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Characterization of *Rhopalosiphum padi takeout-like* genes and their role in insecticide susceptibility

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ABSTRACT

Due to the extensive use of chemical insecticides, the field populations of *Rhopalosiphum padi*, a serious wheat pest worldwide, have developed resistance to insecticides. Therefore, deep understanding of the mechanisms of the aphid's physiological response to insecticides would be of importance for the management of insecticide resistance in pests. Takeout belongs to a protein superfamily found exclusively in insects. Previous research showed that the *takeout* gene had various functions in insect physiology and behavior. However, few studies have explored the functions of *takeout* in insect insecticide susceptibility. The susceptibility of *R. padi* to imidacloprid and beta-cypermethrin was tested. Thirteen *takeout-like* genes were identified based on the genome database of *R. padi*. The number of exons was variable in these *takeout-like* genes, and nine highly conserved amino acids (two Cysteine, two Proline, four Glycine and one Aspartic acid) were identified. Expression levels of *takeout-like-2*, *takeout-like-3*, *takeout-like-5*, *takeout-like-8*, *takeout-like-10* and *takeout-like-11* were significantly increased after imidacloprid treatment; seven genes (*takeout-like-1*, *takeout-like-2*, *takeout-like-5*, *takeout-like-6*, *takeout-like-7*, *takeout-like-8* and *takeout-like-11*) tended to be upregulated after beta-cypermethrin treatment. RNA interference results showed that the mortalities of *R. padi* injected with ds*TOL-2*, ds*TOL-5*, ds*TOL-8*, ds*TOL-10* and ds*TOL-11* were significantly increased after exposure to imidacloprid in comparison with control (injection of ds*GFP*). Under two sublethal concentrations of beta-cypermethrin, the silencing of *takeout-like-2*, *takeout-like-5* and *takeout-like-11* significantly increased the mortalities of *R. padi*. These results provide evidence for the involvement of *takeout-like* genes in insecticide susceptibility of *R. padi*, which improves our understanding the determinant of insecticide susceptibility.

1. Introduction

Takeout proteins belong to a large superfamily of small molecule transporting proteins that were initially discovered in *Drosophila melanogaster* as a clock-regulated gene (Sarov-Blat et al. 2000; So et al. 2000). The takeout proteins are ubiquitous, and homologues of *takeout* have been characterized from a diverse range of insect species, including *Manduca sexta* (Du et al. 2003), *Aedes aegypti* (Bohbot and Vogt 2005), *Myzus persicae* (Ghanim et al., 2006), *Phormia regina* (Fujikawa et al. 2006), *Bombyx mori* (Saito et al. 2006), *Apis mellifera* (Hagai et al. 2007), *Locusta migratoria*(Guo et al. 2011), *Reticulitermes flavipes* (Schwinghammer et al. 2011), *Spodoptera litura* (Lin et al. 2017), *Schizaphis graminum* (Zhang et al. 2019) and *Spodoptera litura* (Jia et al. 2020). Amino acid analysis found that the takeout protein usually contains approximately 250 amino acids, which share a defining domain (hemolymph juvenile hormone-binding protein). The takeout proteins are a type of secreted proteins that bind small lipophilic molecules, and there is a signal peptide and two highly conserved cysteine residues at the N-terminus (Sarov-Blat et al., 2000; So et al. 2000; Fujikawa et al. 2006; Hamiaux et al. 2009). The number of *takeout-like* genes has not been reported in aphid at the genome level.

Takeout genes are found exclusively in insects, and the expression levels of *takeout* genes were usually affected by gender, age, nutrients and hormones (Du et al. 2003; Hagai et al. 2007; Vanaphan et al. 2012). Takeout proteins have been proposed to participate in a diverse range of physiological and biochemical pathways and perform various functions in insect physiology and behavior, including circadian regulation (Sarov-Blat et al. 2000; So et al. 2000; Benito et al., 2010), feeding behavior and locomotor activity (Sarov-Blat et al. 2000; Meunier et al. 2007), courtship (Dauwalder et al. 2002; Saurabh et al. 2018), response to starvation and nutritional status (Justice et al. 2003; Saito et al. 2006), chemosensory detection (Bohbot and Vogt 2005; Fujikawa et al.

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2006; Yoshizawa et al. 2011), longevity (Bauer et al. 2010; Chamseddin et al. 2012), trail-following behavior (Schwinghammer et al. 2011), behavioral phase change (Guo et al. 2011). Interestingly, takeout proteins might be involved in the process of insect response to insecticides as molecular carriers. The *takeout* expression levels could be significantly induced by the insecticides imidacloprid, chlorpyrifos, emamectin benzoate, fipronil and and fluralaner (Ayyanath et al. 2014; Lin et al. 2017; Zhang et al. 2019; Jia et al. 2020). Silencing of the *takeout-like* precursor gene could significantly increase the susceptibility of *Schizaphis graminum* to imidacloprid (Zhang et al. 2019).

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae), is one of the most important wheat pests worldwide. The aphid causes severe economic losses and has a wide host range, including cereal crops and wild grasses (Zhang et al. 2016; Duan et al. 2017; Peng et al. 2017, 2020). Due to the frequent use of chemical insecticides, the field populations of *R. padi* have developed resistance to many insecticides such as abamectin, decamethrin, bifenthrin, imidacloprid, pymetrozine, beta-cypermethrin, thiamethoxam, acetamiprid and malathion (Zuo et al. 2016; Wang et al. 2018). In particular, a moderately resistant strain to pyrethroid insecticides was sampled from Shaanxi province, and a mutation site (M918) in the voltage-gated sodium channel gene was detected, which was associated with pyrethroid resistance (Wang et al. 2020). Therefore, deep understanding of the mechanisms of the aphid's physiological response to insecticides would be of major importance to insecticide resistant management of the pest. In this study, we systematically identified and characterized all the *takeout-like* genes based on *R. padi* genome data. The expression profiles of *takeout-like* genes were measured in *R. padi* treated with three different concentrations of imidacloprid or beta-cypermethrin. Furthermore, we used RNA interference (RNAi) technology to explore the roles of candidate *takeout-like* genes in the insecticide susceptibility of *R. padi*. These data laid a foundation for developing new ways for the integrated control of *R. padi*.

2. Materials and methods

2.1. Insect and insecticide bioassays

The bird cherry-oat aphid population used in experiments was collected from Taian, Shandong Province, China. The *R. padi* was reared in well-ventilated cages (30 cm × 30 cm) covered with mesh gauze (100 mesh) in an artificial chamber. All aphids were reared under a 16:8 light/dark photo regimen at 24 ± 1 °C and on wheat (*Triticum aestivum*, cultivar "Xiaoyan 22") seedlings at densities of 150–300 aphids per cage to ensure sufficient food. The population was reared for more than two years on wheat seedlings without exposure to any insecticides.

We chose two different types of insecticides, including neonicotinoid imidacloprid (95% purity; Jiangsu Changlong Chemical Co., Ltd., Nanjing, China) and pyrethroid beta-cypermethrin (96% purity; Yancheng Nongbo Bio-technology Co., Ltd., Yancheng, China), to conduct the experiments. The two insecticides were dissolved in acetone (10 g/L). Then, they were diluted to corresponding concentrations containing 0.01% (v/v) Triton X-100 (Aladdin, Shanghai, China) for bioassays. The leaf dipping bioassay was used to estimate the insecticide susceptibility of *R. padi* (Zuo et al. 2016). The aphids were divided into a control group, an imidacloprid group and a beta-cypermethrin group. The control group was treated with ddH_2O with 0.01% (v/v) Triton X-100; the treatment groups were performed with five serial concentrations (0.5, 1, 2, 4 and 8 mg/L). Thirty apterous adult aphids were used for each insecticide concentration, and the number of surviving aphids was recorded after 24 h. Three sets of biological replications were established in each treatment.

