypermethrin and CYP321B1-deltamethrin complexes were −6.33, −9.70 and −9.07 kcal/mol, respectively (Fig. 3A, B and C). Chlorpyrifos and cypermethrin were docked within 14.2 Å and 14.3 Å from heme iron by hydrophobic interactions and hydrogen bonds (Fig. 3A, B and Additional file 1: Table S2). Deltamethrin was docked within a distance of 14.0 Å from heme iron by hydrophobic interactions (Fig. 3C and Additional file 1: Table S2). There was only one hydrogen bonding interaction between CYP321B1 residue ARG-104 and chlorpyrifos with a distance of 2.5 Å (Fig. 3A). One hydrogen bonding interaction existed between residue THR-373 (2.2 Å) of CYP321B1 and cypermethrin (Fig. 3B). No hydrogen bonding interaction was found between CYP321B1 and deltamethrin (Fig. 3C). These results indicated that these bonds are discernible.

Overexpression of *CncC/Maf* regulates the expression of *CYP321B1* gene

To explore the regulation mechanism of overexpression of *CYP321B1* in the resistant strain, a 1380 bp upstream promoter sequence was identified by genomic walking approach. Predictive analysis of transcription factor binding sites showed that the promoter region of *CYP321B1* gene contains the binding sites for *EcR*, *P53*, *CncC/Maf*, *Hb*, *BR–C*, *Kr*, *GATA* and *Dfd* (Fig. 4A). Our previous study has shown that the mRNA levels of *CncC* and *Maf* were dramatically upregulated in the resistant strain used in this study, whereas no significant differences in the expression levels of *P53*, *EcR*, *USP*, *Dfd*, *BR–C*, and *Kr* between the resistant and susceptible strains were observed [27]. These results imply that the expression of *CYP321B1* may be mediated by transcriptional factors *CncC* and *Maf*. To further verify this hypothesis, the *CYP321B1* promoter region was ligated into the reporter gene vector and co-transfected into Sf9 cells with the expression vectors containing the open reading frames of *CncC* and *Maf* (Fig. 4B). Compared to the control, the promoter activities of *CYP321B1* were increased by 1.47- and 2.80-fold in the presence of CncC and Maf, respectively. Co-expression of CncC and Maf increased *CYP321B1* promoter activity even more, 4.64-fold. These results demonstrate that upregulated *CncC* and *Maf* enhance the expression of *CYP321B1* gene in the resistant strain.

Identification of *CncC/Maf* binding sites in the *CYP321B1* promoter region

To determine the relevant *CncC/Maf* binding sites, the promoter region of the *CYP321B1* gene was divided into different size fragments to construct promoter truncation, and the fragments were ligated into reporter gene vector (Fig. 5A). Each construct containing truncation of the *CYP321B1* promoter was co-transfected with CncC and Maf constructs. The promoter activities of full-length promoter (Full) and truncation −258 to −1 bp (T3) increased by 4.4- and 4.9-fold respectively in the presence of CncC and Maf (Fig. 5B). In contrast, the promoter activities of truncation −1463 to-1234 bp (T1), truncation −1253 to-238 bp (T2) and truncation core promoter (T4) were not significantly changed by the CncC and Maf. These results confirm that the effective *CncC/Maf* binding site was present in the region between −258 to −1 bp, which is consistent with the prediction of *CncC/Maf* binding site (AATGACAACGCAAAA) located in the region.

Furthermore, we introduced mutations into the *CncC/maf* binding site located in the *CYP321B1* region (Fig. 6A). In the construct M-1, the binding site AAT GACAACGCAAAA was mutated to AAgactAAtttAAAA based on the wild-type WT-1 construct. Similarly, the construct WT-2 contained the binding site AATGAC AACGCAAAA which was replaced with AAgactAAtttAAAA in the construct M-2. A significant decrease in the promoter activity of the construct M1 was observed when compared with that of the wild-type WT-1 (Fig. 6B). Similarly, compared to the wild-type WT-2, the promoter activity of the construct M2 was markedly reduced by 8.6-fold. These results demonstrate that the specific *CncC/Maf* binding site is responsible for the activation of *CYP321B1* promoter.

Cis-acting mutations in the promoter region enhanced the expression of *CYP321B1* gene

To investigate the genetic variation degrees of the promoter region of *CYP321B1* derived from the resistant and susceptible strains, the upstream promoter sequences of *CYP321B1* derived from six resistant and six susceptible individuals were cloned and sequenced. Phylogenetic analysis revealed that the twelve promoter sequences were clustered into three different groups (Fig. 7A). Among them, all six sequences derived from resistant insects are distributed in Type 1, and most of the sequences cloned from susceptible insects are grouped into Type 3. Subsequently, alignment of promoter sequences derived from the resistant and susceptible strains identified several mutations that differentiate the strains (Additional file 1: Fig. S1). To confirm whether these mutations contribute to the increase in the

