Expression patterns, mutation detection and RNA interference of *Rhopalosiphum padi* voltage-gated sodium channel genes

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The voltage-gated sodium channel (VGSC) is the target of sodium-channel-blocking insecticides. Traditionally, animals were thought to have only one VGSC gene comprising a α-subunit with four homologous domains (DI–DIV). The present study showed that *Rhopalosiphum padi*, an economically important crop pest, owned a unique heterodimeric VGSC (H1 and H2 subunits) encoded by two genes (*Rpvgsc1* and *Rpvgsc2*), which is unusual in insects and other animals. The open reading frame (ORF) of *Rpvgsc1* consisted 1150 amino acids, and the ORF of *Rpvgsc2* had 957 amino acids. *Rpvgsc1* showed 64.1% amino acid identity to DI–DII of *Drosophila melanogaster* VGSC and *Rpvgsc2* showed 64.0% amino acid identity to DIII–DIV of *D. melanogaster* VGSC. A M918L mutation previously reported in pyrethroids-resistant strains of other insects was found in the IIS4–S6 region of *R. padi* field sample. The two *R. padi* VGSC genes were expressed at all developmental stages and showed similar expression patterns after treatment with beta-cypermethrin. Knockdown of *Rpvgsc1* or *Rpvgsc2* caused significant reduction in mortality rate of *R. padi* after exposure to beta-cypermethrin. These findings suggest that the two *R. padi* VGSC genes are both functional genes.
F932, L1014) associated with pyrethroid resistance have been identified in several species, including M. persicae, Aphis gossypii, and Sitobion avenae.

Rhopalosiphum padi (Linnaeus) (Hemiptera: Aphididae) is one of the key pests of wheat crops, causing significant damage by direct feeding and by the transmission of plant viruses. This aphid is distributed in wheat-growing areas worldwide. In China, damage by R. padi is increasing annually in wheat-growing regions. Resistance of R. padi to various insecticides, including pyrethroids, has been reported in China.

Despite the economic importance of R. padi and development of insecticide resistance of the pest, the molecular mechanisms underlying its pyrethroid resistance are still unknown. Our objective was to clone and analyze the structural features of the R. padi VGSC gene (Rpvgsc), and assess the expression of Rpvgsc at different developmental stages of the aphid. We analyzed the transcriptional characteristics of the VGSC genes in R. padi after exposure to beta-cypermethrin and detected mutation sites in the IIS4–IIS6 region of Rpvgsc from field populations. We hypothesized that R. padi harbors two Rpvgsc genes, both of which are neotargets. RNA interference (RNAi) was used to test the function of VGSC genes in R. padi.

Results

cDNA cloning and characterization of Rpvgsc genes. We obtained two Rpvgsc genes, Rpvgsc1 and Rpvgsc2, which encoded the H1 and H2 subunits, respectively. The complete open reading frame (ORF) of the Rpvgsc1 was 3450 nucleotides long, encoding 1150 amino acids (~13.03 kDa) (GenBank accession no.: KJ872633). The Rpvgsc2 ORF was 2874 nucleotides long, encoding 957 amino acids (~11.01 kDa) (GenBank accession no.: KP966088). The Rpvgsc1 and Rpvgsc2 ORFs suggested that the R. padi sodium channel was encoded by two unique 2 × 6 TM heteromers. Rpvgsc1 had 64.1% identity to the amino acid sequence of the DI–DII domain of the Drosophila melanogaster para voltage-gate sodium channel gene, whereas the Rpvgsc2 had 64.0% identity to the amino acid sequence of the DIII–DIV domain of the D. melanogaster para voltage-gate sodium channel gene (Fig. 1). The predicted R. padi Rpvgsc1 and Rpvgsc2 possessed the expected features of a voltage-dependent sodium channel (Fig. 1). First, the S4 region of each domain in R. padi Rpvgsc1 and Rpvgsc2 had four to seven basic amino acid residues, arginine (R) or lysine (K), separated by two neutral amino acid residues. These sequence features are characteristics of voltage sensors, which move outward in response to membrane depolarization and initiate the opening of the channel.