2.2. Genome-wide identification of takeout-like genes in R. padi

The annotated takeout-like proteins from Acyrthosiphon pisum, Myzus persicae and Rhopalosiphum maidis downloaded from the NCBI database were used as references to screen the R. padi genome v2 downloaded from the BioInformatics Platform for Agroecosystem Arthropods (BIPAA) (http://bipaa.genouest.org/is/aphidbase/) (Mathers et al. 2017; Chen et al. 2019; Li et al. 2019; Morales-Hojas et al. 2020). The candidate R. padi takeout-like genes were obtained using the blastx algorithm against the nonredundant protein sequence (NR) database of GenBank. The genomic and cDNA sequences of candidate takeout-like genes were extracted from R. padi genome v2 (Morales-Hojas et al. 2020). All candidate sequences were further validated by gene cloning and Sanger sequencing. NCBI BLAST was used to further check the similarities of identified gene sequences. Comparison of the genomic sequence with the mRNA sequence revealed the exon-intron boundary and start-stop codons of each takeout-like gene using MEGA v7 (Kumar et al. 2016). The conserved domain of takeout-like proteins was identified by the NCBI Conserved Domain Search program (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The WebLogo sequence alignment map was generated using an online website (http://weblogo.berkeley.edu/logo.cgi). The neighbor-joining algorithm was used to construct the phylogenetic tree using 218 takeout proteins in MEGA v7, and the tree branches were created from 1000 bootstrap replications (Kumar et al. 2016).

2.3. Expression patterns of takeout-like genes in R. padi treated with different concentrations of insecticides

Based on the abovementioned bioassay results, the three concentrations (LC_{30}, LC_{50} and LC_{80}) of imidacloprid and beta-cypermethrin were chosen for the following experiments. Wheat leaves containing wingless 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 24 h, the surviving aphids were collected in RNase-free tubes and immediately stored in liquid nitrogen.

The samples collected above were ground to extract total RNA using Invitrogen TRIzol Reagent (Life Technologies, Carlsbad, USA). The DNA contamination was removed using a DNA-free Kit (Applied Biosystems, Foster City, CA, USA). The GoScript™ Reverse Transcriptase system kit (Promega, Madison, Wisconsin, USA) was used to synthesize first-strand cDNA with two micrograms of total RNA as template. The quantitative primers are shown in Table S1. The fast-start essential DNA green master (Roche, Basel, Switzerland) was used for quantitative PCR (qPCR) with a total volume of 20 μL as follows: 10.0 μL of SYBR mix (Roche, Basel, Switzerland), 0.8 μL of forward and reverse primers (10 μmol/L), 2.0 μL of cDNA and 6.4 μL of ddH_2O. The qPCR conditions were 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 20 s; with one additional cycle at 72 °C for 10 min. Alpha-tubulin and β-actin genes was used as reference genes (Kang et al. 2016; Zhang et al. 2018). The relative expression levels of the *takeout-like* genes were calculated by the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001). Each sample was evaluated with three technical and biological replicates.

2.4. Determination of the efficiency of RNA interference (RNAi)

The unique nucleotide regions of *takeout-like* genes were analyzed for the synthesis of specific dsRNA. The dsRNA primers of six *takeout-like* genes are shown in Table S1. The dsRNA was synthesized using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). The purity and integrity of dsRNA products were confirmed by agarose gel electrophoresis, and the concentrations of purified dsRNAs were confirmed using a biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany).

Based on our preliminary experiments in RNAi of *takeout-like* genes,

we found that the optimum concentration of dsRNA with the highest interference efficiency was 6.76 μg/μL by comparing the interference efficiencies of four different concentrations of dsRNA (1.69 μg/μL, 3.38 μg/μL, 6.76 μg/μL and 10.14 μg/μL). Thus, we injected 50 nL of dsRNA (6.76 μg/μL) into the suture joining the ventral mesothorax and metathorax of wingless adult aphids 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). Wingless adult aphids injected with double-strand green fluorescent protein (ds*GFP*) were considered as the control group. The injected aphids were reared under the conditions described above, and ten surviving adults were randomly collected at 24, 48, 72 and 96 h posttreatment, respectively. The expression patterns of targeted genes were analyzed at different posttreatment times using qPCR. The experiment was repeated three times to ensure result accuracy.

2.5. Susceptibility to imidacloprid and beta-cypermethrin after RNAi

After injection of ds*GFP*, ds*TOL-2*, ds*TOL-5*, ds*TOL-8*, ds*TOL-10* or ds*TOL-11*, susceptibility of *R. padi* to imidacloprid was measured by the leaf-dipping bioassay described earlier. Based on the determination of the RNAi efficiency, we performed the bioassay in the corresponding time for the highest interference efficiency of the respective *takeout-like* dsRNA. Two different concentrations (LC_{30} and LC_{50}) of imidacloprid were used to conduct the insecticide bioassays. After injection of ds*GFP*, ds*TOL-2*, ds*TOL-5*, ds*TOL-7*, ds*TOL-8* or ds*TOL-11*, susceptibility of *R. padi* to beta-cypermethrin was measured under two different sublethal concentrations (LC_{30} and LC_{50}) of beta-cypermethrin conditions. The mortality of dsRNA-injected adult aphids was calculated 24 h post-treatment. We randomly chose thirty injected adult aphids to conduct the insecticide bioassays in each treatment, and the experiments were performed five times. The *R. padi* without injection and injected with ds*GFP* was considered the control group.

2.6. Statistical analysis

All data statistical analyses were performed with SPSS v.20 (IBM-SPSS, Armonk, NY, USA). Comparisons of the expression patterns of 13 *takeout-like* genes in different treatments were subjected to one-way ANOVA followed by Tukey's honestly significant difference (HSD) test ($P < 0.05$). Student's *t*-test was used to compare the interference efficiencies of *takeout-like* genes ($P < 0.05$). The percentages of mortality were log-transformed to meet the assumptions of normality and homoscedasticity required for these analyses. The student's t-test ($P < 0.05$) was used to compare the significant differences between control and treatment groups.

3. Results

3.1. Susceptibility of R. padi to imidacloprid and beta-cypermethrin

The results of insecticide bioassays are shown in Table 1. The toxicity regression equations and slope ± SE of imidacloprid on *R. padi* were Y = 4.71 + 1.91× and 1.91 ± 0.17, respectively. The slope ± SE of beta-cypermethrin was 2.00 ± 0.18 (Table 1). The median lethal concentration values (LC_{50}) of imidacloprid and beta-cypermethrin were 1.42 (95% Confidence limit, 1.18–1.67) and 1.21 (95% Confidence limit, 1.00–1.43) mg/L, respectively.

3.2. Characteristics and gene structure of takeout-like genes

Thirteen *takeout-like* genes were identified and named *takeout-like-1* – *takeout-like-13*(Fig. S1). All sequences were deposited in GenBank. The accession numbers are *takeout-like-1* (MT435101), *takeout-like-2* (MT435102), *takeout-like-3* (MT435103), *takeout-like-4* (MT435104), *takeout-like-5* (MT435105), *takeout-like-6* (MT435106), *takeout-like-7* (MT435107), *takeout-like-8* (MT435108), *takeout-like-9* (MT435109), *takeout-like-10* (MT435110), *takeout-like-11* (MT435111), *takeout-like-12* (MT435112), and *takeout-like-13* (MT435113). The exon-intron boundary results showed that *takeout-like-2* and *takeout-like-5* had four exons, and *takeout-like-7* and *takeout-like-10* had five exons; the other nine *takeout-like* genes had six exons based on comparisons of genomic and nucleotide sequences (Fig. 1a). Common conserved domain analysis showed that these proteins belonged to the hemolymph juvenile hormone binding protein (JHBP) family (Fig. 1b). The alignment of takeout-like protein sequences from *R. padi* is shown in Fig. 1S.

Alignment of the 13 deduced amino acid sequences showed that takeout-like proteins had two highly conserved amino acid cysteines (C) at the N-terminus, two highly conserved prolines (P) and four highly conserved glycines (G) in the middle, and a highly conserved asparagine (N) at the C-terminus (Fig. 2). The phylogenetic tree was constructed by comparing 218 takeout/takeout-like proteins from other insect species with the 13 takeout-like proteins from *R. padi* (Fig. 3). Takeout-like-8, takeout-like-9, takeout-like-11 and takeout-like-12 clustered together, and the four proteins were highly homologous intraspecies; takeout-like-3 and takeout-like-7 clustered together; takeout-like-2 and takeout-like-5 also clustered together; the other proteins did not cluster together and were in different branches; the corresponding takeout-like proteins from different aphid species were clustered into one branch, indicating that these takeout-like genes are conserved, at least in aphid.

3.3. The expression levels of takeout-like genes at sublethal concentrations

The relative transcriptional levels of the 13 *takeout-like* genes were compared among *R. padi* treated with different concentrations of imidacloprid (Fig. 4). The expression levels of *takeout-like-2* ($F = 38.19$; $P < 0.001$), *takeout-like-3* ($F = 5.40$; $P = 0.025$), *takeout-like-5* ($F = 370.59$; $P < 0.001$), *takeout-like-7* ($F = 5.46$; $P = 0.024$), *takeout-like-8* ($F = 11.24$; $P < 0.01$), *takeout-like-9* ($F = 15.12$; $P < 0.01$), *takeout-like-10* ($F = 14.55$; $P < 0.01$), *takeout-like-11* ($F = 85.44$; $P < 0.001$) and *takeout-like-12* ($F = 10.39$; $P < 0.01$) were significantly varied in *R. padi* with sublethal concentrations of imidacloprid; compared with the control group, the expression levels of *takeout-like-2*, *takeout-like-5*, *takeout-like-8*, *takeout-like-10*, and *takeout-like-11* were significantly increased after the three different concentrations of imidacloprid treatment; the expression of *takeout-like-3* in *R. padi* treated with LC_{80} (3.92 mg/L) imidacloprid was significantly higher than that of the control group. In conclusion, there were six *takeout-like* genes upregulated after imidacloprid treatment.

The qRT-PCR results indicated that there were significantly different expression levels of *takeout-like-1* ($F = 17.04$; $P < 0.001$), *takeout-like-2* ($F = 38.93$; $P < 0.001$), *takeout-like-5* ($F = 43.24$; $P = 0.025$), *takeout-like-6* ($F = 5.52$; $P = 0.024$), *takeout-like-7* ($F = 7.75$; $P < 0.01$), *takeout-like-8* ($F = 12.19$; $P < 0.01$), *takeout-like-9* ($F = 33.11$; $P < 0.001$), *takeout-like-10* ($F = 8.15$; $P < 0.01$), *takeout-like-11*

Table 1
Insecticide bioassays of apterous *Rhopalosiphum padi* adults to imidacloprid and beta-cypermethrin.

Insecticides	Slope ± SE	χ^2	*P*-value	LC_{30} (mg L-1) (95% CL)	LC_{50} (mg L-1) (95% CL)	LC_{80} (mg L-1) (95% CL)
Imidacloprid	1.91 ± 0.17	3.08	1.00	0.76 (0.58–0.93)	1.42 (1.18–1.67)	3.92 (3.28–4.91)
Beta-cypermethrin	2.00 ± 0.18	4.13	0.99	0.66 (0.50–0.83)	1.21 (1.00–1.43)	3.20 (2.72–3.89)

χ^2, Chi-square; LC, the mortality in corresponding concentration; CL: Confidence limit.

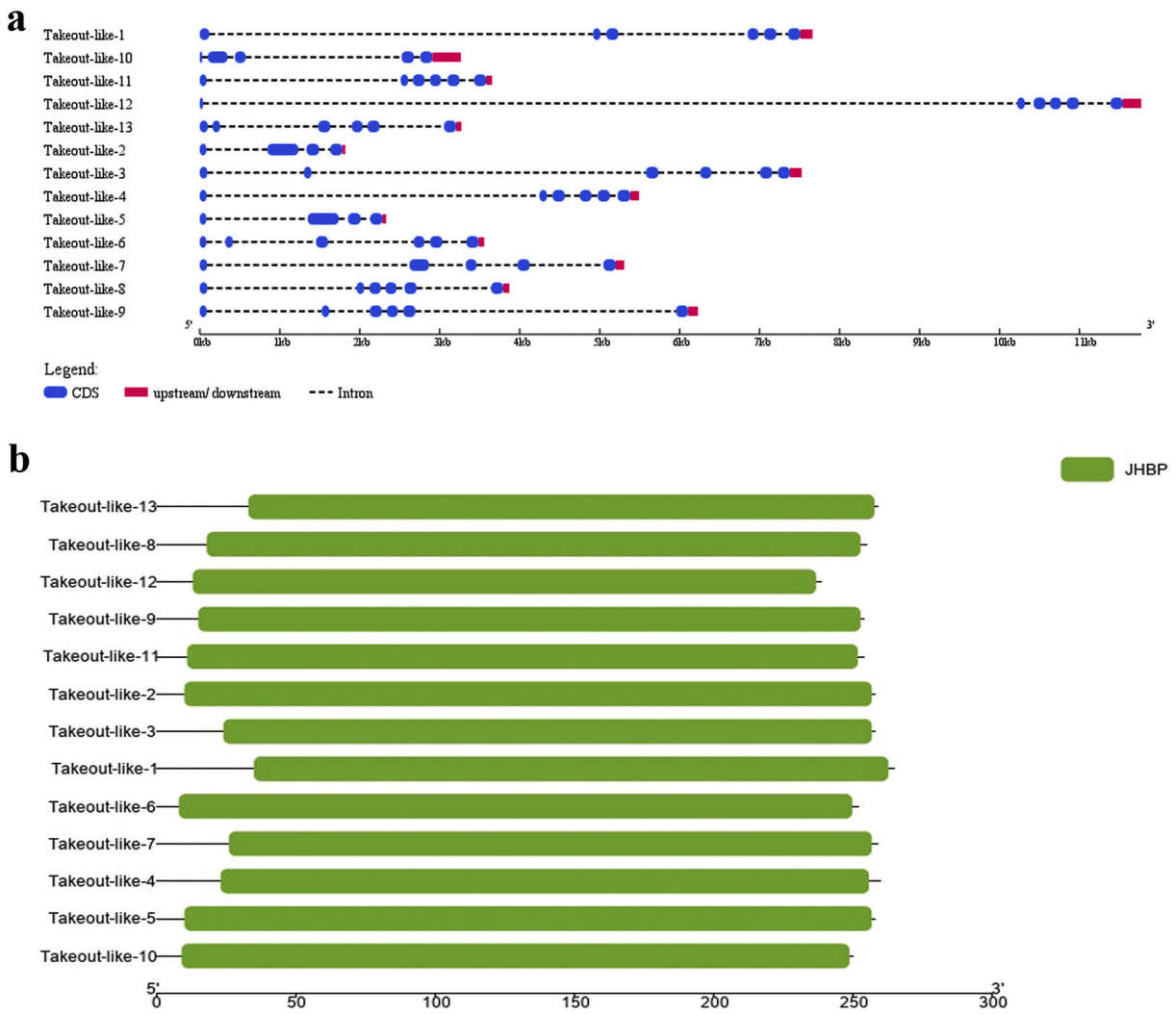

Fig. 1. Gene structure analysis of 13 *takeout-like* genes from *Rhopalosiphum padi* based on genomic and mRNA sequences (a); analysis of the conserved domains in 13 takeout-like proteins (b).

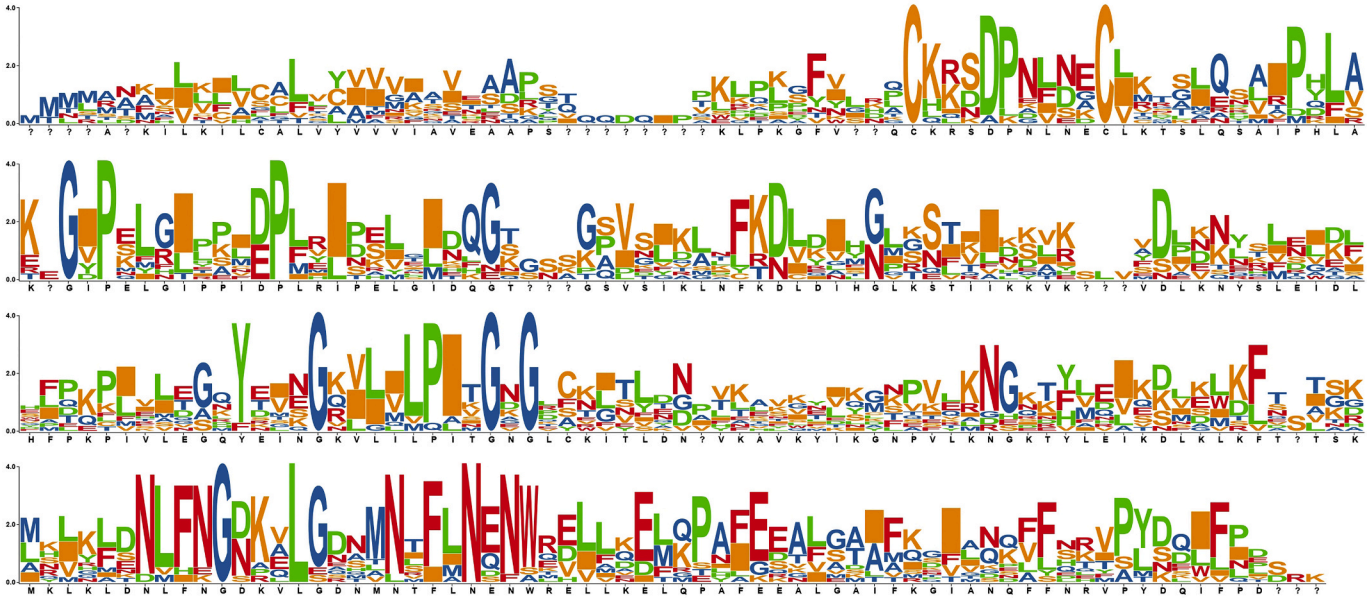

Fig. 2. WebLogo sequence alignment map of 13 takeout-like proteins from *Rhopalosiphum padi*.

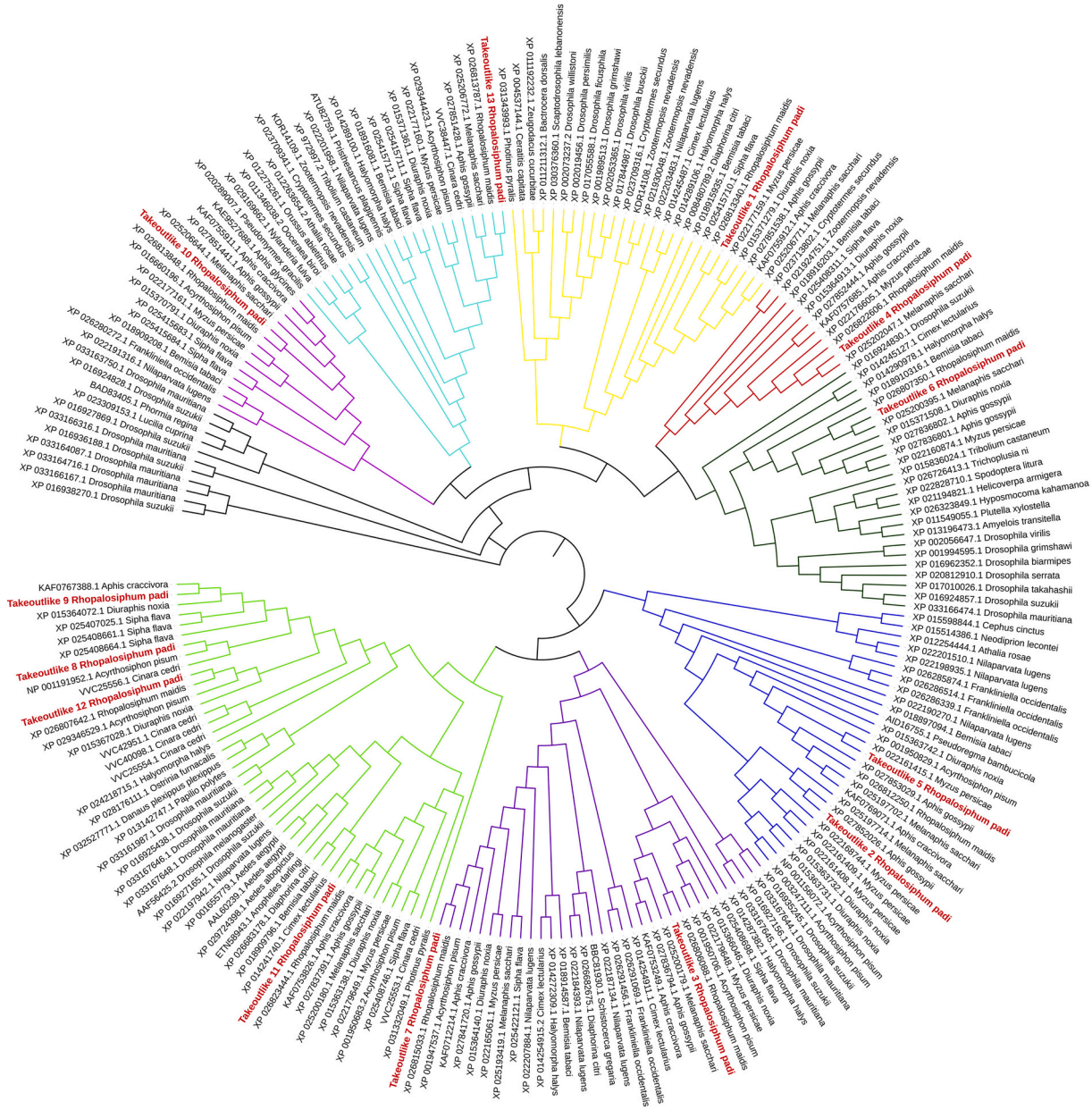

Fig. 3. Phylogenetic analysis of takeout-like proteins from different insect species. All of the takeout-like proteins which can be found in NCBI Database (https://www.ncbi.nlm.nih.gov/) for each of the species were included in the analyses. The phylogenetic tree is based on aligned amino acid sequences using MEGA5. Numbers above branches represent values (%) based on 1000 replicates.

(F = 48.12; $P < 0.001$) and *takeout-like-13* (F = 11.03; $P < 0.01$) in *R. padi* treated with different concentrations of beta-cypermethrin (Fig. 5). In addition, the transcript level of *takeout-like-1* in *R. padi* treated with LC_{30} (0.66 mg/L) beta-cypermethrin was significantly higher than in the control group; the expression levels of *takeout-like-2*, *takeout-like-5*, *takeout-like-6* and *takeout-like-11* were significantly increased after treatment with LC_{50} (1.21 mg/L) beta-cypermethrin; the six genes (*takeout-like-2*, *takeout-like-5*, *takeout-like-6 takeout-like-7*, *takeout-like-8*, and *takeout-like-11*) tended to be upregulated following LC_{80} (3.20 mg/L) beta-cypermethrin treatment in *R. padi*.

3.4. The RNAi efficiency of takeout-like genes

We found that a total of nine genes (*takeout-like-1*, *takeout-like-2*, *takeout-like-3*, *takeout-like-5*, *takeout-like-6*, *takeout-like-7 takeout-like-8*, *takeout-like-10*, and *takeout-like-11*) had significantly increased expression after insecticide treatment based on the abovementioned qPCR results. These upregulated genes were chosen for further functional studies. However, the RNAi efficiencies of *takeout-like-1*, *takeout-like-3*, and *takeout-like-6* were too low; we did not further analyze the effects of the three genes on the insecticide susceptibility of *R. padi*. Injection of dsTOL-2, dsTOL-5, dsTOL-7, dsTOL-8, dsTOL-10 and dsTOL-11 was used to suppress the expression of *takeout-like-2*, *takeout-like-5*, *takeout-like-7*, *takeout-like-8*, *takeout-like-10* and *takeout-like-11*. qPCR analysis was conducted to show the time-dependent suppression after injection of dsRNA. The *takeout-like-5* gene expression level significantly decreased (reduced by 51.01%; $P < 0.001$) 24 h after injection of dsTOL-5 compared with control (injection of dsGFP) (Fig. 6a). The interference efficiencies of *takeout-like-2*, *takeout-like-7*, *takeout-like-10* and *takeout-like-11* were significant from 24 to 48 h, and the relative expression levels of these four genes were suppressed by 54.66% (*takeout-like-2*; $P < 0.001$), 46.23% (*takeout-like-7*; $P = 0.024$), 62.53% (*takeout-like-10*; $P < 0.01$) and 43.10% (*takeout-like-11*; $P < 0.01$) at 48 h (Fig. 6a, b). The expression of *takeout-like-8* in the treatment group was

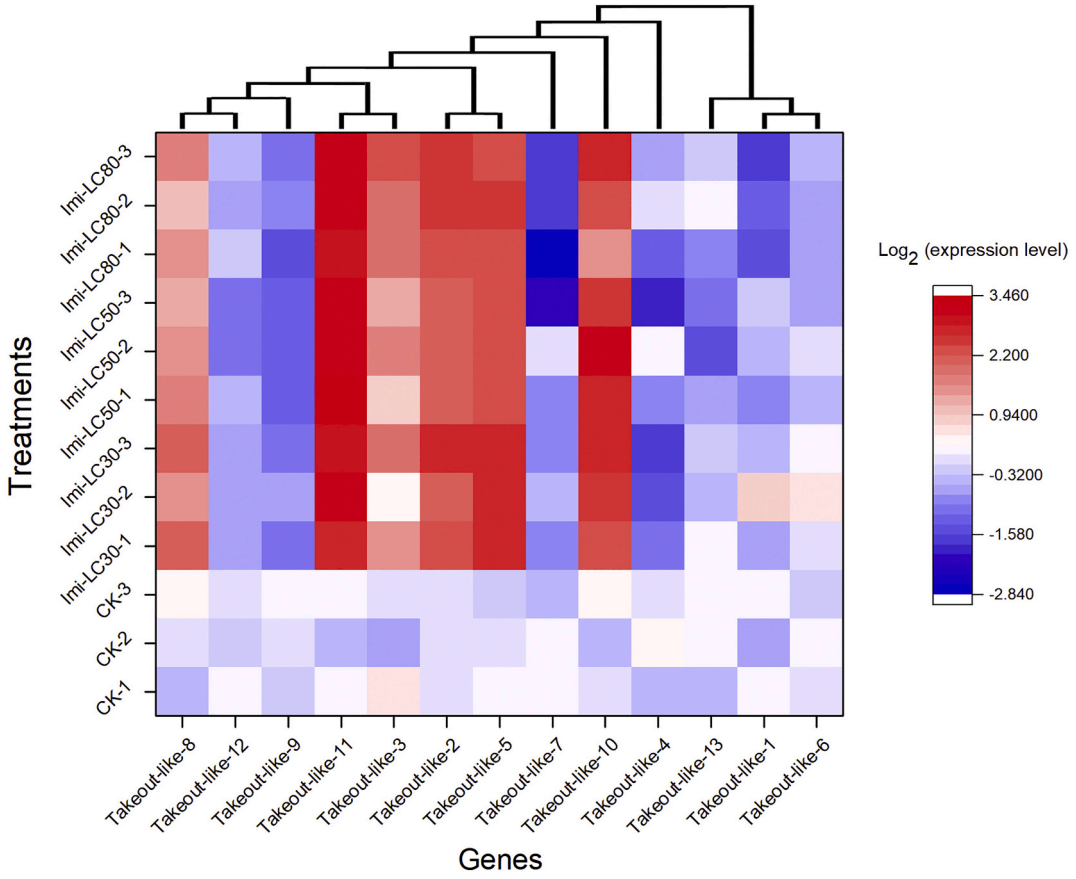

Fig. 4. Heatmap showing the relative expression levels of 13 *takeout-like* genes from *R. padi* treated with three different sublethal concentrations of imidacloprid. CK represents aphid treated with distilled water containing 0.01% (*v*/v) Triton X-100 and 0.01% acetone; Imi-LC_{30}, Imi-LC_{50} and Imi-LC_{80} represent the aphid treated with LC_{30}, LC_{50} and LC_{80} concentrations of imidacloprid, respectively.

significantly decreased in comparison with the control group at 48 h (reduced by 45.80%; P = 0.032) (Fig. 6b).

3.5. The role of takeout-like genes in the insecticide susceptibility of R. padi

Based on the RNAi efficiency determination results showed above, the insecticide treatments were performed 24 h after the ds*TOL-5* injection, and 48 h after the ds*TOL-2,* ds*TOL-7,* ds*TOL-8,* ds*TOL-10* or ds*TOL-11* injection, respectively. After injections of ds*TOL-2* ($P < 0.01$), ds*TOL-5* ($P < 0.001$), ds*TOL-8* ($P = 0.013$), ds*TOL-10* ($P = 0.010$) and ds*TOL-11* ($P = 0.013$), the mortalities of *R. padi* were significantly increased compared with that of ds*GFP* injection after exposure to imidacloprid (LC_{30}), respectively (Fig. 7a). Similarly, the mortalities in *R. padi* exposed to imidacloprid (LC_{50}) after injection with ds*TOL-2* ($P < 0.001$), ds*TOL-5* ($P < 0.001$), ds*TOL-8* ($P < 0.01$), ds*TOL-10* ($P < 0.01$) and ds*TOL-11* ($P < 0.01$) were significantly higher than those in the control group (injection of ds*GFP*) (Fig. 7b). The silencing of *takeout-like-2* resulted in a significant increase in mortality of *R. padi* under beta-cypermethrin (LC_{50}) ($P < 0.001$); however, no significant difference was found in mortality between the treatment group (injection of ds*TOL-2*) and the control group (injection of ds*GFP*) under beta-cypermethrin (LC_{30}) ($P = 0.058$) (Fig. 8a, b). Following knockdown of *takeout-like-5* (LC_{30}: $P = 0.031$; LC_{50}: $P < 0.001$) and *takeout-like-11* (LC_{30}: $P = 0.031$; LC_{50}: $P < 0.001$), the mortality rate of *R. padi* was significantly increased compared with that of ds*GFP* injection after exposure to sublethal concentrations of beta-cypermethrin. RNA interference of *takeout-like-7* (LC_{30}: $P = 0.88$; LC_{50}: $P = 0.29$) and *takeout-like-8* (LC_{30}: $P = 0.89$; LC_{50}: $P = 0.090$) could not significantly affect the mortality of *R. padi* under two sublethal concentrations of beta-cypermethrin (Fig. 8a, b).

4. Discussion

The research on the sensitivity of *R. padi* to insecticides mainly focuses on detoxification enzymes, including cytochrome P450 monooxygenases (cytochrome P450s), carboxylesterases (CarEs) and glutathione S-transferases (GSTs) (Zhang et al. 2017; Balakrishnan et al. 2018; Wang et al. 2018; Wang et al. 2020). In recent years, some nondetoxification enzyme genes have been reported to be involved in insect sensitivity to insecticides, such as *GATA binding protein 2* and *takeout-like* genes (Lin et al. 2017; Zhang et al. 2019; Jia et al. 2020). In the present study, we found that there were 13 *takeout-like* genes in *R. padi*. The expressions of some *takeout-like* genes were significantly increased under three sublethal concentrations of imidacloprid or beta-cypermethrin treatment. Furthermore, knockdown of *takeout-like-2*, *takeout-like-5* or *takeout-like-11* could significantly decrease the survival of *R. padi* under imidacloprid or beta-cypermethrin treatment. The interference of *takeout-like-8* and *takeout-like-10* genes could significantly affect the susceptibility of *R. padi* to imidacloprid. These results indicated that the *takeout-like* genes were involved in the susceptibility of *R. padi* to insecticides.

Takeout/takeout-like proteins had two highly conserved Cys residues at the N-terminal position, which was important for the disulfide bond formation (Sarov-Blat et al. 2000; Dauwalder et al. 2002; Du et al. 2003; Fujikawa et al. 2006). Furthermore, juvenile hormone-binding protein (JHBP) and takeout protein belonged to the same superfamily, and two Cys residues in the N-terminal region of JHBP have been shown to be able to affect the binding ability with ligands (juvenile hormone) (Wojtasek and Prestwich 1995). Hamiaux et al. (2009) first proved that takeout proteins are ligand carriers by direct experimental evidence and found that an almost completely hydrophobic region at

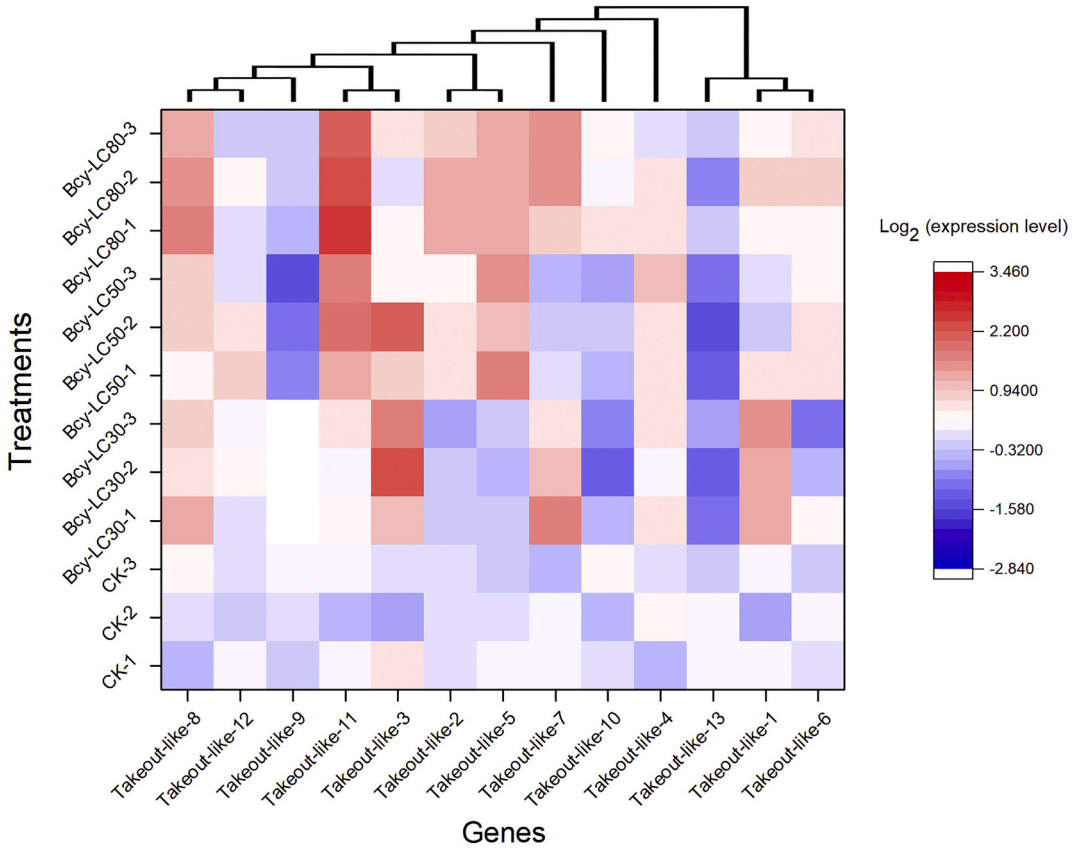

Fig. 5. Heatmap showing the relative expression levels of 13 *takeout-like* genes from *R. padi* treated with three different sublethal concentrations of beta-cypermethrin. CK represents aphid treated with distilled water containing 0.01% (v/v) Triton X-100 and 0.01% acetone; Imi-LC_{30}, Imi-LC_{50} and Imi-LC_{80} represent the aphid treated with LC_{30}, LC_{50} and LC_{80} concentrations of beta-cypermethrin, respectively.

the termini site played a vital role in the ligand binding and release. Then, the ligand binding properties of takeout protein were further verified in *Epiphyas postvittana* (Hamiaux et al. 2013) and *Schistocerca gregaria* (Sugahara et al. 2020). There were some highly conservative amino acid residues, including two Cys and three Gly, in *takeout/JHBP* genes of different insect species (Saito et al. 2006). Moreover, the 18 different takeout proteins from *Spodoptera litura* also contain some highly conserved amino acids: two Cys, two Gly, one Pro, and one Tyr (Lin et al. 2017). In this study, nine highly conserved amino acids (two Cys, two Pro, four Gly and one Asp) were found in 13 takeout-like proteins from *R. padi* (Fig. 2).

Spraying chemical insecticides is a commonly used means to manage insect populations. Insects have carried out a series of defensive strategies to survive under insecticide treatment conditions. Some research results showed that the expression of *takeout/takeout-like* genes was induced by some insecticides. A significant increase in the expression of *takeout-like* gene from second instar *Myzus persicae* was found after exposure to 0.025, 0.1, 0.25 and 2.5 μg/L imidacloprid, respectively; in *Myzus persicae* adults, there were 2.6- and 3.0-fold up-regulations of *takeout-like* genes at 0.025 and 10 μg/L of imidacloprid (Ayyanath et al. 2014). The transcripts of *SlTO4, SlTO10* and *SlTO15* from *S. litura* were significantly increased after treatment with the insecticides chlorpyrifos or emamectin benzoate. After being treated with fipronil, the expression levels of *SlTO4* and *SlTO10* in *S. litura* were induced at a high fold level (Lin et al. 2017). Zhang et al. (2019) compared the gene expression profiles of control and imidacloprid-treated *Schizaphis graminum* baes based on transcriptome data and found that the *takeout-like precursor* gene, one of 20 highly differentially expressed genes, had a significant increase in expression level after imidacloprid treatment. Analysis of RNA-seq data showed that protein takeout in *Spodoptera litura* was significantly up-regulated in response to fluralaner (Jia et al. 2020). Similar results were also observed in *R. padi*; the expression levels of six *takeout-like* genes (*takeout-like-2*, *takeout-like-3*, *takeout-like-5*, *takeout-like-8*, *takeout-like-10* and *takeout-like-11*) were significantly increased after imidacloprid treatment; the seven genes (*takeout-like-1*, *takeout-like-2*, *takeout-like-5*, *takeout-like-6*, *takeout-like-7*, *takeout-like-8* and *takeout-like-11*) tended to be upregulated after beta-cypermethrin treatment. These results indicated that *takeout-like* genes might be involved in the response of *R. padi* to exogenous insecticides.

RNAi was used to further clarify the function of the *takeout-like* genes in the sensitivity of *R. padi* to insecticides. Our results showed that the mortalities of *R. padi* injected with ds*TOL-2*, ds*TOL-5*, ds*TOL-8*, ds*TOL-10* and ds*TOL-11* were significantly increased after exposure to imidacloprid, suggesting that these five genes played important roles in the imidacloprid susceptibility of *R. padi*. Furthermore, the silencing of *TOL-2*, *TOL-5* and *TOL-11* significantly increased the mortalities of *R. padi* under sublethal concentrations of beta-cypermethrin, which indicated that the three genes might be involved in susceptibility of beta-cypermethrin in *R. padi*. Crystal structure analysis of takeout proteins showed that these proteins have a role in binding ligands and function through binding and transportation in insect species (Hamiaux et al. 2013; Sugahara et al. 2020). In *Helicoverpa armigera*, takeout proteins might endogenously perform some functions that act through hydrophobic receptors in lymphocytes (Lin et al. 2017). The two insecticides (imidacloprid and beta-cypermethrin) tested in this study are small hydrophobic molecules, it is possible that the takeout-like proteins could bind with imidacloprid or beta-cypermethrin; increased expression of *takeout-like* genes might be helpful to bind more chemicals and decrease their toxicity, though the mechanism by which the takeout proteins involved with the insecticide susceptibility remain unclear and further studies investigating the mechanisms are required. In *S.*

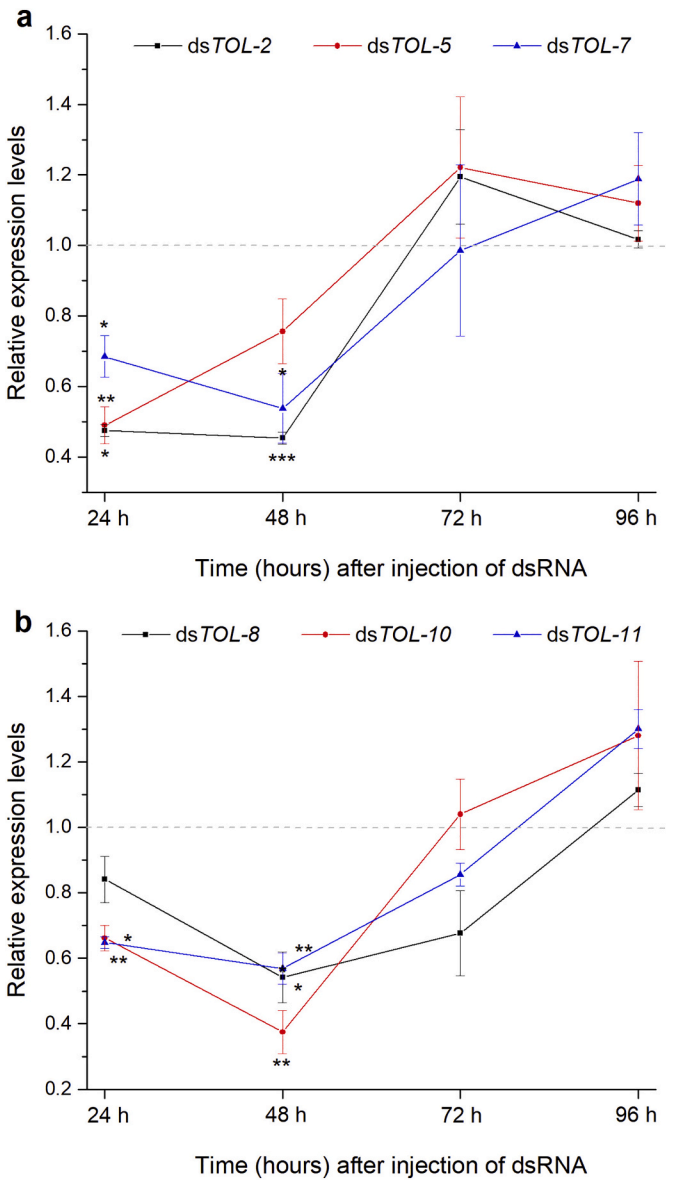

Fig. 6. Relative expression levels of *takeout-like-2*, *takeout-like-5* and *takeout-like-7* after injection of ds*TOL-2*, ds*TOL-5* and ds*TOL-7*, respectively (a), and relative expression levels of *takeout-like-8*, *takeout-like-10* and *takeout-like-11* after injection of ds*TOL-8*, ds*TOL-10* and ds*TOL-11*, respectively (b). Data are shown as the means ± SE. Asterisks at the sides of the bars indicate that the values were significantly different (* $P < 0.05$; ** $P < 0.01$; *t*-test). The expression of all *takeout-like* genes was normalized to the control group (injection of ds*GFP*).

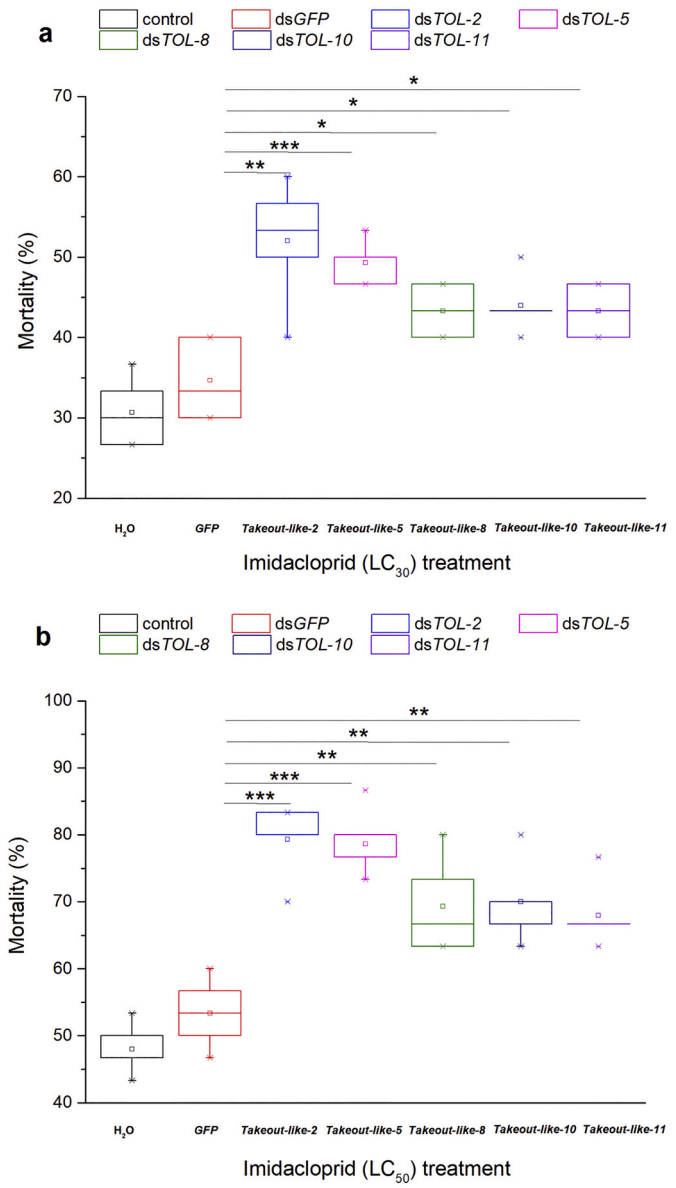

Fig. 7. The mortality of *R. padi* injected with ds*GFP*, ds*TOL-2*, ds*TOL-5*, ds*TOL-8*, ds*TOL-10* and ds*TOL-11* under two sublethal LC_{30} (a) and LC_{50} (b) concentrations of imidacloprid. Uninjected aphids and *R. padi* injected with ds*GFP* were used as control groups. Asterisks on the tops of the bars indicate that the values were significantly different (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test).

graminum, the survival rate was significantly decreased following feeding on ds*takeout-like* for 24 h compared with the control (Zhang et al. 2019). Moreover, *takeout* are circadian clock-regulated output genes, which are thought to be involved in different aspects of insect physiology and behavior. In our unpublished results (Peng and Chen, unpublished), we showed that the circadian clock genes could significantly affect the insecticide susceptibility of *R. padi*, the expression levels of *takeout-like* genes, and some detoxification enzyme activity, however, whether or how the *takeout-like* genes affect the susceptibility of *R. padi* to insecticides by regulating the activity of detoxification enzymes needs further verification.

To a certain extent, different insecticides had diverse effects on the expression of *takeout-like* genes, including *takeout-like-1*, *takeout-like-3*, *takeout-like-6*, *takeout-like-7* and *takeout-like-10,* in *R. padi*. Moreover, the silencing of *takeout-like-8* had a different effect on the susceptibility of *R. pad* to imidacloprid and beta-cypermethrin. These results might be due to the differences in the three-dimensional structure between imidacloprid and beta-cypermethrin. Interestingly, the silencing of *takeout-like-2*, *takeout-like-5* and *takeout-like-11* could significantly affect the susceptibility of *R. pad*, regardless of whether the insects were under imidacloprid or beta-cypermethrin treatment. Recently, due to the extensive use of chemical insecticides, the field populations of *R. padi* have developed moderate resistance to many insecticides in many provinces of China (Zuo et al. 2016; Wang et al. 2020). Therefore, it is of great significance to find new methods to effectively control the population density of the pests and to improve the efficiency of pest control.

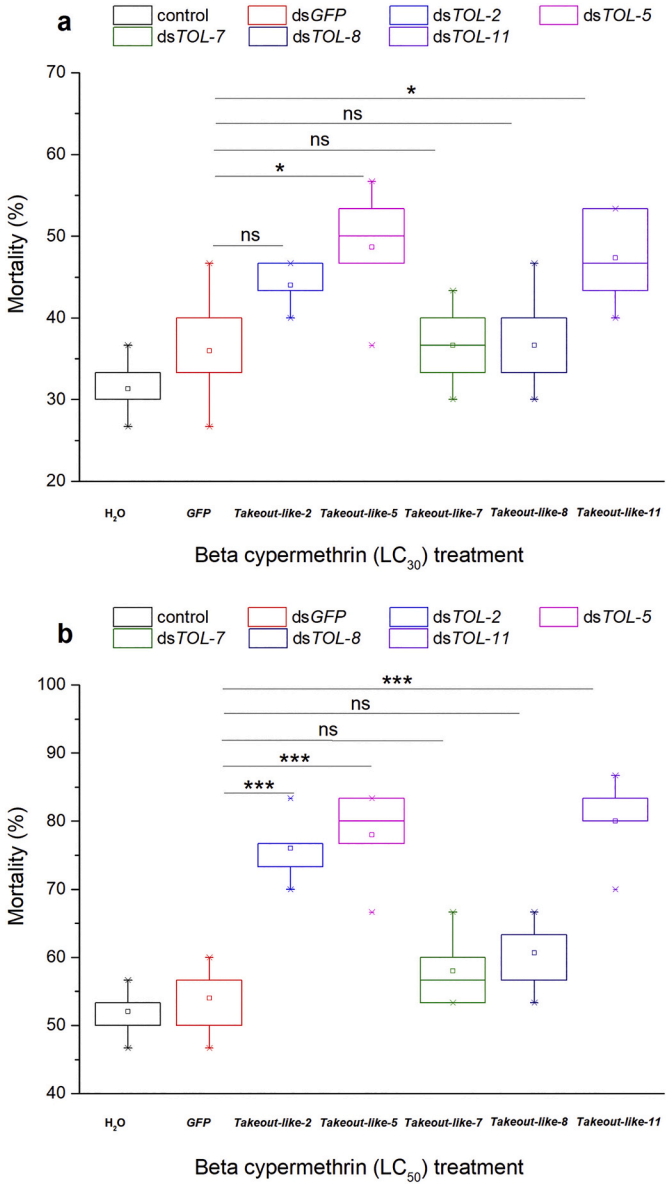

Fig. 8. The mortality of *R. padi* injected with ds*GFP*, ds*TOL-2*, ds*TOL-5*, ds*TOL-7*, ds*TOL-8* and ds*TOL-11* under two sublethal LC_{30} (a) and LC_{50} (b) concentrations of beta-cypermethrin. Uninjected aphids and *R. padi* injected with ds*GFP* were used as control groups. Asterisks on the tops of the bars indicate that the values were significantly different (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t-test).

5. Conclusions

In conclusion, the expression levels of some *takeout-like* genes were significantly induced by imidacloprid and beta-cypermethrin, and *takeout-like* genes have been shown to play an important role in *R. padi* susceptibility to imidacloprid and beta-cypermethrin. However, the mechanism by which the *takeout-like* genes regulate insecticide susceptibility remains unclear. Further studies on binding between takeout-like proteins and insecticides are needed, which may be important for understanding the insecticide susceptibility mechanism of aphids.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2020.104725.

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