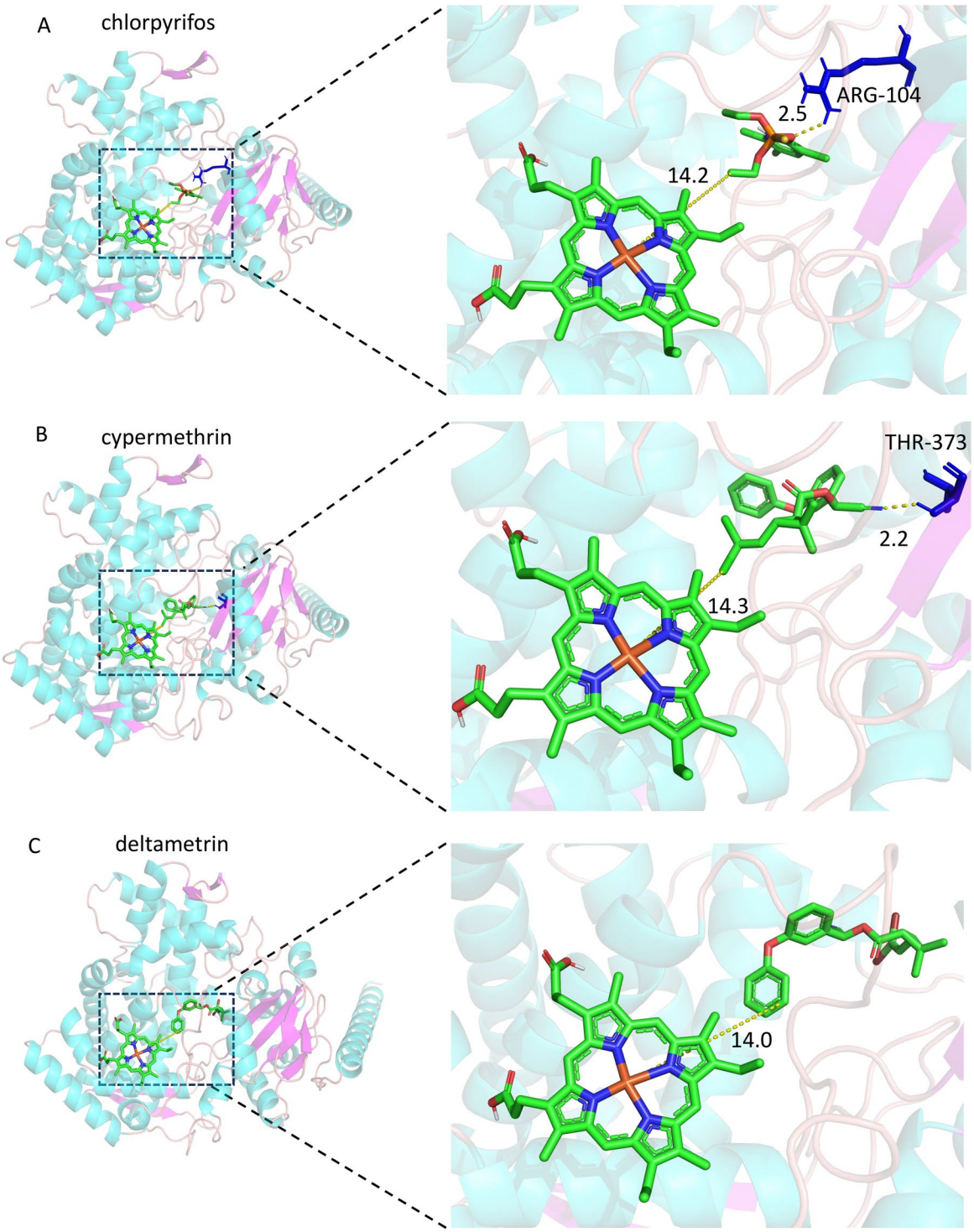

Fig. 3 Homology modeling and insecticides docking. Binding modes of chlorpyrifos (**A**), cypermethrin (**B**) and deltamethrin (**C**) to CYP321B1 protein. The residues of P450 protein interacting with insecticides were represented in blue sticks

promoter activity of *CYP321B1* in the resistant strain, the promoter sequences from the resistant and susceptible strains were constructed into the promoter vector. Compared to the susceptible strain, a 2.1-fold increase in the promoter activity of *CYP321B1* from the resistant strain was observed (Fig. 7B), suggesting that higher

A

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-1380 GTCCAGTTACAGCTGTACACAATATTCCAAAGTAACAGCCTCGTATAGAGGTCAAAATTCAATGATCTATAAATAACATAAGTAATAATTTATCCTTAAA
                                                          EcR
-1280 TAATAAAGTTTATATGCCATGTAATTGTCTTGGTGGCCCAGAGGTTGAAGCACCCGCTTCTCATGCATGAGGGTGCGAAACCTGGCAAGTAACAGTGTGA
                                  P53                                 CncC/Maf
-1180 TTTTTTCCAAGTTATAGGTACTTTCTATGATTATTTAGACACCATTGACAAACGGTGAAGGAAAACATCGTGAGGAAACCTGGACTTATAATTTCTAAAT
          Hb
-1080 TATAAGTTTGAAAACGACAATCCGCCTTGAGCAAGCGTGCTAATTAATGCTCAACCCTTCTCCTTGTGGGAAGAGGCCTTTGTGTTGTATTTATTTTTAC
                BR-C                                KR                                                Hb
 -980 ATTAAGCACATACACACTCAATAGTTCATTTACGTACTTACTTATTATTTTTTCTGGCGTCTTTTCTTTATTATAGGGTTGGCGTTAAATGTTACCACCG
 -880 TACTCAGAGTCGTTAAATGTCAATTCAAGAATTAGGAATTTCAATTTAGGAATTAATAAAGACAGGATACTTAGGAATAATTTAAGTAATCACTATTTTG
 -780 GGTGGTTTTAGTACTGAGTTTGAAACAACAGTAATTTATAATCGATGTTTTAACAATAGGCTTACTTGACGATTTTGGAATGATTAAACAATAACCGATT
 -680 TTTGACATGTCGCAATAACAATTAAATCAATGTCAAATTCAAAATCATTTATTCCAACTAAACCATAAATAGGTAATTTTGAAACATCAAGATAGAATCA
 -580 GTCTGTTTCTCAGTGAAGCTAGGTGCACATTCCAAATTGAAGATTCGAATGGAGAAGAAAGAGCAAGAAACTCCATCTGGCACTAAAGAGATTGCCTCAA
 -480 TTTTGTGCGTACTGCATTTATTGCGTTACTTGTGCGATAATATCTTAGAACATGCTCTAATATTTTAAGAAAAATATTGATCAAACTGGTTTTATTTACA
                                                GATA
 -380 TATCTAAATTTGCAAATTATCAACGGGGTATTTACCATTAACGCTCTTAATAAAACAAAAATACTGTTCAACACTATTGTTAGTATTTGTTTAGCCATTA
 -280 ATAAGGCTCCGAGTAAATAAAAGATAAATAACTAAAATCGAATATATGTAATTATTACCGCCCATATAATAACAATGACAACGCAAAATATCTCGTAGGA
                                                                                  CncC/Maf
 -180 TAACAAAAATGTTCGAGACTACAAATAGATAAGACAAGTCGCAACCTGTCGAGTTCAAATAAATCATTACCGTATCAGTTGTAAACATCAAGCGTATTAT
                                                          Dfd
  -80 AAAAAGAAGCGTCCGACGGAGGCTCATCATCAGTCACAACTAAACACTTAACGAGCTTAGACAAGTTCAGACAACCAAACATGTTGTCCACAGCTATACT
                                Inr                                                  +1
  +21 CGCTATTACCAT
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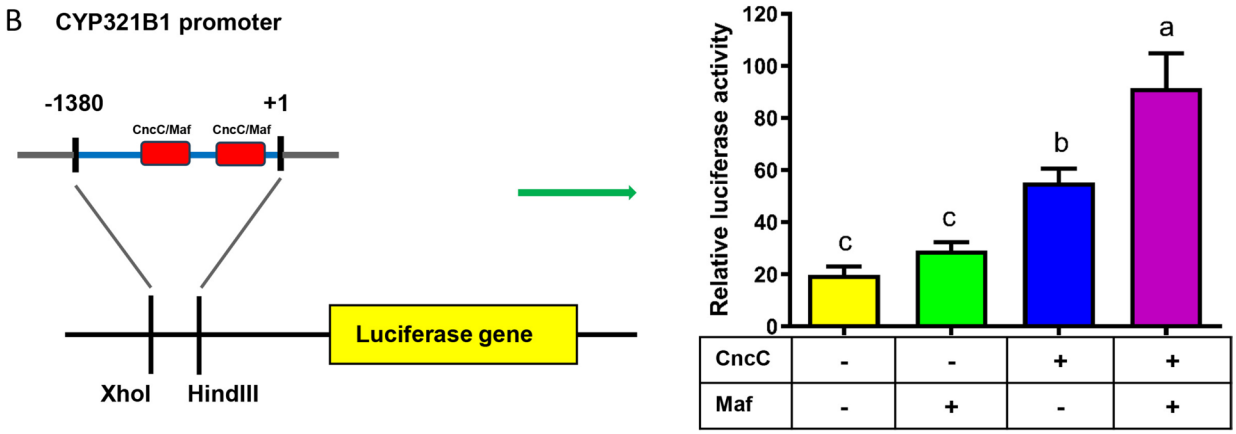

Fig. 4 Overexpression of *CncC* and *Maf* increase the promoter activity of *CYP321B1*. *A* Prediction of transcription factor binding sites in the promoter region of *CYP321B1*. The nucleotides are numbered relative to the translation start site (ATG) indicated by + 1, with sequence upstream of it preceded by "-". The transcription start sites (Inr) and putative transcription factor binding sites are underlined. **B** Promoter activities of *CYP321B1* in the presence of CncC and/or Maf. The data are presented as the mean ± SD. Different letters above the bars indicate significant differences based on ANOVA followed by Tukey's HSD multiple comparison test ($p < 0.05$)

promoter activity is also one of the reasons for the overexpression of *CYP321B1* in the resistant strains.

To further identify the region of the promoter that is responsible for the differences in promoter activity between the resistant and susceptible strains, a series of truncations (−1463 ~ −1131, −1130 ~ −689, −688 ~ −354, −353 ~ −168 and −167 ~ −1) were constructed. As shown in Fig. 7C, the transcriptional activities of P (−1463/−1), P (−1130/−1), P (−688/−1) and P (−353/−1) showed significant differences between the resistant and susceptible strains. In contrast, no difference in the promoter activity of P (−167/−1) was observed. These results indicate that the mutations contributing to the difference in the promoter activity were located in the fragment located between − 353 to − 167 bp. To further identify this region, two additional truncations (−258/−1 and −210/−1) were constructed and assayed in Sf9 cells. The luciferase assays showed that these two truncations remain the obvious difference in the promoter activity in the resistant and susceptible strains, suggesting that the mutations responsible for the difference in the activity of *CYP321B1* promoter from the resistant and susceptible strains were located in the fragment, −258 ~ −210 (Fig. 7D). Subsequently, analysis of resistant and susceptible promoter sequences revealed that five mutation sites were found in the fragment from −258 to −210 (Fig. 8A).

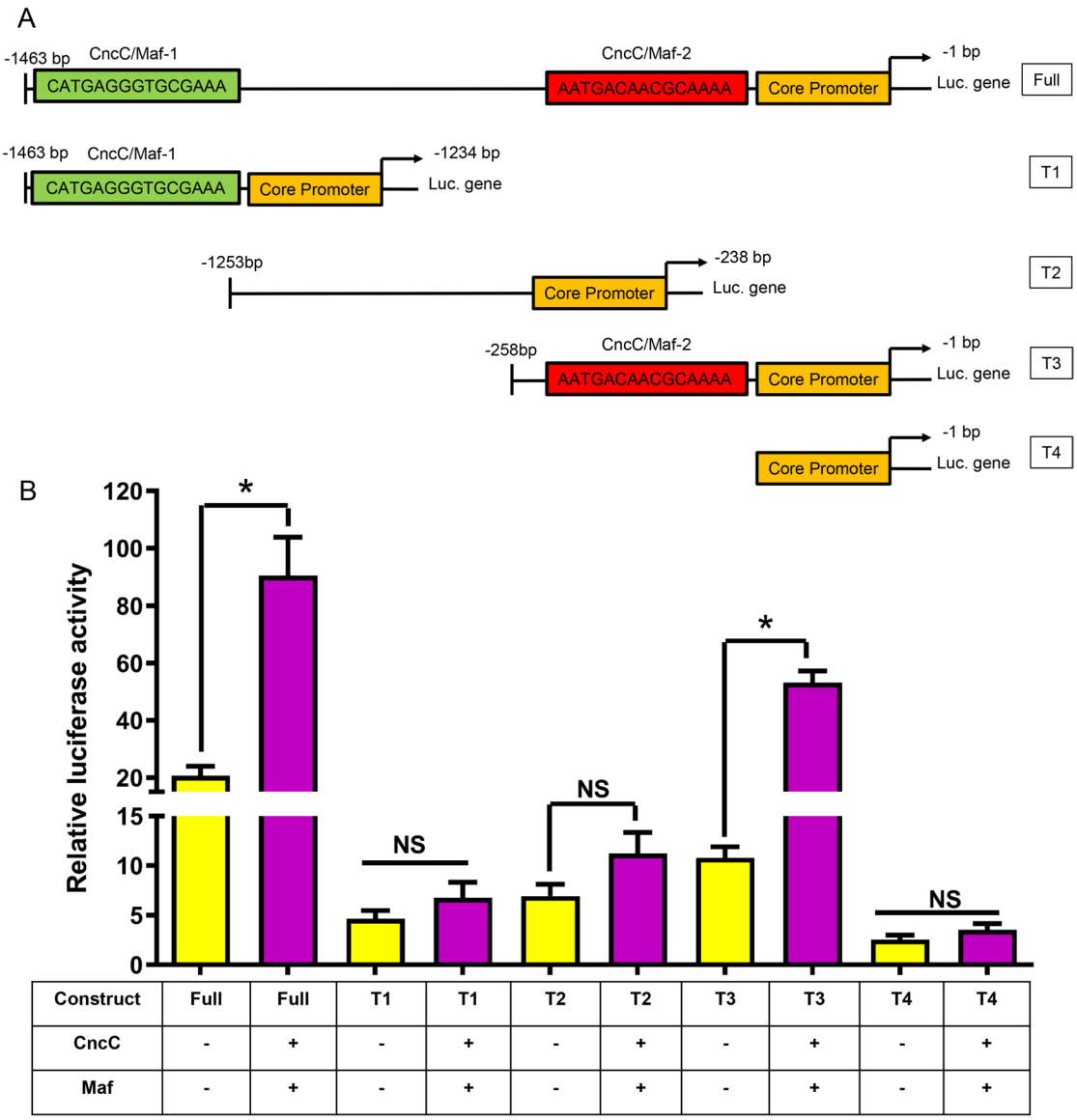

Fig. 5 *CncC* and *Maf* regulate the expression of *CYP321B1* by binding to a specific site present in the region between −258 to −1 bp. **A** Schematic representation of the *CYP321B1* promoter truncations. **B** Analysis of *CYP321B1* promoter truncations. The cells were harvested at 48 h after transfection and the luciferase activity was quantified. Significant differences in the luciferase activity were identified using Student's t-test and are indicated using an asterisk. A value of $p < 0.05$ was considered statistically significant

To verify if these mutations can affect promoter activity, P (−258/−1)-R was selected as standard and two mutation constructs P (−258/−1)-R-M1 and P (−258/−1)-R-M2 were generated to recover these mutations. This result showed that the reporter activity of the mutant construct (M2) was significantly decreased compared with the control construct P (−258/−1)-R, indicating that the *cis*-acting element resulting from mutation at the M2 site can significantly upregulate the expression of *CYP321B1* gene (Fig. 8B). However, the mutant construct M1 has on significant effect on the promoter activity. Surprisingly, the M2 mutation is located in a predicted binding site of the POU/homeodomain transcription factor, *Nubbin*, indicating that this transcription factor may regulate the overexpression of *CYP321B1* in the resistant strain.