Second, a conserved insect MFM motif, which is conserved in seven basic amino acid residues, arginine (R) or lysine (K), separated by two neutral amino acid residues. These sequence features are characteristics of voltage sensors, which move outward in response to membrane depolarization and initiate the opening of the channel.

Third, a channel selectivity filter motif (DENS) was found in the linker between domains III and IV in the IIS4–IIS6 region of the Rpvgsc1 and Rpvgsc2 genes (Fig. 1). The predicted R. padi Rpvgsc1 and Rpvgsc2 possessed the expected features of a voltage-dependent sodium channel (Fig. 1). First, the S4 region of each domain in R. padi Rpvgsc1 and Rpvgsc2 had four to seven basic amino acid residues, arginine (R) or lysine (K), separated by two neutral amino acid residues. These sequence features are characteristics of voltage sensors, which move outward in response to membrane depolarization and initiate the opening of the channel.

Mutations in the IIS4–IIS6 region of the R. padi Rpvgsc gene. The IIS4–6 region (~1640 bp) of the R. padi VGSC gene was amplified by RT-PCR using the RpNa primers (Table 1). Three introns (intron 1, ~131 bp; intron 2, ~67 bp; and intron 3, ~1010 bp) were present in this region. No R. padi field samples had mutation in the exon of the IIS4-6 region except that only one individual from the HNN population showed a single base difference, which caused a super-\( \text{kdr} \) mutation (M918L) (Fig. 3). This base change (ATG to TTG) appeared as a mixed peak in the sequence chromatograms (Fig. 4), indicating a heterozygous allele. No other mutation sites (M918, L925, T929, and F932) previously reported in other insect VGSCs were detected in the samples.

Expression of Rpvgsc genes at different developmental stages of R. padi. qRT-PCR analysis showed that Rpvgsc1 and Rpvgsc2 were expressed at all developmental stages of R. padi. The highest expression level of Rpvgsc1 was found in the 3rd instar and was 1.14-fold of that in the adult aphid (Fig. 5). The lowest expression level was found in the 1st instar and was significantly lower than those at all other developmental stages \( (p < 0.05) \) (Fig. 5). There were no significant differences among Rpvgsc1 gene expression levels in the 2nd, 3rd, 4th, and adult. The highest expression level of Rpvgsc2 was found in 4th instar R. padi, and the lowest expression level was found in the 3rd instar. There was no significant difference in the Rpvgsc2 expression level among the 1st, 2nd, 4th, and adult (Fig. 5). In addition, the Rpvgsc1 and Rpvgsc2 expression levels differed significantly in the 3rd instar.

Expression of Rpvgsc genes upon exposure to beta-cypermethrin. Compared with the control, the Rpvgsc1 and Rpvgsc2 expression levels were influenced by exposure to LC\(_{10}\) and LC\(_{30}\) concentrations of beta-cypermethrin. When exposed to 0.3987 mg/L beta-cypermethrin, the transcription levels of Rpvgsc1 and Rpvgsc2 peaked at 24 h and were 1.70-fold and 1.38-fold, respectively (Fig. 6A). There was no significant difference in Rpvgsc1 expression levels among the treatment time points. The transcription level of Rpvgsc2 at 24 h was significantly higher than that at 12 h and 36 h (Fig. 6A). Upon exposure to 0.9280 mg/L beta-cypermethrin, the transcription levels of Rpvgsc1 (at 24 h) and Rpvgsc2 (at 12 h) peaked; both were around 1.37-fold of that for the control (Fig. 6B). The transcription levels of Rpvgsc1 and Rpvgsc2 at 36 h were significantly lower than those at 12 h and 24 h (Fig. 6B). Upon exposure to 0.3987 mg/L and 0.9280 mg/L beta-cypermethrin, there was no significant difference in Rpvgsc1 and Rpvgsc2 expression throughout the treatment period (Fig. 6).

RNAi of H1 and H2 subunit genes and the effect on R. padi susceptibility to beta-cypermethrin. The silencing efficiencies of the two dsRNAs on H1 and H2 genes were examined using real-time quantitative PCR. Injection of H1-dsRNA and H2-dsRNA was effective in silencing the respective transcription of H1 and H2.
Figure 1. Alignment of deduced amino acid sequences of the *R. padi* voltage-gate sodium channel gene H1 and H2 subunits with sodium channels of *D. melanogaster* and with the H1 and H2 subunits of the *A. pisum* and *M. persicae* sodium channels. Transmembrane helices from different domains are indicated by bars above the sequences. Identical amino acids are highlighted in gray. The apparent evolutionary sequence duplication in the DII-DIII intracellular loop to generate the novel 5′ end for H2 is highlighted in black. The MFM motif is indicated by an asterisk (*). Basic amino acid residues in helix 4 are indicated by a black box. Triangles mark the positions of the sodium selectivity filter motif (DENS). The amino acid sequences far from the six transmembrane segments (S1–S6) are indicated by the suspension point lines. (−), deletion; Dm para, VGSC amino sequences of *Drosophila melanogaster* (GenBank accession No.: AAB59195.1); Ap H1, amino sequences of *Acrithosiphon pisum* VGSC H1 subunit (XP_008183365.1); Mp H1, amino sequences of *Myzus persicae* VGSC H1 subunit (CBI71141.1); Rp H1, amino sequences of *Rhopalosiphum padi* VGSC H1 subunit (KJ872633); Ap H2, amino sequences of *Acrithosiphon pisum* VGSC H2 subunit (XP_001949648.2); Mp H2, amino sequences of *Myzus persicae* VGSC H2 subunit (CBI71142.1); Rp H2, amino sequences of *Rhopalosiphum padi* VGSC H2 subunit (KP966088).
subunit genes. The H1 subunit gene mRNA levels decreased dramatically (reduced by 54.8%) at day 1 after injection of H1-dsRNA compared with the GFP-dsRNA injection, and expression of H2 subunit gene was reduced by 42.3%, indicating significant cross-suppressions in the transcript levels between H1 and H2 subunit genes. The RNAi effect of H1-dsRNA was greatly decreased at day 2 and day 3, and H1 subunit genes mRNA levels were similar to that in the control (Fig. 7A). Injection of H2-dsRNA also induced reduction in the transcript levels of both H1 and H2 subunit genes in R. padi (Fig. 7B). The transcript levels of H2 subunit gene were significantly reduced at day 3 after H2-dsRNA injection, with significant cross-suppressions in the transcript levels between H1 and H2 subunit genes at day 3, and the effect was still strongly at day 4.

RNAi of H1 and H2 subunit genes significantly decreased the susceptibility of R. padi to beta-cypermethrin (Fig. 8). After knockdown of H1 subunit gene, the mortality rate (38.7%) of the survivals was significantly lower than that of dsGFP injection and that of control (naive insect without any injection) after exposure to LC50 concentration of beta-cypermethrin (p < 0.05) (Fig. 8A). Similar result was observed after knockdown of H2 subunit gene (Fig. 8B).

Figure 2. Phylogenetic analysis of Rpvgsc with other VGSC genes from arthropods and mammalians.
Values at the nodes indicate bootstrap support values based on 1000 replicates. Branches with bootstrap values above 50% were collapsed. GenBank accession numbers or Gene IDs are as follows: Drosophila ananassae (XP_001966146.1), Drosophila melanogaster (AAB59195.1), Drosophila grimshawi (XP_001992511.1), Bactrocera dorsalis (AEX08661.1), Musca domestica (CAA65448.1), Anopheles gambiae (CAM12801.1), Aedes aegypti (ACB37023.1), Culex pipiens pallens (BAI77918.1), Culex quinquefasciatus (CAM31893.1), Plutella xylostella (BAF37096.2), Helicoverpa zeae (ADJ80418.1), Heliothris virescens (AAC26517.1), Cnaphalocrocis medinalis (AGH70334.1), Danaus plexippus (EH74501.1), Bombyx mandarina (ACD80425.1), Bombyx mori (NP_001136084.1), Blattella germanica (AAX47484.1), Periplaneta americana (ACX44801.1), Nasonia vitripennis (XP_008204157.1), Apis cerana (AEGY6607.1), Camponotus floridanus (EFN61422.1), Harpegnathos saltator (EFN86793.1), Cimex lectularius (ACI43362.1), Myzus persicae (CBI71141.1 and CBI71142.1), Acrithosiphon pisum (XP_008183365.1 and XP_001949648.2), Rhopalosiphum padi (KJ872633 and KP966088), Tetranychus urticae (AFU35097.1), Tetranychus cinnabarinus (AFR6409.1), Homo sapiens (NP_066287.2), Mus musculus (NP_001092768.1). The variable regions between the H1 and H2 subunits of the three aphid species—R. padi, A. pisum, and M. persicae—were excluded, and the two subunits were combined for better alignment. Bootstrapping of the ML analysis was conducted using 1000 replicates.
Table 1. Primers used for RT-PCR, quantitative PCR and RNA interference of Rpvgsc.

| Fragment | Forward (5′ to 3′) | Reverse (5′ to 3′) |
|----------|--------------------|--------------------|
| H1-1     | CGTCCTGTATCGTGTCGXTGCT | TATGCTGGAGTTGGGCCATTAT |
| H1-2     | TGCTGACCGATCTGGATTTT | ATCCCGAATAGTGCAAGCA |
| H1-3     | AACCTGAGGATAGGAAGCGT | CAAAGACGACTAGAACAGAG |
| H1-4     | GTTGCCATTCCTCTGTTCTT | CTTTGGCTGACAGGTTAG |
| H1-5     | GCCGAGGGGATGATGCCAGG | ATACAGGAGTTGGTGTAGCC |
| H1-6     | GAACTGCTGGCTGAACTTG | TACACCTGGGACAGTATAG |
| H1-7     | TGCCATCGTGACAGTGAACG | TACACCTGGGACAGTATAG |
| Full H1  | GCCGAGGGGATGATGCCAGG | ATACAGGAGTTGGTGTAGCC |
| H2-1     | CGGCTGACCGATTCTTATCC | AACAGGCTGAGGACAGA |
| H2-2     | TGCTGACCGATTCTTATCC | AACAGGCTGAGGACAGA |
| H2-3     | GCCGAGGGGATGATGCCAGG | ATACAGGAGTTGGTGTAGCC |
| H2-4     | GAACTGCTGGCTGAACTTG | TACACCTGGGACAGTATAG |
| Full H2  | GCCGAGGGGATGATGCCAGG | ATACAGGAGTTGGTGTAGCC |
| QH1      | CGGCTGACCGATTCTTATCC | AACAGGCTGAGGACAGA |
| QH2      | CGGCTGACCGATTCTTATCC | AACAGGCTGAGGACAGA |

Figure 3. Nucleotide and amino acid sequences of the IIS4–IIS6 region from the susceptible R. padi clone (RpSS) and a field population (HNN). The single codon change ATG to TTG causing the super-kdr substitution M918L is boxed. The positions of the PCR and sequencing primers are indicated by arrows. The positions of the three introns (intron 1, ~131 bp; intron 2, ~67 bp; and intron 3, ~1010 bp) are indicated by vertical arrows.
Discussion
Sodium channels are targeted by pyrethroids. Due to the intensive use of pyrethroids, pyrethroid resistance, which involves reduced target-site sensitivity, has developed in many pest populations in the past several decades. In this study, we cloned and characterized sodium channel genes from the important wheat pest, R. padi, and found that the R. padi para sodium channel was encoded by two separate genes. qRT-PCR analysis showed that the expression of the two genes varied at different R. padi developmental stages, and exposure to beta-cypermethrin affected the expression of both genes. The transcript levels of the two separate genes could be significantly suppressed by injection of their respective dsRNA. Knockdown each of the two genes significantly reduced the mortality rate of R. padi after exposure to LC50 concentrations of beta-cypermethrin.

Rpvgsc is an unique heterodimeric voltage-gated sodium channel encoded by two separate genes, each of which comprises two unique 2 × 6 TM heteromers and has a reasonable degree of identity with known para...
widely accepted hypothesis is that the primordial Ca\textsuperscript{2+} channel gave rise to the Na\textsuperscript{+} channel by further divergence following gene duplication. Based on sequence similarities, Strong et al.\textsuperscript{29} suggested that the two-domain channel consisting of domains I/III and II/IV, each of which then duplicated to result in the first four-domain Ca\textsuperscript{2+} channel, emerged because of the original duplication event. Phylogenetic analysis suggested that the VGSC of aphids forms an independent branch, which is differentiated from other channel genes of insecta and arachnida species, but that it shares a common ancestor gene with the channel genes of other insecta species, suggesting that the aphid VGSC was orthologous to VGSC of other insects and that aphid heterodimeric assembly had arisen by structural modification of an ancestral 4 × 6 TM invertebrate Nav channel.\textsuperscript{7} It is possible that gene fission resulted in this modification, which occurred in a duplication of part of the domain II–III linker region in the ancestral gene of aphids.\textsuperscript{7}

The *Apis mellifera* para-like sodium channel is encoded by one gene located on the LG9 chromosome (NC_007078: 5110044–5069614), and the pea aphid *A. pisum* para-like sodium channel is encoded by two genes located on scaffold 318 ([GL349938: 191764–191391 (III–IV) and 175077–144603 (I–II)], respectively. The two genes are transcribed in opposite directions, which suggests that the aphid para-like sodium channel is encoded by two genes. Modification of the aphid para-like sodium channel gene was caused by chromosome inversion (Fig. 9). *Rpvgsc1* and *Rpvgsc2* expression profiles were different in different stages. Knockdown one of the two genes could cause significant cross-suppressions of another one. On the other hand, the *Rpvgsc1* and *Rpvgsc2* expression levels were affected by treatment with two concentrations of beta-cypermethrin. Knockdown *Rpvgsc1* or *Rpvgsc2* could
significantly reduce the susceptibility of *R. padi* to beta-cypermethrin, indicating the decrease of the primary (i.e. voltage-gated sodium channel) targets of pyrethroid reduce the effect of the chemical in overstimulation of the aphid nerve that eventually caused death. *Rpvgsc1* and *Rpvgsc2* transcription was up- and down-regulated at different treatment time points after exposure to beta-cypermethrin, which may maintain the ion equilibrium status of the body of *R. padi*. The aforementioned results suggested that *Rpvgsc1* and *Rpvgsc2* were both functional, and function together rather than individually. It is possible that expression of two genes was regulated by the different regulatory factors. In different development stages of *R. padi*, the regulatory factors could regulate the corresponding gene differently, resulting in varied expression of two genes at a same stage. Further investigation is required to analyze the detailed co-expression patterns and the functions of *Rpvgsc1* and *Rpvgsc2* in *R. padi*, as well as to elucidate the adaptive evolution of voltage-gated sodium channel in *R. padi* and other aphid species.

**Materials and Methods**

**Insects.** A susceptible *R. padi* clone (RpSS) was reared in the laboratory for >3 years at 22°C ± 1°C with a 16 h light/8 h dark cycle and 80% relative humidity. To identify mutations, apterous *R. padi* adults were collected from wheat (*Triticum aestivum* L.) in seven regions of various provinces of China (Table 2). One aphid was collected per plant, and the distance between the sampled plants was ≥30 m. The samples were preserved in absolute ethanol and transported to the laboratory for DNA extraction.
Rpvgsc gene cloning. Total RNA was extracted from 15 apterous adult females from RpSS strain using 500 μL TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Takara, Kyoto, Japan) to remove genomic DNA contamination. The purity and concentration were determined using a biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany). cDNA was synthesized from 2 μg of total RNA at 42 °C for 90 min with oligo dT-adaptor primers using an M-MLV reverse transcriptase cDNA Synthesis Kit according to the instructions of the manufacturer (Promega, Madison, WI, USA).
**Rpvgsc** was amplified from cDNA. PCR was performed using gene-specific primers designed based on the nucleotide sequences of *vgsc* from *M. persicae* (accession nos. FN601405.1 and FN601406.1) and *A. pisum* (accession nos. XM_001949613.3 and XM_008185143.1) (Table 1). The amplification strategies and sequence-specific primers used for cloning **Rpvgsc** are shown in Fig. 10 and Table 1. We amplified 10 fragments (fragments H1-1 to H1-6 and H2-1 to H2-4) covering the open reading frames of two separate voltage-gated sodium channel genes. Then, two pairs of primers (Full H1 and Full H2; Table 1) were used to confirm the full length of the two genes, separately. PCR was performed in a volume of 25 μL using standard procedures with 2 μL of each primer (10 mmol L\(^{-1}\)), 2.5 μL of 10× buffer (Mg\(^{2+}\) Plus), 4 μL of dNTPs (0.2 mmol L\(^{-1}\) each), 2 μL cDNA as the template, 0.25 μL of Takara LA-Taq DNA polymerase (LA Taq; Takara Bio, Dalian, China) (5 U/μL), and 12.25 μL of ddH\(_2\)O, which resulted in more efficient amplification and higher fidelity than conventional Taq DNA polymerase, under PCR conditions of 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 52–62 °C for 30 s, and 72 °C for 1–4 min, and a final 10 min at 72 °C. The PCR products were purified using a Wizard PCR Preps kit (Promega, Madison, WI). The purified fragments were then cloned into the pGEM-Teasy Vector (Promega) and transformed into *Escherichia coli* JM109. Finally, five positive clones were randomly chosen for bidirectional sequencing on an Applied Biosystems 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) using the commercial services of Sangon Biotech (Shanghai, China).

**Detection of mutations in the IIS4–IIS6 region of **Rpvgsc.** Ten individuals from each of the seven regions were selected randomly as well as from the RpSS colne. Genomic DNA was extracted from single aphids using the DNeasy Tissue Kit following the manufacturer’s recommendations (Qiagen, Hilden, Germany). DNA was eluted using TE buffer and stored at –20 °C. The RpNa primer pair (Table 1) was used to amplify the IIS4-6 region of the *R. padi* sodium channel gene. PCR amplification, gel extraction, cloning, and plasmid purification were performed according to the aforementioned methods. Three positive clones from each sample were selected randomly for bidirectional sequencing on an Applied Biosystems 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) using the commercial services of Sangon Biotech (Shanghai, China).

**Phylogenetic analysis.** A rooted tree was generated using MEGA 5.0, with the maximum likelihood (ML) method according to the Jones–Taylor–Thornton (JTT) model\(^{32}\). The alignments of protein sequences were generated by Clustal X\(^{23}\). VGSC of *Homo sapiens* (NP_066287.2) and *Mus musculus* (NP_001092768.1) were used as outgroups. The variable regions between the H1 and H2 subunits of the three aphid species—*R. padi*, *A. pisum*, and *M. persicae*—were excluded, and the two subunits were combined for better alignment. Bootstrapping of the ML analysis was conducted using 1000 replicates.

**Rpvgsc expression in **R. padi** according to developmental stage.** For analysis of **Rpvgsc** expression at different developmental stages, individuals (5 mg) of each stage from RpSS strain were used to isolate total RNA as described above. Three independent extracts were generated. The first-strand cDNA of each RNA sample for RT-qPCR was synthesized using 1.5 μg of total RNA using an M-MLV reverse transcriptase cDNA Synthesis Kit (Promega, