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Chemosensory proteins participates in insecticide susceptibility in *Rhopalosiphum padi*, a serious pest on wheat crops

X. Peng[*], M. J. Qu†, S. J. Wang[*], Y. X. Huang[*], C. Chen[1] and M. H. Chen[*]

[*]Northwest A&F University, State Key Laboratory of Crop Stress Biology for Arid Areas, Key Laboratory of Crop Pest Integrated Pest Management on the Loess Plateau of Ministry of Agriculture and Rural Affairs, Northwest A&F University, Yangling 712100, Shaanxi, China.

†Shandong Academy of Agricultural Sciences, Shandong Peanut Research Institute, Qingdao 266100, Shandong, China

Corresponding author: Maohua Chen, Northwest A&F University, Yangling 712100, Shaanxi Province, China.

E-mail: maohua.chen@nwsuaf.edu.cn; Tel: +86-029-87091853

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Abstract

Rhopalosiphum padi is a worldwide agricultural pest. Chemosensory proteins (CSPs) are considered to be a type of transporters which can bind chemicals from external environments. Previous researches showed that the expression of some insect *CSPs* were significantly increased after exposure to insecticides, and CSPs were involved in insecticide resistance or susceptibility. However, the role of CSPs in the susceptibility and response of *R. padi* to insecticides is still unknown. In this study, we identified eight *CSP* (*RpCSP*) from *R. padi* by genome-wide investigation. Seven *RpCSP* genes had two exons, while *RpCSP7* had three exons. qPCR analyses showed that the mRNA levels of the eight *RpCSP* genes were significantly affected by imidacloprid and beta-cypermethrin in different post-treatment periods. Molecular docking predicted that there were hydrogen bonding sites which played key roles in binding of *RpCSP4*, *RpCSP5*, *RpCSP6*, *RpCSP7* and *RpCSP10* with imidacloprid and beta-cypermethrin. Knockdown of *RpCSP4*, *RpCSP5*, *RpCSP6* and *RpCSP10* by RNA interference significantly increased the aphid mortality under two sublethal concentrations of imidacloprid. Mortalities under two sublethal concentrations of beta-cypermethrin conditions were significantly higher after injection of *R. padi* with ds*CSP4* and ds*CSP6*. The results indicate that some *RpCSP* genes are involved in the insecticide susceptibility of *R. padi*.

***Keywords*:** molecular docking; beta-cypermethrin; imidacloprid; survival rate; chemosensory proteins; bird cherry-oat aphid

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Introduction

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), which is a worldwide agricultural pest, is abundant in different developmental stages of wheat (Peng *et al.*, 2017). Apart from direct feeding by sucking plant liquid and nutrients, the aphid also transmits the barley yellow dwarf virus (BYDV), causing severe economic losses (Schliephake *et al.*, 2013). Because of global warming conditions and increases in the annual frequency of extreme high temperature events, damage caused by *R. padi* has been increasing annually (Cao, 2006; Ma *et al.*, 2015; Peng *et al.*, 2020b). In recent years, insecticide application is the primary approach to control cereal aphids, and various types of insecticides have been used to control *R. padi* in China. Imidacloprid, one of the neonicotinoid insecticides, is widely used against aphids, whiteflies, and planthoppers (Jeschke and Nauen, 2008). Beta-cypermethrin, one of the pyrethroid insectcides, is one of the most commonly used options for controlling aphids on cereals in China (Zuo *et al.*, 2016a). However, serious ecological and environmental problems caused by the massive use of insecticides have recently attracted much attention. Moreover, the use of insecticides can cause other problems, such as insecticide resistance (Bass *et al.*, 2014). Our previous research analyzed the regional susceptibilities of 12 *R. padi* populations to 10 different insecticides in China. The results showed there were moderately resistant populations to abamectin, decamethrin, bifenthrin and imidacloprid, and low resistance populations to pymetrozine, beta-cypermethrin, thiamethoxam, acetamiprid and malathion (Zuo *et al.*, 2016b).

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Chemosensory proteins (CSPs) belong to a group of small soluble transporting proteins. Since *CSPs* were first discovered in *Drosophila melanogaster*, they have been attracting much interest (McKenna *et al.*, 1994). Currently, CSPs have been discovered in many insect taxa (Pelosi *et al.*, 2018). The number of *CSP* genes in different insect taxa is variable (Pelosi *et al.*, 2006; Xu *et a*l., 2009; Fan *et al.*, 2011; Pelosi *et al.*, 2018). Through analyzing genomic and transcriptomic data of aphid, the total number of *CSPs* in *Myzus persicae*, *Aphis glycines* and *Acyrthosiphon pisum* are 9, 9 and 13, respectively (Zhou *et al.*, 2010; Mathers *et al.*, 2017; Wenger *et al.*, 2017). The genome data of *R. padi* have been published by Thorpe *et al.* (2018) and Ramiro *et al.* (2020); however, an analysis of CSPs on the genome level has not been reported in *R. padi.*

Previous researches showed that chemosensory proteins could not only distinguish and selectively bind hydrophobic chemicals from external environments (Sánchez-Gracia *et al.*, 2009; Pelosi *et al.*, 2018; Peng *et al.*, 2020a), but also had some other miscellaneous functions, such as solubilization of chemicals in other tissues (Laughlin *et al.*, 2008; Zhou *et al.*, 2013) and insecticide binding (Lin *et al.*, 2018a, 2018b; Xiong *et al.*, 2019; Ingham *et al.*, 2020). Insecticide treatment can significantly affect the expression levels of some *CSP* genes in *Bemisia tabaci* (Liu *et al.*, 2014, 2016), *Bombyx mori* (Xuan *et al.*, 2015), *Plutella xylostella* (Bautista *et al.*, 2015), *Apis cerana* and *A. mellifera* (Li *et al.*, 2017, 2019), *Spodoptera litura* (Lin *et al.*, 2018a, 2018b) and *Tribolium castaneum* (Xiong *et al.*, 2019). Further analysis of the relationship between *CSPs* and insecticide

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sensitivity showed that knockdown of *SlituCSP18* decreased the survival rates of *Spodoptera litura* after feeding on chlorpyrifos (Lin *et al.*, 2018a). RNA interference targeting of *TcCSP10* significantly increased the mortality rate of *T. castaneum* after exposure to dichlorvos or carbofuran (Xiong *et al.*, 2019). More specifically, Ingham *et al.* (2020) proved that sensory appendage protein (SAP2), a member of the CSP family, has a vital role in pyrethroid resistance of *Anopheles gambiae*; the silencing of *SAP2* fully restored mortality of the mosquitoes, whereas the overexpression of *SAP2* resulted in increased pyrethroid resistance. Currently, there are no researches about the effects of insecticides on the *CSP* genes in aphids, and the functions of *CSP*s in aphids fighting against insecticides are still unknown.