Trans-acting factors *CncC/Maf* and *cis*-acting mutations synergistically contribute to the increase in the expression of *CYP321B1* in the resistant strain

To authenticate whether the changes in both *cis*-acting elements and *trans*-acting factors coordinately control

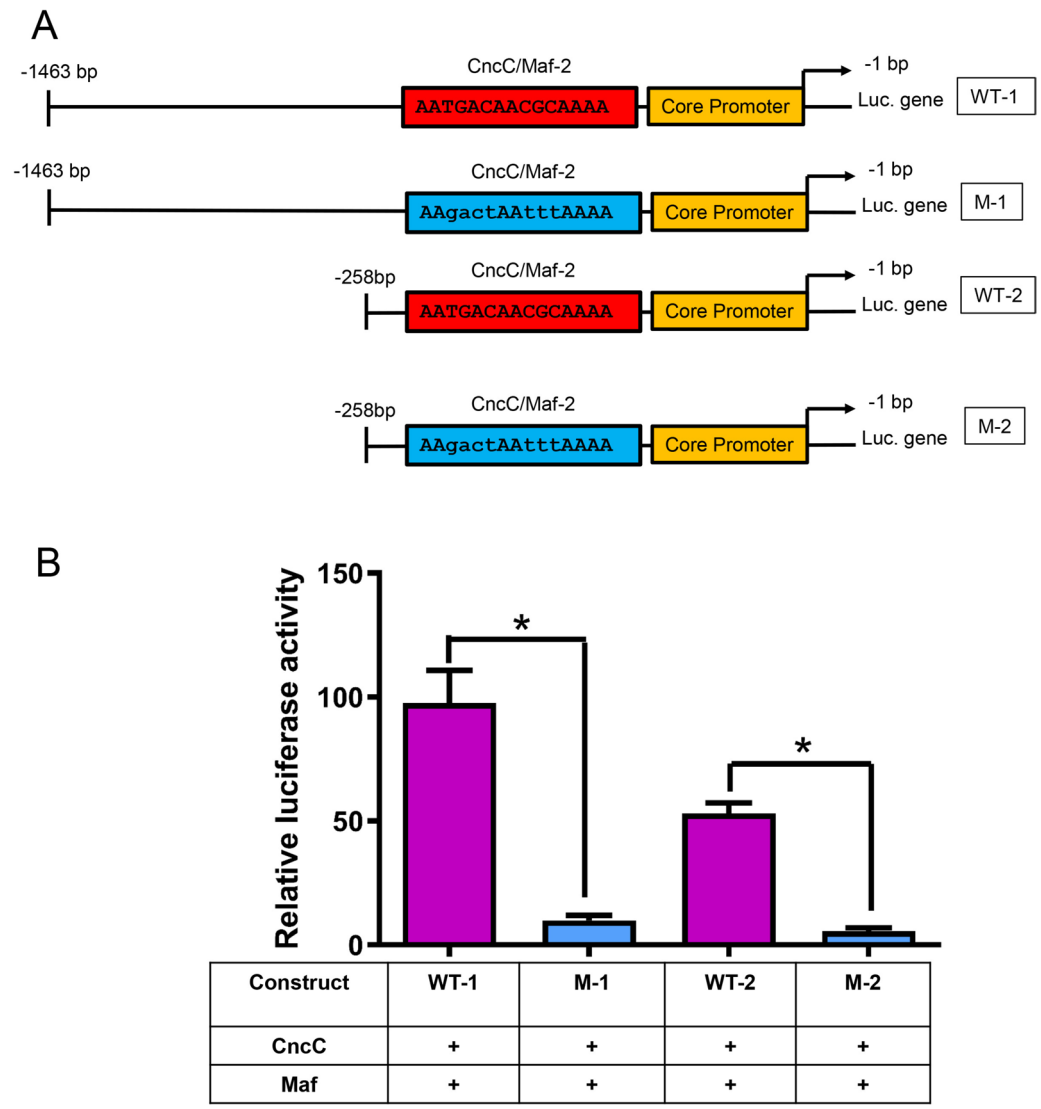

Fig. 6 Mutations in *CncC/Maf* binding sites block the ability of these transcription factors to regulate the expression of *CYP321B1*. **A** Schematic representation of mutated *CYP321B1* promoter constructs. **B** Analysis of *CYP321B1* promoter with the mutation of *CncC/Maf* binding sequence. The *CYP321B1* promoter constructs with or without mutations were transfected with *CncC/Maf* constructs into sf9 cells. The cells were harvested at 48 h after transfection, and the luciferase activities were measured. The data were shown as the mean ± SD of three independent transfections. The results were analyzed using Student's t-test and significant differences ($p < 0.05$) are indicated with an asterisk

the expression of *CYP321B1* gene, the CncC and Maf constructs, and the promoter constructs with (DNA 5'-R) or without the Nubbin binding site (DNA5'-S) were co-transfected and assayed in sf9 cells. The promoter constructs DNA5'-S co-transfected with empty vector pIB served as the control. Compared to the control, the DNA5'-R construct showed a 1.9-fold increase in the promoter activity in the absence of CncC and Maf, and then overexpression of CncC and Maf enhanced the promoter activity of DNA5'-S and DNA 5'-R by 4.3- and 8.4-fold, respectively (Fig. 8C). Amazingly, the increase in the promoter activity mediated by both *cis*-acting mutations and CncC/Maf overexpression (8.4-fold) were much greater than the sum of the individual effect (1.9-fold + 4.3-fold). These results demonstrate that overexpression of *trans*-acting factors *CncC/Maf* and *cis*-acting mutations in the promoter region synergistically regulate the overexpression of *CYP321B1* gene in the resistant strain.

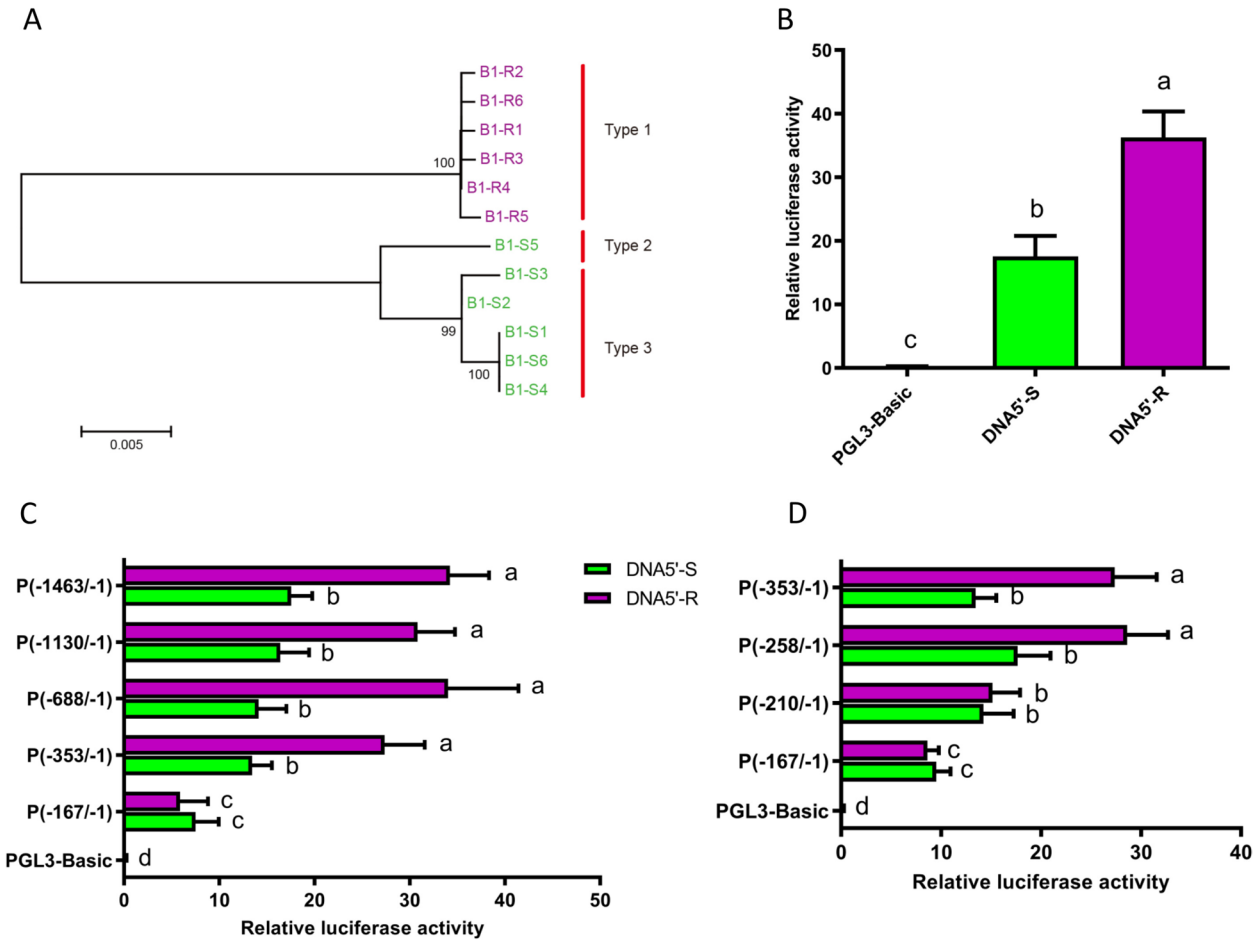

Fig. 7 *Cis*-acting mutations in the promoter region enhanced the expression of *CYP321B1* gene. **A** Phylogenetic relationship of *CYP321B1* promoter sequences from the susceptible and resistant strains. The phylogenetic tree was inferred using maximum likelihood using MEGA5. Nodes with distance bootstrap values (1000 replicates) are shown. **B** Analysis of the activity of the *CYP321B1* resistant promoter (DNA5′-R) and susceptible promoter (DNA5′-S). Error bars display SD. Letters to the right of bars denote significant differences at $p < 0.05$ (ANOVA with post-hoc Tukey HSD). **C** The activity of progressive 5′ deletion constructs of *CYP321B1* promoter from the susceptible and resistant strains. Error bars display SD. Letters to the right of bars denote significant differences at $p < 0.05$ (ANOVA with post-hoc Tukey HSD). **D** The luciferase activity of progressive 5′-deletion constructs from −353 to −167 of *CYP321B1* promoter. The average relative luciferase activity and standard errors of three independent experiments are presented. Different letters on the right of error bars indicate significant differences based on ANOVA with Tukey's HSD multiple comparison test ($p < 0.05$)

Discussion

Cytochrome P450s are the primary enzyme family that is associated with resistance to insecticides in insect species [28–30]. Although the importance of CYP450s in conferring resistance has been well known, the underlying transcriptional regulatory mechanisms of these P450 genes remain largely unknown. In the present study, we determine that upregulation of *CYP321B1* results in resistance to chlorpyrifos, permethrin and deltamethrin in the resistant strain. Two transcription factors *CncC* and *Maf* were overexpressed in the resistant strain and we demonstrate that these regulate the expression of *CYP321B1* by binding to one specific site in the promoter region of this P450 gene. In addition, we identify that *cis*-acting mutations in the promoter region of *CYP321B1* create a cis-regulatory element that facilitates binding of a POU/homeodomain transcription factor *Nubbin*, enhancing the expression of this P450 gene. Altogether, these data demonstrate that the combined action of two genetically independent mechanisms synergistically results in the overexpression of *CYP321B1* in the resistant strain of *S. exigua* (Fig. 9).

Insect P450s are known to play an important role in detoxifying insecticides and plant toxins [1, 31–34]. Constitutive overexpression of cytochrome P450 genes has been found in insecticide-resistant strains and has been

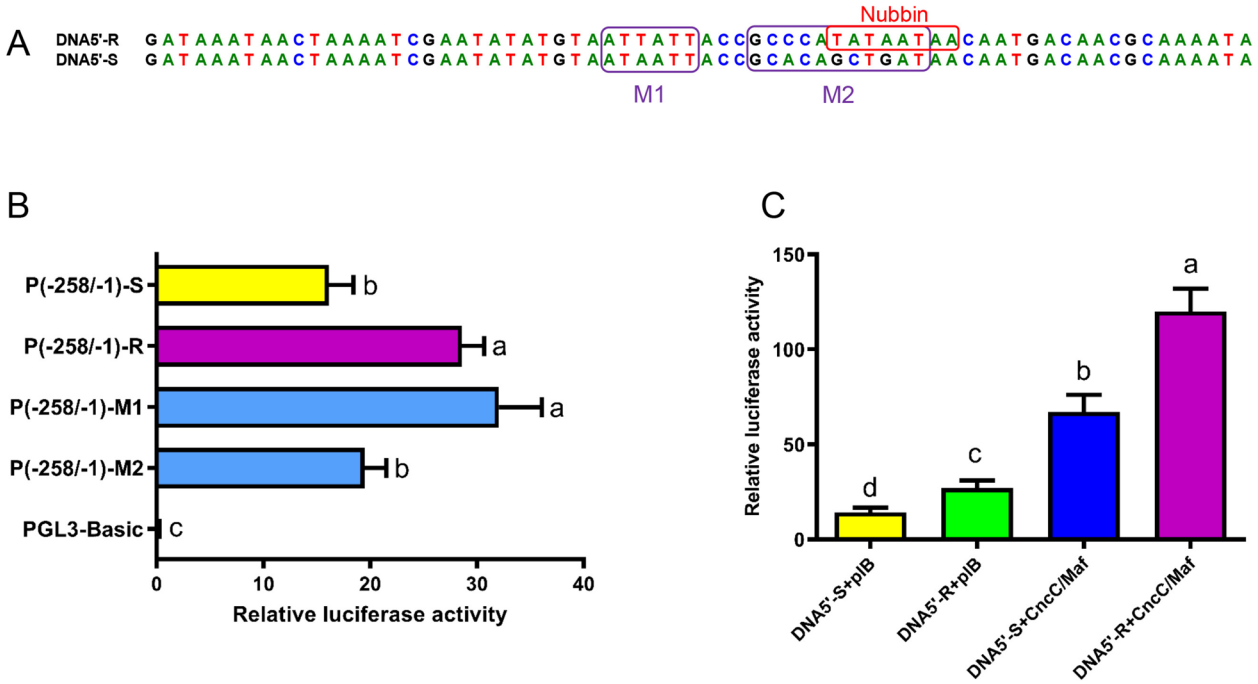

Fig. 8 Creation of a new binding site for the POU/homeodomain transcriptional regulator, *Nubbin*, resulting from the mutations in the *CYP321B1* promoter region from the resistant strain, enhances the transcriptional level of this gene. **A** Alignment of *CYP321B1* promoter sequences from the resistant and susceptible strains. Two mutation regions (M1 and M2) are marked under the corresponding nucleotides. **B** The luciferase activity of recovery mutation constructs in the region from −258 to −210. Data are the mean ± SD of three independent assays. Different letters on the right of error bars indicate significant differences based on ANOVA with Tukey's HSD multiple comparison test ($p < 0.05$). **C** Transcription factor *CncC/Maf* and *cis*-acting mutation coordinately enhance the promoter activity of *CYP321B1* gene. The promoter constructs (DNA5'-R and DNA5'-S) were transfected with or without *CncC/Maf* constructs into sf9 cells. Data are the mean ± SD of three independent assays. The data were analyzed using Tukey's HSD. Letters a, b, c and d denote significant differences at $p < 0.05$

proven to be involved in insecticide resistance [35–37]. For example, upregulation of *CYP6G1* gene in *D. melanogaster* confers resistance to DDT and imidacloprid [38]. Similarly, the *CYP6M2*, overexpressed in a resistant population of *A. gambiae*, results in the resistance to DTT and pyrethroids [39]. Finally, overexpression of *CYP6CM1* leads to the cross-resistance between imidacloprid and pymetrozine in *Bemisia tabaci*. A previous study has documented that *CYP321B1* may play an important role in chlorpyrifos and β-cypermethrin detoxification in *Spodoptera litura*, however, the relevant metabolic analysis is not conducted [40]. In this study, transgenic strains of *D. melanogaster* expressing CYP321B1 showed higher levels of tolerance to chlorpyrifos, cypermethrin and deltamethrin than the control flies with the same genetic background. Furthermore, recombinant P450 protein metabolized these insecticides. Our results determine that the increased expression of *CYP321B1* is associated with resistance to chlorpyrifos, cypermethrin and deltamethrin in vivo and in vitro. These results suggest that the functional roles of *CYP321B1* in conferring resistance to insecticides are highly conserved in lepidopteran pests and will contribute to investigating the functional role of CYP321B genes in insecticide resistance in other pests. Simultaneously, these studies indicate that insect P450s exhibit significant flexibility in metabolizing structurally diverse insecticides belonging to different mode of action classes. The beet armyworm has developed high resistance and cross-resistance to insecticides [41], which may not only be caused by a certain CYP450. Although our previous study has reported that *CYP321A8* confers cross-resistance to insecticides [27], a new P450 gene, *CYP321B1*, which not only metabolize pyrethroid insecticides but also degrade organophosphate chlorpyrifos is found in this study. These results suggest that *CYP321B1* may be one of the reasons for the cross-resistance between organophosphate and pyrethroid insecticides, and can be used as a biomarker and target for management of *S. exigua* insecticide resistance in fields.

In mammals, *Nrf2* is identified as an important regulation factor responsible for the activation of P450 genes involved in the detoxification of xenobiotic compounds [15]. Similarly, Nrf2 homolog, *CncC* and its heterodimer partner *Maf* regulate the expression of multiple P450 genes coding for enzymes that are associated with

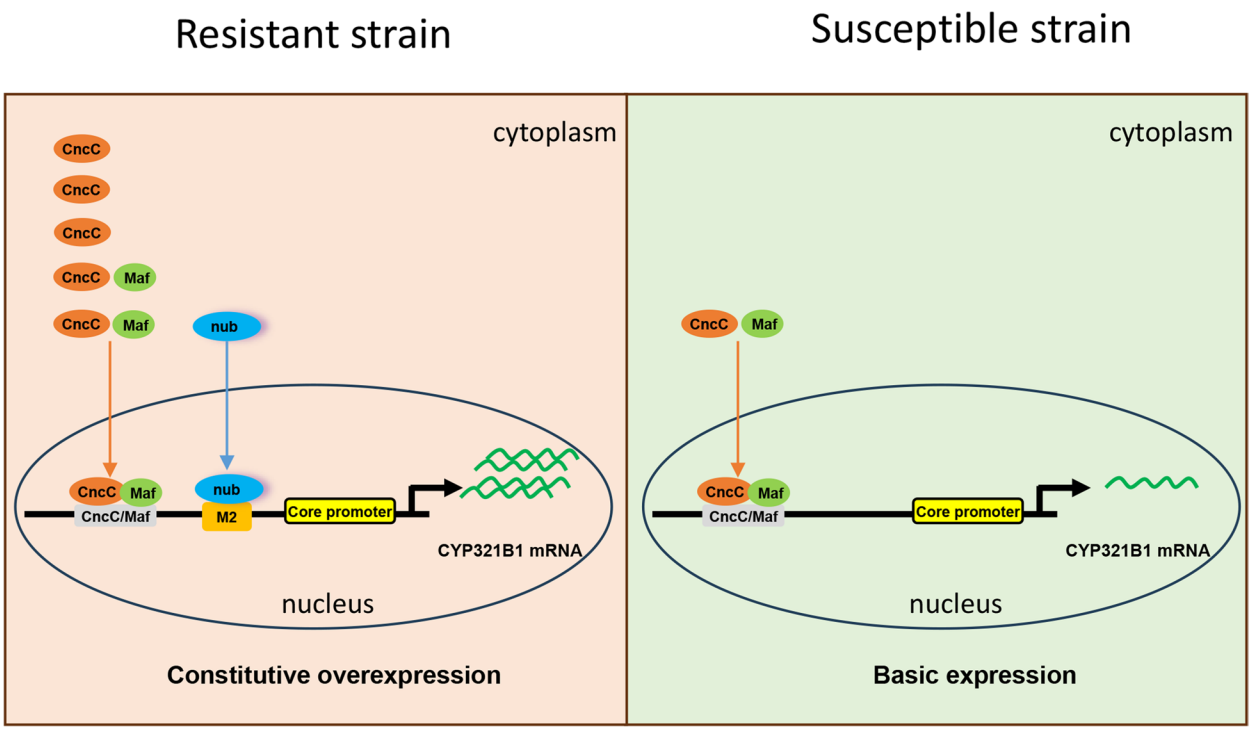

Fig. 9 Schematic representation of *trans*-acting factors and *cis*-acting mutation involved in the overexpression of *CYP321B1* gene conferring resistance to insecticides in *S. exigua*. In the resistant strain, constitutive overexpression of *trans*-acting factors *CncC* and *Maf*, and *cis*-acting mutations in the resistant promoter creating the binding site for the POU/homeodomain transcription factor *Nubbin* synergistically enhance the expression of *CYP321B1* gene associated with insecticide resistance; In the susceptible strain, *CncC* and *Maf* only regulate the basic expression of *CYP321B1* gene, and there is no binding site for *Nubbin*. Therefore, the expression level of *CYP321B1* gene is low which makes the larvae susceptible to insecticides

metabolism and detoxification of insecticides in insects. For example, *CncC* and *Maf* are constitutively overexpressed in a fenpropathrin-resistant *T. cinnabarinus* strain, and increase the expression of *CYP391A1*, *CYP391B1* and *CYP392A28*, which result in the resistance to this insecticide [21]. Similarly, *CncC* and *Maf* enhance the expression of four cytochromes P450 genes (*CYP6BJa/b*, *CYP6BJ1v1*, *CYP9Z25*, and *CYP9Z29*), which are involved in imidacloprid resistance in *Leptinotarsa decemlineata* [42]. Our previous study has shown that the transcriptional levels of *CncC* and *Maf* are upregulated in the resistant strain used in this paper [27]. Here, we determine that *CncC* and *Maf* regulate the expression of *CYP321B1* by binding to one specific regulatory element in the promoter region of this P450. In addition, we have previously shown that the increased expression levels of three p450 genes (*CYP321A8*, *CYP321A16* and *CYP332A1*), which confer resistance to insecticides in *S. exigua*, are regulated by these two key regulatory factors, *CncC* and *Maf* [27, 43]. These data suggest that the functional roles of *CncC/Maf* in mediating the expression of a series of detoxification genes conferring resistance to insecticides were conserved in insects. Therefore, these transcription factors can serve as new target genes to screen for inhibitors of detoxification genes for pest control. In this study, the *CncC/Maf* binding site was determined by reporter gene assays and site-directed mutagenesis analyses. This result is very important for improving the accuracy of in silico prediction of the binding sites of these transcription factors in insects.

In this study, we also identify that *cis*-acting mutations in the promoter region of *CYP321B1* from the resistant strain enhance the transcriptional level of this P450 gene. A few studies have documented that insertions/deletions or mutations in the promoter region contribute to insecticide resistance by increasing the expression of P450 genes. For example, a single nucleotide substitution in the promoter region of *CYP9M10* enhances the expression of this gene in *Culex quinquefasciatus* [44]. Similarly, the changes of *cis*-regulatory elements result in the upregulation of *CYP6P9a* and *CYP6P9b* involved in resistance to pyrethroid insecticides in the major African malaria vector *Anopheles funestus* [45]. Finally, multiple *cis*-acting mutations in the promoter region lead to the overexpression of *CYP6FU1*, which is responsible for

deltamethrin resistance in *L. striatellus* [46]. Together with the results of our study, these findings illustrate that changes in *cis*-acting mutations in the promoter region often confer resistance to insecticides by controlling the overexpression of the P450 genes associated with the metabolism of insecticides in insects. Meantime, these mutations can act as markers for identification of the frequency and distribution of resistance, and further design effective integrated pest management programmes. In this study, we indicate that creation of a new binding site for the POU/homeodomain transcriptional regulator, *Nubbin*, resulting from the mutation in the *CYP321B1* promoter region from the resistant strain, enhances the transcriptional level of this gene compared with the susceptible strain. In *D. melanogaster*, *Nubbin* regulates the expression of genes that are involved in immune, differentiation, stress responses and metabolism [47]. Here, we showed that *Nubbin* may act as a regulator of P450 genes in *S. exigua*.

Although few studies have reported that *cis*- and/or *trans*-acting factors regulate the expression of P450 genes that are associated with insecticide resistance, how these regulatory mechanisms interact, is unclear. For example, a 15 bp insert in the promoter region of *CYP6D1* enhances the transcriptional level of this P450 gene involved in resistance to pyrethroid insecticides in housefly [48]. Further studies showed that an unknown *trans*-acting factor located in chromosome 2 also results in the increased expression of *CYP6D1,* however, whether this unknown *trans*-acting factor and the 15 bp insert in the promoter region act in combination to regulate the expression of *CYP6D1* has not been identified [49]. In this study, our results showed that the combined effect of *cis*- and *trans*-acting factors was much greater than the sum of their individual effects, suggesting that *cis*- and *trans*-acting factors regulate the expression of *CYP321B1* in a synergistic manner. Altogether, these studies suggest that insect pests have evolved two independent regulatory mechanisms that work in concert to resist insecticides. The beet armyworm has developed high resistance to various groups of chemical insecticide classes, and even cross-resistance was found in the filed populations [41]. Although our previous study has indicated that *CncC/Maf* and mutations in the promoter region control the expression of *CYP321A8* [27], new transcription factor and regulatory mechanism contributing to insecticide resistance is reported in this study. These results suggest that insect pests have evolved complex and diverse regulatory mechanisms that contribute to the adaptation of resistant populations to insecticides and there may be other regulatory mechanisms that need to be further explored.

Conclusions

In summary, the current study identified upregulation of *CYP321B1* confers resistance to chlorpyrifos, cypermethrin and deltamethrin in the resistant strain. Furthermore, constitutive overexpression of transcription factors *CncC /Maf* is associated with the enhanced expression of *CYP321B1* by binding to a special binding site in the promoter region of this P450 gene. In addition, *cis*-acting mutations in the promoter region of *CYP321B1* creating a *cis*-regulatory element that recruits the binding of the transcription factor *Nubbin*, results in the upregulated expression of this P450 gene. Finally, these results present the evidence that *cis*- and *trans*-acting factors synergistically upregulate the expression of P450 gene conferring resistance to insecticides in an agriculturally important insect pest and provide us with more insights into the design of effective integrated pest management strategies for the pest control.

Methods

Insect strains

The Wuhan susceptible strain of *S. exigua* used in this study was obtained from Wuhan Kernel Bio-pesticide Company, Hubei, China and the chlorpyrifos resistant strain was collected from *Allium fistulosum* in Huizhou, Guangdong province, China [27]. According to our previous method [26], the resistant strain was selected for continuous generations by exposing neonate larvae to chlorpyrifos at a LC_{70} concentration. Larvae were reared on an artificial diet at 25 °C under a 16-h light/8-h dark photoperiod with a relative humidity of 60 ± 5% [25].

DNA/RNA extraction and cDNA synthesis

Total RNAs were extracted from larvae using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. First-strand cDNAs were synthesized using the Supermo III RT Kit (BioTeKe, Beijing, China) according to the operation manual and stored at −25 °C. An Insect DNA Kit (Omega Bio-Tek Inc., Norcross, Georgia, USA) was utilized to isolate the *S. exigua* Genomic DNA.

Functional expression of CYP321B1 in Sf9 cells

CYP321B1 was expressed in Sf9 cells using a baculovirus expression system according to our previous approaches [27]. All the primers are shown in Additional file 1: Table S1. Empty pFastBacHTA vector served as a negative control. Bacmids were transfected into sf9 cells using FUGEGE transfection reagent (Promega, Madison, WI, USA), and virus titer was tested by plaque assay and qPCR [50]. Various multiplicity of infection ratios was used to determine the optimal conditions. After 48 h,

the infected cells were collected and washed with 0.1 M potassium phosphate buffer (PH 7.4, with 20% glycerin), and the microsome of the membrane fraction was prepared according to standard procedures [51] and stored at − 80 °C. The concentration of total protein was quantified by the Bradford method using bovine serum albumin as the standard [52]. P450 content was measured in reduced samples using CO-difference spectra [53].

Enzyme activities to model substrate

The fluorescent P450 model probe substrate7-ethoxy coumarin (ECOD) was used to identify the O-dealkylation of recombinant P450s as described previously [54]. The assay was carried out at 30 °C in 0.1 M potassium phosphate buffer (pH 7.4) with 0.1 mg microsomes, 100 mM ECOD and 500 μM cumene hydroperoxide (CuOOH) for a final volume of 200 μL. Microsomes and substrate ECOD were incubated at 30 °C for 5 min before adding CuOOH. The fluorescence was measured in a TECAN microplate reader (Infinite™ M200) at 380 nm excitation, 460 nm emission and 30 °C for 15 min. P450 activity was calculated based on the 7-hydroxycoumarin standard curve and was expressed as mean pmoles of 7-OH per pmole of microsomal protein/min ± SD.

HPLC analysis of insecticide metabolism

For chlorpyrifos, cypermethrin and deltamethrin metabolism, in vitro reactions were carried out according to our previous methods [27]. The non-insertion microsomes served as the control. The reactions were performed at 30 °C with 700 rpm/min oscillation for 1.5 h, and stopped by adding 500 μL acetonitrile and incubated for further 20 min. Finally, the quenched reactions were centrifuged at 16,000 g for 10 min and 200 μL supernatant was transferred to HPLC vials and analyzed immediately by reverse phase HPLC using a C18 column (Ameritech Technology, USA, 4.6 × 250 mm in length, 5 μm particle size) with 80% methanol, 90% methanol and 82% acetonitrile as the mobile phase for chlorpyrifos, cypermethrin and deltamethrin, respectively, with a flow rate of 1 ml min^{-1}. The quantity of chlorpyrifos, cypermethrin and deltamethrin remaining in the samples was determined at a monitoring absorbance wavelength of 289, 230 and 240 nm, respectively. The insecticide was quantified by peak integration and calculated based on the standard curves.

Construction of transgenic *D. melanogaster* and bioassays

The UAS-CYP321B1 strain was constructed according to the previous methods [27]. UAS-CYP321B1 flies were generated and crossed with Act5C-GAL4 lines and the offspring were used in the insecticide bioassays. The mRNA level of *CYP321B1* was analyzed by RT-PCR and qRT-PCR. All primers are shown in in Additional file 1: Table S1. For insecticide bioassays, each assay needed ten adult flies (2–5 days old) and the progeny from the w1118 and Act5C-GAL4 strains served as controls. The flies (2–5-day-old) were added to each vial with 10 ml corn meal medium containing 0.05 mg/L chlorpyrifos, 0.4 mg/L deltamethrin and 0.6 mg/L permethrin. At least six replicates were used for each experiment.

Homology modeling and docking

Three-dimensional structures of insecticides (chlorpyrifos, cypermethrin and deltamethrin) were obtained from the PubChem servers. The P450 protein model was generated by using SWISS-MODEL. AutoDock v4.2 software was used to perform the molecular docking between P450 protein and insecticides [55]. The modeling and docking results were visualized in PyMOL.

Cloning and sequencing the 5′-flanking regions

Four different restriction enzymes including DraI, EcoRV, PvuII, and StuI, were used to digest the genomic DNAs according to the operation manual of the Universal Genome Walker Kit (Clontech, Palo Alto, CA, USA). Genome Walker adaptors were ligated into the DNA fragments using T4 DNA ligase. The target sequences were amplified using LATaq polymerase (Takara, Japan), and ligated into the PMD-19 T vectors and sequenced. The primers are shown in Additional file 1: Table S3. All the sequences have been deposited in GenBank (Accession nos: MK327549 and MK327550). The putative TF binding sites in 5′-flanking regions were predicted and analyzed by the online software JASPAR and ALLGEN [46, 56].

Reporter and expression constructs

Promoter region sequences (DNA5′-R and DNA5′-S) were amplified and inserted into the firefly luciferase reporter vector PGL3-Basic (Promega, Madison, WI, USA) between the XhoI and KpnI restriction sites. Various promoter truncations were amplified using the full promoter region DNA as a template. A Mut Express II Fast Mutagenesis Kit was utilized to generate the mutated promoter truncations following the manufacturer's instructions [57]. The open reading frames from *CncC* and *Maf* were ligated into the expression vector, pIB/V5-His (Invitrogen, San Diego, CA, USA). The primers used in this study are shown in Additional file 1: Table S4.

Luciferase reporter assays

The luciferase reporter assays were performed according to the previous description [58]. The sf9 cells were seeded in 24-well cell culture plates with a density of

4 × 105 cells/well. 0.5 μg of the promoter construct, 0.5 μg expression plasmid of CncC or/and Maf and 0.02 μg of pRL-CMV (Promega, Madison, WI, USA) were transfected using 2 μL of FUGENE transfection reagent (Promega, Madison, WI, USA) following the operation manual. 2 μL of FUGENE transfection reagent was used to transfect a mixture of 1 μg promoter constructs (DNA5'-R, DNA5'-S, promoter truncations and mutated promoter truncations) and 0.02 μg pRL-CMV. After 48 h post-transfection, the cells were harvested and the luciferase activities were measured.

Statistical analysis

These data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and were presented as the mean ± standard deviation (SD). Student's t-test was carried out for the comparison of two samples. One-way ANOVA with Tukey's HSD tests was used to determine the statistical significance of differences among more than two groups. A value of $P < 0.05$ was considered statistically significant.

Abbreviations

Nrf2	Nuclear factor erythroid 2 related factor 2
AREs	Antioxidant response elements
NQO1	Quinone oxidoreductase-1
UGT	UDP-glycosyltransferase
HO-1	Heme oxygenase 1
CncC	Cap 'n' Collar isoform-C
Maf-S	Muscle aponeurosis fibromatosis
HPLC	High-performance liquid chromatography
ECOD	Substrate7-ethoxy coumarin
CuOOH	Cumene hydroperoxide
SD	Standard deviation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-025-02228-5.

Additional file 1: Table S1. Primers for the eukaryotic expression of P450 genes from *S. exigua*. Table S2. The primers used for cloning 5'-flanking regions. Table S3. The primers used for reporter and promoter constructs. Figure S1. Alignment of upstream sequences of *CYP321B1* gene from susceptible and resistant strains of *S. exigua*.

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Not applicable.

Authors' contributions

Conceptualization, J.Y.S.; Methodology, B.H., Y.T.Z., X.Z.C, A.J.T and J.Y.S.; Investigation, B.H. and Z.P.X.; Visualization, B.H., Y.T.Z. and C.R.; Writing – Original Draft, B.H. and J.Y.S.; Writing – Review & Editing, B.H., J.Y.S., A.J.T and K.T.L.; Funding Acquisition, B.H., A.J.T and J.Y.S. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in the manuscript and Supplementary material. Supplementary material contains all primers used to amplify gene sequences and construct expression vectors. *S. exigua* genomic sequences have been deposited in GenBank (Accession nos: MK327549 and MK327550) [59, 60].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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