In this study, our objective was to investigate the role of *CSP* genes in insecticide susceptibility of *R. padi*. We hypothesized that *R. padi* CSPs participates in insecticide susceptibility by binding to the chemicals. We tested the susceptibility of *R. padi* to imidacloprid and beta-cypermethrin. Their *RpCSPs* were identified by analysis of the genome data of *R. padi*, and the expression profiles of the *RpCSP*s were investigated at different posttreatment periods of *R. padi* exposure to imidacloprid and beta-cypermethrin. The interactions of CSPs and insecticides were predicted by molecular docking and the roles of *RpCSP*s in the insecticide susceptibility of *R. padi* were investigated. Our study provides knowledge for studying the correlation of olfactory systems with insecticides and the function of CSP proteins on the adaption of aphids to stress conditions.

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Results

Susceptibility of R. padi to imidacloprid and beta-cypermethrin

The toxicity regression equation of imidacloprid on *R. padi* was Y = 4.59 + 1.90X (Fig. S1a). The toxicity regression equation of beta-cypermethrin was Y = 4.75 + 2.01X (Fig. S1b). The sublethal concentration values (LC_{40}) of imidacloprid and beta-cypermethrin were 1.21 (95% confidence limit, 1.00–1.44) and 1.00 (95% confidence limit, 0.80–1.19) mg/L, respectively. The LC_{60} of imidacloprid and beta-cypermethrin were 2.24 (95% confidence limit, 1.91–2.64) and 1.78 (95% confidence limit, 1.52–2.08) mg/L, respectively. The results are shown in Figure S1.

Characteristics and gene structure of the RpCSP genes.

Before the genome of *R. padi* was published, we had cloned seven CSP genes through RT-PCR and Rapid amplification of cDNA ends (RACE) technology. After the new *R. padi* genome was published, we found a novel *RpCSP* gene (*RpCSP10*) that had not been cloned before (Thorpe *et al.*, 2018; Ramiro *et al.*, 2020). Analysis of the eight deduced amino acid sequences showed that the RpCSPs had a typical four-cysteine motif in the conserved domain (Fig. 1a). Comparisons of genomic and nucleotide sequences indicated seven of the *RpCSP* genes had only two exons, while *RpCSP7* had three exons (Fig. 1b). The alignment of the four-cysteine motif sequences revealed the RpCSPs had

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a shared conserved proline (P) between the second and third "C" (Fig. 1c). Seven RpCSPs contained a common conserved olfactory specific protein D (OS-D) domain, and RpCSP7 contained the OS-D superfamily domain (Fig. 1d). Phylogenetic analysis indicated that the *RpCSP* genes clustered in different clades (Fig. 1e). Among them, *RpCSP5*, *RpCSP8* and *RpCSP10* clustered with one homologous *CSP*s from *Bemisia tobaci* in one branch. Interestingly, *RpCSP6* and SAP2 (AAL84186.1), which had a vital role in pyrethroid resistance of *A. gambiae* were clustered together (Ingham *et al.,* 2020).

Expression patterns of RpCSPs in different periods under sublethal conditions

The mRNA levels of eight *RpCSP* genes in *R. padi* exposed to sublethal concentration (LC_{60}) of imidacloprid and beta-cypermethrin are shown in Figure 2. The expression levels of *RpCSP1* (2.60±0.42; $P = 0.021$) and *RpCSP2* (1.53±0.09; $P = 0.0065$) in *R. padi* exposed to imidacloprid were significantly increased 48 h posttreatment compared with that of the control group. The expression of *RpCSP4* in *R. padi* was significantly affected by insecticides treatment; a significant increase in *RpCSP4* expression was observed at 6 h (1.68±0.21; $P = 0.040$) and 48 h (1.38±0.10; $P = 0.022$) after imidacloprid treatment compared with that of the control group. After exposure to beta-cypermethrin, the transcripts of *RpCSP4* at 3 h (1.17±0.05; $P = 0.042$) and 24 h (1.90±0.19; $P = 0.011$) were significantly higher than those in the control treatment. After imidacloprid treatment, the expression of *RpCSP5* at 12 h (3.35±0.40; $P = 0.0084$) and 48 h (2.86±0.17; $P < 0.001$)

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was significantly higher than in the control. No significant difference was found in *RpCSP5* between the beta-cypermethrin treated group and control. A significant decrease in *RpCSP6* expression was observed at 3 h after imidacloprid (0.52±0.02; P = 0.0048) and beta-cypermethrin (0.59±0.10; P = 0.029) treatment compared with that of the control group. After exposing *R. padi* to two insecticides, the expression levels of *RpCSP6* at 24 h (imidacloprid: P = 0.0010; beta-cypermethrin: P = 0.0053) and 48 h (imidacloprid: P = 0.0011; beta-cypermethrin: P = 0.0034) were significantly higher than that of the control. The mRNA levels of *RpCSP6* were significantly higher at 12 h for beta-cypermethrin (3.19±0.39; P = 0.0062) than that of the control. The expression levels of *RpCSP7* in *R. padi* exposed to beta-cypermethrin were significantly increased in 3 h (1.30±0.07; P = 0.032) and 48 h (1.39±0.09; P = 0.0031) posttreatment compared with those of the control group. After exposing aphids to imidacloprid, a significant increase in *RpCSP7* (1.90±0.27; P = 0.031) and *RpCSP8* (1.44±0.07; P = 0.031) expression was observed at 48 h compared with that of the control group. Compared with the control group, the *RpCSP10* gene tended to be upregulated following imidacloprid treatment in *R. padi*, and no significant changes were detected in *RpCSP10* expression between the beta-cypermethrin treated group and control.

Molecular docking study

To better understand the effect of RpCSPs on insecticide susceptibility of *R. padi*, the 3D

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homology models of RpCSPs were constructed, followed by analyses of the binding pose of the CSP-imidacloprid complex and CSP-beta-cypermethrin complex. The sequence similarities and the Global Model Quality Estimation (GMQE) values of the CSP protein and homologous protein template are shown in Table S2. *RpCSP*8 cannot be used for molecular docking study because the similarity between RpCSP8 protein and the corresponding template is less than 30%. The 3D conformer structures of imidacloprid and beta-cypermethrin were downloaded from the ZINC website and are shown in the attachment. The potential binding sites of RpCSP protein with imidacloprid or beta-cypermethrin were calculated continuously for a total of 100 times using AutoDock Tools. The lowest negative binding energy value was considered to be the optimal binding position of the RpCSP and insecticide, as shown in Table S3.

As shown in Figure 3, a hydrogen bond was found between Asn91 (nitrogen atom) of RpCSP4 and imidacloprid (oxygen atom), and the distance of the hydrogen bond was 3.2 Å (Fig. 3a). In 3D conformer structure, there was a hydrogen bond between Tyr36 (nitrogen atoms) and beta-cypermethrin (oxygen atom) with a 2.9 Å distance (Fig. 3b). There was a hydrogen bond between Gln73 of *Rp*CSP5 and imidacloprid with a 2.9 Å distance (Fig. 3c). A hydrogen bond (2.9 Å) was found between His61 (nitrogen atom) and beta-cypermethrin (oxygen atom) (Fig. 3d). In 3D conformer structure of the RpCSP6-imidacloprid complex, two hydrogen bonds could be found between Asn32 and Lys90 of RpCSP6 with imidacloprid; the distances of the hydrogen bonds were 3.1 and

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2.8 Å, respectively (Fig. 3e). A hydrogen bond could be found between Asn34 (2.7 Å) of RpCSP6 with beta-cypermethrin (Fig. 3f). In both the detailed planar and 3D conformer structures, a hydrogen bond could be found between Ser85 of RpCSP10 with imidacloprid; however, the distances of the hydrogen bond were different in the two types of conformer structures (Fig. 3g, 4). There was a hydrogen bond between Tyr35 of RpCSP10 and beta-cypermethrin with a 2.95 Å distance in the detailed planar conformer structure, however, no hydrogen bond was found in 3D conformer structure (Fig. 3h, 4).

A detailed planar binding plot between RpCSP4 protein and imidacloprid was obtained using Ligplot+ Version 2.1; there was a hydrogen bond between Asn91 and imidacloprid with a 3.18 Å distance; imidacloprid was located in one region composed of 9 residues, including Ala35, Tyr36, Thr37, Thr38, Tyr40, Glu71, Asn91, Thr93 and Gln94 (Fig. 4a). In the detailed planar conformer structure, beta-cypermethrin was located in one region composed of 8 residues (Pro34, Ala35, Tyr36, Thr37, Thr38, Tyr40, Glu71 and Glu74); two hydrogen bonds were found between Tyr36 and Pro34 with beta-cypermethrin; the distances of the two hydrogen bonds were 2.94 and 2.80 Å, respectively (Fig. 4b). Imidacloprid was located in one region composed of 9 residues, and a hydrogen bond could be found between Gln73 (2.92 Å) with imidacloprid in the detailed planar conformer structure (Fig. 4c). In the detailed planar conformer structure of the RpCSP5-beta-cypermethrin complex, a hydrogen bond (2.90 Å) was found between His61 and beta-cypermethrin which was located in one region composed of 8 residues

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(Phe43, Leu46, Ser60, His61, Lys63, Pro70, Val72 and Gln74) (Fig. 4d). Three hydrogen bonds could be found between Tyr30 (3.05 Å), Gly87 (3.20 Å) and Lys90 (2.85 Å) of RpCSP6 with imidacloprid (Fig. 4e). Compared with the 3D conformer structure, an additional hydrogen bond (3.15 Å) was found between Lys25 of RpCSP6 and beta-cypermethrin in the detailed planar structure (Fig. 4f). In detailed planar structure of the RpCSP10- imidacloprid complex, imidacloprid was located in one region composed of 9 residues, and a hydrogen bond could be found between Ser85 with imidacloprid, the distance of the hydrogen bond was 2.83 Å (Fig. 4g). A hydrogen bond was found in the detailed planar conformer structure, and beta-cypermethrin was located in one region composed of 10 residues (Fig. 4h).

The binding positions of the RpCSP1 and the insecticide are shown in Figure S2. A hydrogen bond could be found between Gln99 (3.2 Å) with imidacloprid in the 3D (Fig. S2a) and detailed planar (Fig. S2b) conformer structures. The combination situations of RpCSP2 and beta-cypermethrin were different in the detailed planar and 3D conformer structures (Fig. S3). There was a hydrogen bond (2.0 Å) between Tyr125 of RpCSP7 and imidacloprid in the 3D conformer structure (Fig. S4a). In the detailed planar conformer structure, imidacloprid was located in one region composed of 9 residues; a hydrogen bond (2.80 Å) was found between Tyr125 with imidacloprid (Fig. S4b). No hydrogen bond was found between RpCSP7 and beta-cypermethrin in the 3D conformer structure (Fig. S4c). A hydrogen bond could form between Val82 (2.82 Å) with beta-cypermethrin (Fig.

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S4d). Because a target protein sequence that had high similarity with RpCSP8 was not found (lower than thirty percent), we did not further analyze the interactions of RpCSP8 and insecticides using molecular docking.

The role of RpCSPs in the insecticide susceptibility of R. padi

The RNA interference efficiency of three *RpCSP* genes (*RpCSP4*, *RpCSP5* and *RpCSP6*) was determined in our previous research (Peng *et al*., 2020b). Because the RNAi efficiency of *RpCSP1*, *RpCSP2, RpCSP7* and *RpCSP8* were too low, we did not further analyze the function of these four CSP genes in the insecticide susceptibility of *R. padi*. The *RpCSP10* gene expression level was significantly decreased (reduced by 43.72%; P = 0.044) on day 2 after injection of dsCSP10 compared with the control (injection of dsGFP) (Fig. 5a). The silencing of *RpCSP10* resulted in a significant increase in mortality of *R. padi* treated with two different concentrations of imidacloprid (Fig. 5b). After knockdown of *RpCSP10*, the mortality rate of *R. padi* was significantly increased compared with that of dsGFP injection after exposure to LC_{40} (P = 0.0025) or LC_{60} (P = 0.013) imidacloprid; RNA interference of *RpCSP10* could not significantly affect the mortality of *R. padi* in response to the two different concentrations of beta-cypermethrin.

RNAi of the *RpCSP4*, *RpCSP5* and *RpCSP6* genes could also affect the mortality of *R. padi* treated with different concentrations of insecticides (Fig. 6). Compared to aphids injected with dsGFP, the mortalities of *R. padi* injected with dsCSP4 were significantly

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increased after exposure to LC_{40} (P < 0.001) or LC_{60} (P = 0.0026) imidacloprid and LC_{40} (P = 0.0020) or LC_{60} (P = 0.042) beta-cypermethrin (Fig. 6a). After knockdown of *RpCSP5*, the mortality rate of *R. padi* was significantly increased compared with that of dsGFP injection after exposure to LC_{40} (P = 0.0019) or LC_{60} (P = 0.011) imidacloprid. Silencing of the *RpCSP5* gene did not affect the survival of the aphids exposed to LC_{40} (P = 0.13) or LC_{60} (P = 0. 42) beta-cypermethrin (Fig. 6b). The mortalities of *R. padi* injected with dsCSP6 were significantly increased compared with the control group after injection of dsGFP and treatment with two different concentrations of imidacloprid (LC_{40}: P < 0.001; LC_{60}: P = 0.0015) and beta-cypermethrin (LC_{40}: P = 0.0014; LC_{60}: P = 0.0077) (Figure 6c).

Discussion

Previous numerous studies focused on the canonical functions of CSPs in chemosensory systems, attempting to elucidate how CSP detected and combined with chemical volatile molecules (Yi *et al*., 2013; Pelosi *et al*., 2018; Li *et al*., 2019). However, in recent years, several miscellaneous functions from behavior to some physiological and biological processes beyond the chemosensory have been reported, including reducing inflammation (Mans *et al*., 2007), pheromone delivery (Iovinella *et al*., 2011), modulating the behavioral phase change (Guo *et al*., 2011), survival and reproduction (Gong *et al*., 2012), integument and moulting process of larvae (Cheng *et al*., 2015), innate immunity (Liu *et al*., 2016), carriers of visual pigments (Zhu *et al*., 2016). Interestingly, some

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chemosensory genes including odorant binding proteins (OBPs) and CSPs were found to be significantly upregulated in some insect species after treatment with insecticides (Liu *et al*., 2014; Bautista *et al*., 2015; Xuan *et al*., 2015; Li *et al*., 2017; Pelosi *et al*., 2018). Further research showed that CSPs might be vital players in binding, sequestering and masking insecticides in some insect taxa (Lin *et al*., 2018a, 2018b; Xiong *et al*., 2019). It is possible that the CSP proteins are responsible for the level of insecticide sensitivity in other insect species. In this study, we identified eight *RpCSP* genes in *R. padi* based on analysis of genome data. The expression levels of some *RpCSP* genes were significantly affected by imidacloprid and beta-cypermethrin at different posttreatment periods. The interaction of *RpCSP4*, *RpCSP5*, *RpCSP6*, *RpCSP7* and *RpCSP10* with imidacloprid and beta-cypermethrin were predicted using molecular docking. Moreover, the *RpCSP4*, *RpCSP5*, *RpCSP6* and *RpCSP10* genes were involved in imidacloprid susceptibility of *R. padi*, and *RpCSP4* and *RpCSP6* played an important role in the level of beta-cypermethrin sensitivity. These results strongly indicated that some *RpCSP* genes have an important role in susceptibility to insecticides.

With the massive use of insecticides for pest management, the rapid adaptation of insects to the insecticides is occurring. Analysis of the relationship between insecticides and chemosensory systems found that some chemosensory genes underwent dramatic upregulation in insects following exposure to sublethal concentrations of insecticides. The expression of most *CSPs* was significantly increased after exposure to abamectin in most

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tissues of *B. mori* adult females (Xuan *et al*., 2015). In *B. tabaci* treated with sublethal concentrations of neonicotinoid thiamethoxam, some *CSPs* underwent dramatic upregulation (Liu *et al*., 2014, 2016). After treatment with permethrin, the transcripts of *CSP8* and *OBP13* were significantly increased in the diamondback moth, *P. xylostella* (Bautista *et al*., 2015). The expression of most of the *OBP* and *CSP* genes have been reported to be upregulated in *S. litura* after it was treated with three insecticides, chlorpyrifos, emamectin benzoate and fipronil (Lin *et al*., 2018a). The insecticides dichlorvos and carbofuran could significantly induce the expression of *TcCSP10* and *TcOBPC01* in *Tribolium castaneum* (Xiong *et al*., 2019). In this study, all *RpCSP* genes in *R. padi* tended to be upregulated following imidacloprid treatment, and *RpCSP4*, *RpCSP6* and *RpCSP7* were significantly induced by beta-cypermethrin, which suggests the *RpCSPs* might be related to the response of *R. padi* to exogenous toxic insecticides. Moreover, these results indicated that the different insecticides shared some common and discrepant effects on the regulation of the expression of *RpCSP* genes in *R. padi*. Different insecticides have different mechanisms of action, resulting in distinct effects on insects. Imidacloprid acting on insect nicotinic acetylcholine receptors mediates fast cholinergic synaptic transmission in the central nervous system (Matsuda *et al*., 2001).The targets of beta-cypermethrin are the voltage-gated sodium channels of insect neurons, and this insecticide can disrupt normal nerve signaling, leading to paralysis and death (Soderlund and Bloomquist, 1989). Thus, application of imidacloprid and

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beta-cypermethrin could result in discrepant effects on the expression of the *RpCSP* genes. A similar phenomenon was also found in *S. litura*; no significant difference was found in the mRNA level of *SlituCSP19* between emamectin benzoate treatment and the control group, while the expression of *SlituCSP19* was significantly induced after treatment with chlorpyrifos or fipronil (Lin *et al*., 2018a). Furthermore, we found that the expression of the *RpCSP*s was variable at five different time points after sublethal exposure to imidacloprid or beta-cypermethrin, and some *RpCSP* genes were significantly induced after treatment with insecticides. In agreement with our observations, thiamethoxam exposure could also cause an increase of *BtabCSP1* expression at 4 and 24 h in *B. tabaci* following insecticide treatment (Liu *et al*., 2014). The transcripts of *TcCSP10* and T*cOBPC01* in *T. castaneum* were significantly increased at 24, 36, 48 and 60 h after exposure to sublethal concentrations of dichlorvos or carbofuran (Xiong *et al*., 2019). These results strongly indicated that the delayed response to *CSP* genes expression in insects could be because of the time needed for the exogenous toxic insecticides to infiltrate into the insects (Liu *et al*., 2014; Xia *et al*., 2015).

In recent years, a large volume of literature has shown that insect chemosensory proteins can bind to plant volatiles, environmental chemical compounds, and sex pheromone analogs (Iovinella *et al*., 2013; Pelosi *et al*., 2018). However, few studies have reported the binding of CSP proteins to insecticides. To further verify the direct combinations of *RpCSPs* and insecticides, molecular docking was used to predict the

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binding. The results showed that hydrogen bonds could be formed between *RpCSP4*, *RpCSP5*, *RpCSP6*, *RpCSP7* or *RpCSP10* with imidacloprid or beta-cypermethrin in both the detailed planar and 3D conformer structures. Therefore, these results suggested that there are direct interactions between *RpCSPs* and imidacloprid or beta-cypermethrin. Similar results have also been reported for the tea geometrid moth, *Ectropis oblique*, the interaction process of neonicotinoid imidacloprid with *EoblGOBP2* was demonstrated through multiple physicochemical lines of evidence (Li *et al.*, 2017). *BtabCSP2* and *BtabCSP3* in *B. tabaci* were able to directly bind to a common direct contact insecticide molecule (Liu *et al.*, 2016). There was direct binding between CSPs and insecticides in *S. litura* as shown with a fluorescence competitive binding assay; *SlituCSP18* could bind with chlorpyrifos and fipronil; and *SlituCSP6* could bind to three pesticides chlorpyrifos, emamectin benzoate and fipronil (Lin *et al.*, 2018b). Moreover, the expression levels of some *CSPs* in Bt-resistant populations were higher than those of Bt-sensitive populations in *Diatraea saccharalis*, suggesting that some *CSP* genes might be involved in the insect resistance response (Guo *et al.*, 2012). In *S. litura*, the silencing of *SlituCSP18* could result in a significantly decrease in survival rates after feeding on chlorpyrifos (Lin *et al.*, 2018a). Knockdown of *TcCSP10* significantly increased the mortality rate of *T. castaneum* exposed to dichlorvos or carbofuran (Xiong *et al.*, 2019). Sensory appendage protein (SAP2) also have vital role in the pyrethroid resistance of *A. gambiae* (Ingham *et al.*, 2020). In the present study, the mortality of *R. padi* in response to imidacloprid following

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the injection of dsCSP4, dsCSP5, dsCSP6 or dsCSP10 was significantly increased, suggesting that these four genes play a vital role in imidacloprid susceptibility of *R. padi*. Moreover, the mortality rate of *R. padi* after treatment with beta-cypermethrin increased significantly after injection of dsCSP4 or dsCSP6, which indicated that the two genes have an important role during the response to beta-cypermethrin treatment. Based on the above-mentioned results from several other insect speices as well as from *R. padi* in this study, we speculate that *Rp*CSP proteins might act as buffers by binding, sequestering and masking toxic insecticide molecules in *R. padi*, which was also discussed in previous publications (Lin *et al*., 2018a; Pelosi *et al*., 2018). Under insecticide treatment, the expression levels of some *RpCSP* genes were significantly increased; *Rp*CSP proteins combining with the insecticide might be helpful to reduce the amount of insecticides that is available to the corresponding target sites, which is beneficial for aphids to resist insecticides; increase of some specific *Rp*CSP proteins might be more helpful for *R. padi* to resist insecticides. When the expression of *RpCSP* is reduced by RNAi, the aphids became more sensitive to insecticides with increased mortality; the higher insecticide efficiency was possibly resulted from greater bioavailability of the chemicals caused by lower insecticide binding by the proteins. In *A. gambiae*, a member of the CSP (SAP2) is involved in pyrothroid resistance by increasing the expression of the gene, which is probably owing to high-affinity binding of SAP2 to pyrethroid insecticides (Ingham *et al*., 2020). Interestingly, we found that the expression of some CSP genes were significantly

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increased in pyrethroid resistant strain of *R. padi* compared with those in susceptible strain (Peng and Chen, unpublished data). Further studies are needed to investigate the molecular mechanisms of CSP involved in insecticide susceptibility and insecticide resistance in *R. padi*.

In conclusion, all of the results in this study indicated that the role of *RpCSPs* might be protective, because increasing their expression levels decreased the toxicity of imidacloprid or beta-cypermethrin by their binding to it to sequester and mask the insecticide, and this mechanism eventually contributes to their defenses against exogenous insecticides, which could be considered a kind of insecticide resistance (Lin *et al.*, 2018a, 2018b). The mortalities of *R. padi* after treatment with imidacloprid increased significantly after injection of dsCSP5 and dsCSP10; however, no significant effect was found after treatment with beta-cypermethrin, which validated the previous results that *RpCSP* genes might play different roles in responses to different insecticide treatments. The mechanism of how *R. padi* resists the insecticides imidacloprid and beta-cypermethrin is not completely known. Therefore, understanding the role of *RpCSPs* and especially their interactions with insecticides will be helpful to understand the insecticides resistance mechanism of aphids.

Experimental procesure

Insect rearing

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The *R. padi* population used in this study was originally sampled from Xianyang, Shaanxi Province, China. The population was subsequently maintained on *Triticum aestivum* (cultivar "Xiaoyan 22") using cages (42 cm by 42 cm by 42 cm) covered with mesh gauze (100 mesh) at 24 ± 1 °C and 70 ± 5% relative humidity (RH) with a photoperiod of 16:8 h (light/dark). The *R. padi* population was reared for more than four generations on wheat seedlings without exposure to any insecticides. To ensure sufficient food for the aphids, the wheat seedlings were replaced every seven days.

2.2. Identification of chemosensory protein genes based on genome data

The available CSP proteins from *Acyrthosiphon pisum*, *Aphis glycines*, *Myzus persicae* and *Rhopalosiphum maidis* which could be downloaded from the NCBI database, were used as references to screen the *R. padi* genome (Zhou *et al.*, 2010; Mathers *et al.*, 2017; Thorpe *et al.*, 2018; Wenger *et al.*, 2017; Chen *et al.*, 2019). The candidate *R. padi* CSPs were confirmed using the blastx algorithm against the nonredundant protein sequence (NR) database of GenBank. The DNA and mRNA sequences of candidate *RpCSP* genes in *R. padi* were extracted from the genome and transcriptome dataset (Thorpe *et al.*, 2018; Ramiro *et al.*, 2020). All candidate CSP sequences were further validated by cloning and sequencing. The similarities of the identified nucleotide and protein sequences were further checked using NCBI BLAST. MEGA v7 was used to identify the exon-intron boundaries and start-stop codons of each *RpCSP* (Kumar *et al.*,

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2016). The conserved domain of the CSPs was identified by the NCBI Conserved Domain Search program. The phylogenetic analysis of all CSPs in *R. padi* was performed using the maximum likelihood algorithm in MEGA v7.

Insecticide bioassays

The insecticides imidacloprid (95% purity; Jiangsu Changlong Chemical Co., Ltd, Nanjing, China) and beta-cypermethrin (96% purity; Yancheng Nongbo Biotechnology Co., Ltd, Yancheng, China) were used in these bioassays. Triton X-100 was purchased from Aladdin (Shanghai, China). The two insecticides were prepared in acetone (10 g/L) and diluted to the corresponding concentrations containing 0.01% (v/v) Triton X-100 for the bioassays. Based on our previous research, the leaf dipping bioassay was adopted to assess the insecticide susceptibility of *R. padi* (Zuo *et al*., 2016a, 2016b).Approximately 30 newly emerged adult apterous aphids were used for each insecticide concentration, and 5 serial concentrations (0.5, 1, 2, 4 and 8 mg/L) were used for each insecticide. The carrier (ddH_2O with 0.01% (v/v) Triton X-100) was used as a control. Three replications were carried out per treatment.

Expression patterns of RpCSPs

To clarify the expression patterns of the *RpCSP* genes under insecticide treatments, sublethal concentrations (LC_{60}) of imidacloprid and beta-cypermethrin were used based

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on the abovementioned bioassay results. Wheat leaves containing newly emerged adult aphids were dipped into the insecticide solution for 10 s each. Then, we removed the wheat leaves from the dilutions and used dry filter paper to absorb the residual droplets on the leaves. The control has been mentioned above. The aphids were reared in uniform environments as described above. After the leaf dipping bioassay, we collected the surviving aphids at 3, 6, 12, 24, and 48 h posttreatment and immediately immersed them in liquid nitrogen. To exclude the possible influence of the circadian rhythm on the expression levels of the *RpCSP* genes, we simultaneously collected all of the aphids.

Total RNA was extracted from the samples using Invitrogen TRIzol Reagent (Life Technologies, Carlsbad, USA). A DNA-free Kit (Applied Biosystems, Foster City, CA, USA) was used to eliminate any DNA contamination. The quality and concentration of RNA were detected by 1% agarose gel electrophoresis and a Nanodrop 1000 instrument (Thermo Scientific, Lithuania). First-strand cDNA was synthesized from two micrograms of total RNA from each sample using a reverse transcriptase (Promega, Madison, Wisconsin, USA) following the manufacturer's protocol. The quantitative primers (Table S1) were designed by Primer Premier 6 software, and the specificity of these primers was verified before quantitative PCR (qPCR). qPCR was carried out on a Rotor Q thermocycler (Qiagen, Hilden, Germany) using a fast-start essential DNA green master (Roche, Basel, Switzerland). The qPCR efficiency of these primers was analyzed using five-fold continuously diluted cDNA. The melting curve from 55 to 95 °C with increments of 0.5 °C

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every 30 s was used to further ensure the specificity and consistency of the amplified product. RNase-free water instead of the templates was used as a blank control in each run and for each gene. Three technical and biological replicates were performed for all treatments. Alpha-tubulin and β-actin genes were used to normalize the expression levels of the *RpCSP* genes were relatively stable in different treatments and biological replicates (Kang *et al*., 2016; Zhang *et al*., 2018). The relative expression patterns of the *RpCSP* genes were calculated by the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). Comparisons of the expression levels of the *RpCSPs* in different treatments were subjected to one-way ANOVA followed by Tukey's honestly significant difference (HSD) test ($P < 0.05$).

Molecular modeling and docking

Because the X-ray crystal structures of CSPs from *R. padi* were not available in the protein data bank, the three-dimensional (3D) structures of the CSPs were built by homology modeling. The modeled structures of the CSPs from *R. padi* were obtained employing online Swiss-model software (https://swissmodel.expasy.org/), and the optimal templates obtained for the modeling targets were selected based on a low E-value, a high sequence identity and query coverage. The 3D structures of imidacloprid and beta-cypermethrin were obtained using the online ZINC website (http://zinc.docking.org). The two small molecules were energy optimized using the ChemOffice CS Chem3D 8.0

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software (PerkinElmer, Waltham, MA, USA) by methods of molecular mechanics (MM2) before docking study. The genetic algorithm of AutoDockTools-1.5.6 was employed for a molecular docking study. The binding poses of the CSP-imidacloprid complex and CSP-beta-cypermethrin complex were obtained with the searching algorithm of AutoDock Tools Optimizer and the energetic evaluation of AutoDock Tools Score. Then, the optimized binding pose and hydrogen bonds were displayed by PyMOLsoftware (DeLano *et al.*, 2002). Moreover, the optimized binding pose and hydrogen bonds were also analyzed by Ligplot+ Version 2.1 to obtain the detailed planar binding plot between the macromolecule and ligand (Laskowski *et al.*, 2011).

Determining the efficiency of RNA interference

Sequence-specific dsRNA primers of *RpCSP* genes (Table S1) were designed, and the amplified target fragments for RNA interference (RNAi) were different from the region for qRT-PCR. The PCR products were separated and purified with a Gel Extraction Kit (Promega, Madison, WI, USA) and used to synthesize dsRNA using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA) following the manufacturer's instructions. Double-strand green fluorescent protein (dsGFP) was synthesized and served as a control. The concentrations of purified dsRNA were examined using a biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany), and their purity and integrity were verified by agarose gel electrophoresis. Then, the purified dsRNAs

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were stored at −80 °C until use.

We selected newly emerged adult aphids to evaluate the RNA interference efficiency. Fifty nanoliters of dsRNA (8.89 μg/μL) was injected into the suture joining the ventral mesothorax and metathorax using an automatic nanoliter injector (Märzhäuser, Wetzlar, Germany) equipped with a microglass needle prepared using a P-97 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Newly emerged adult aphids were injected with dsGFP as a control. The injected aphids were reared under the conditions described earlier, and ten surviving adults were randomly collected at 24 h, 48 h and 72 h posttreatment. RNA was extracted according to the methods described above, and the transcriptional levels of the targeted genes were measured by qPCR. Three replications were carried out per treatment. The data on the expression levels of the transcripts were analyzed by one-way ANOVA. The significant differences were examined using t-tests ($P < 0.05$).

Insecticide bioassays after RNAi

To assess the role of the *RpCSP* genes in aphid insecticide sensitivity, newly emerged adult aphids were injected with dsRNA. Two days after dsRNA injection, the aphids injected with dsGFP, dsCSP4, dsCSP5, dsCSP6 or dsCSP10 were respectively treated with two different concentrations (LC_{40} or LC_{60}) of imidacloprid and beta-cypermethrin. The method of the bioassays was as described above, and aphid mortality was assessed

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24 h posttreatment. Thirty injected adult aphids from each treatment were considered as a replication and five replications were carried out per treatment. Data on the percentages of mortality were log-transformed to meet the assumptions of normality and homoscedasticity required for these analyses. T-tests ($P < 0.05$) were used to compare the significant differences between the control and treatment groups.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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Figure legends

Figure 1. Amino acid sequence alignment of eight *RpCSPs* from *Rhopalosiphum padi* (a), comparison of genomic and nucleotide sequences of the RpCSP genes (b), sequence alignment between the second and third "C" (c), the analysis of conserved domains in RpCSPs (d) and phylogenetic analysis of eight RpCSPs from *R. padi* with those from other insect species (e). The phylogenetic tree is based on aligned amino acid sequences using MEGA5. Small icon above the branches represent values (%) based on 1,000 replicates.

Figure 2. Expression patterns of *RpCSPs* from *R. padi* in different periods under sublethal concentration (LC_{60}) of imidacloprid and beta-cypermethrin conditions. Asterisks on the tops of the bars specify that the values were significantly different (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test).

Figure 3. Molecular docking analysis for the binding of RpCSP4 (a, b), RpCSP5 (c, d), RpCSP6 (e, f) and RpCSP10 (g, h) from *R. padi* with imidacloprid (a, c, e, g) or beta-cypermethrin (b, d, f, h) using 3D binding mode. The 3D structures of imidacloprid and beta-cypermethrin were obtained using the online ZINC website (http://zinc.docking.org). The two small molecules were energy optimized using the ChemOffice CS Chem3D 8.0 software (PerkinElmer, Waltham, MA, USA) by methods of molecular mechanics (MM2) before docking study. The dimensions of the grids were set with overall dimensions of 100 x 100 x 100 points based on the protein

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volume. The genetic algorithm within 10 independent run with 2.5×10^7 number of evaluations was employed for a molecular docking study using AutoDockTools-1.5.6. The binding poses of the CSP-imidacloprid complex and CSP-beta-cypermethrin complex were obtained with the searching genetic algorithm of AutoDock Tools Optimizer and the energetic evaluation of AutoDock Tools Score. The optimized binding pose and hydrogen bonds were displayed by PyMOLsoftware (DeLano *et al*., 2002).The optimized binding pose and hydrogen bonds were also analyzed by Ligplot+ Version 2.1 to obtain the detailed planar binding plot between the macromolecule and ligand (Laskowski *et al*., 2011).

Figure 4. Molecular docking analysis for the binding of RpCSP4 (a, b), RpCSP5 (c, d), RpCSP6 (e, f) and RpCSP10 (g, h) from *R. padi* with imidacloprid (a, c, e, g) or beta-cypermethrin (b, d, f, h) using the detailed planar binding mode. The analyses methods are the same as those explained in Figure 3.

Figure 5. Relative expression levels of *RpCSP10* (a) from *R. padi* at different times after injection of dsGFP or dsCSP10. The mortality of *R. padi* injected with dsGFP or dsCSP10 after exposure to two different concentrations (LC_{40} and LC_{60}) of the two insecticide (imidacloprid and beta-cypermethrin) treatments (b). Asterisks on the tops of the bars specify that the values were significantly different (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test).

Figure 6. The mortality of *R. padi* injected with dsCSP4 (a), dsCSP5 (b) or dsCSP6 (c)

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after exposure to two different concentrations (LC_{40} and LC_{60}) of the two insecticide (imidacloprid and beta-cypermethrin) treatments. *R. padi* injected with dsGFP was considered as the control. Asterisks on the tops of the bars specify that the values were significantly different (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test).

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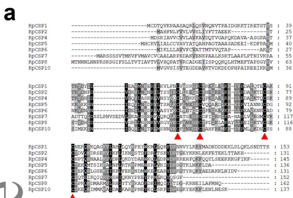

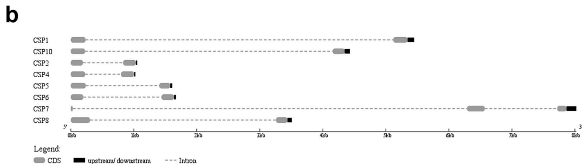

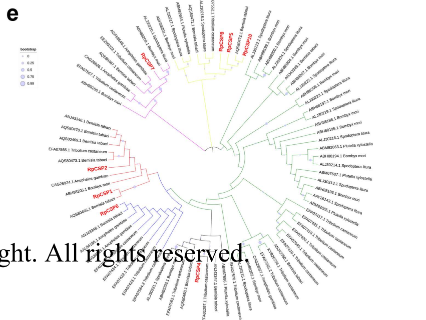

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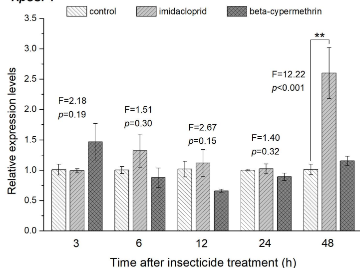

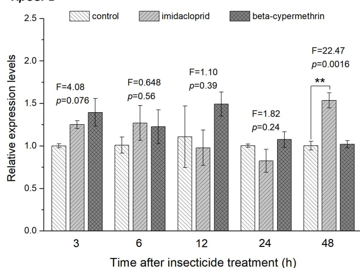

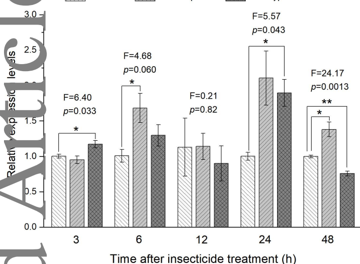

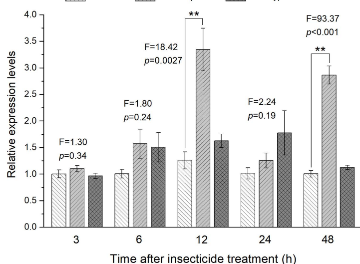

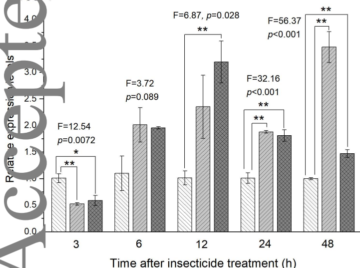

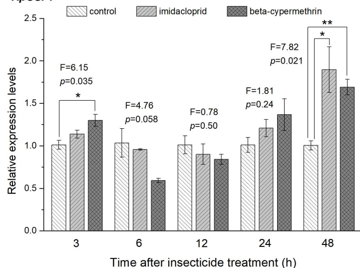

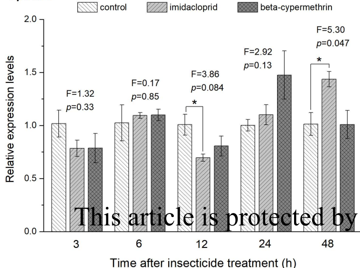

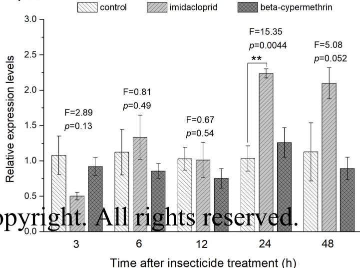

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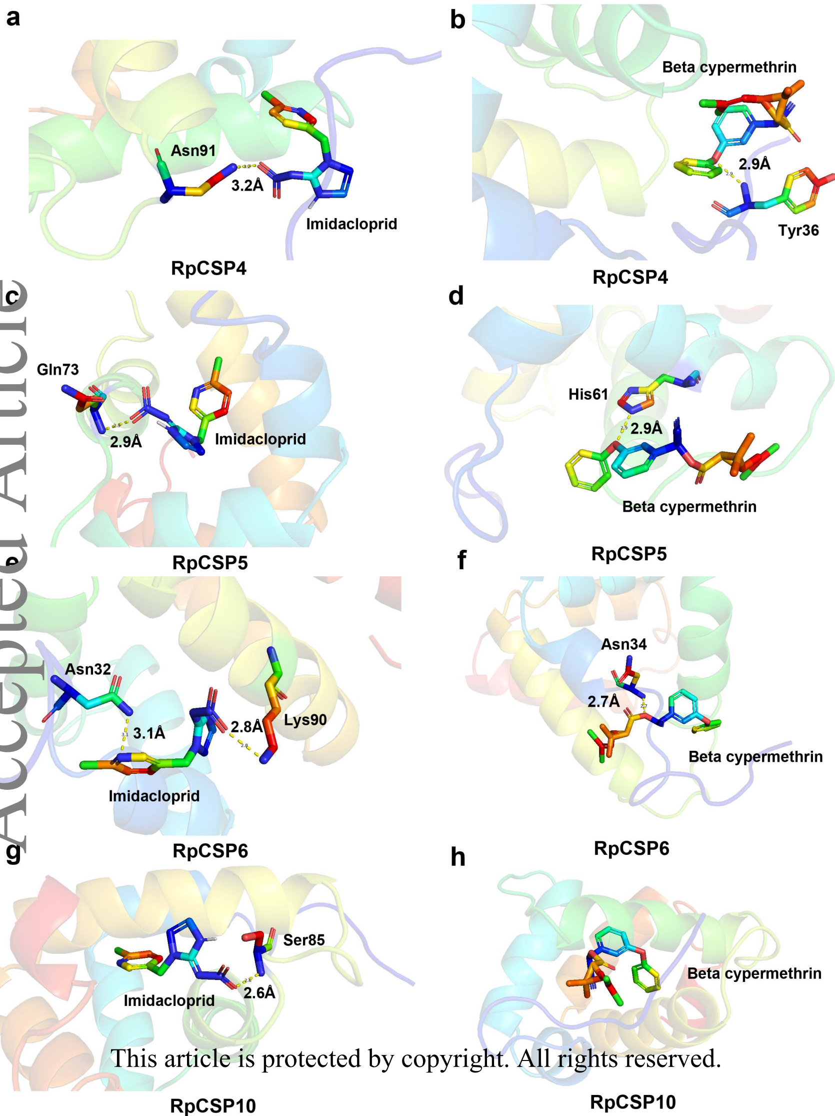

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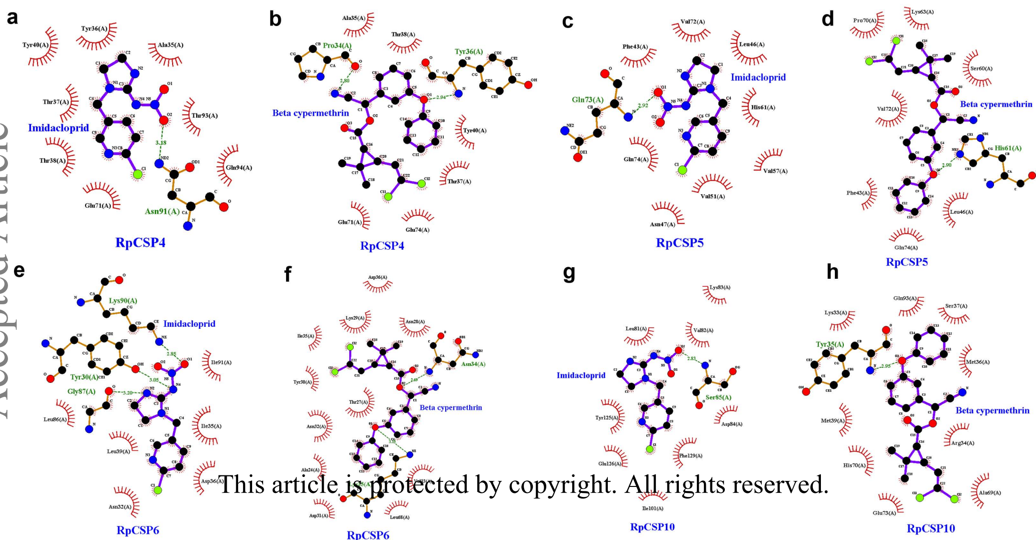

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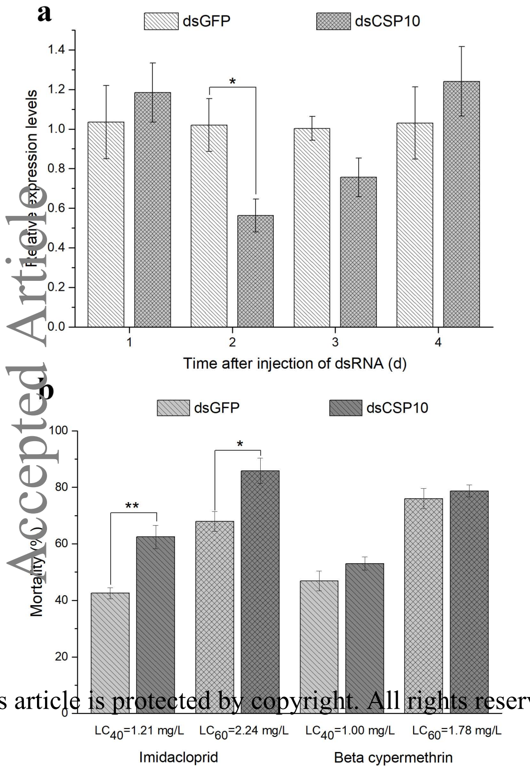

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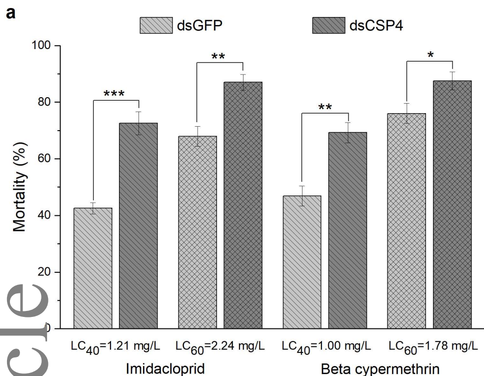

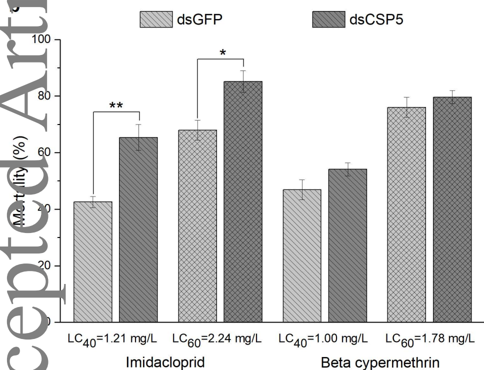

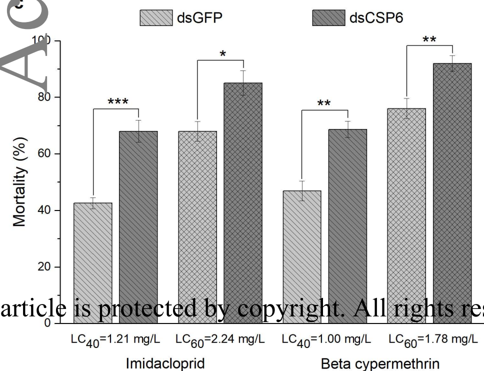

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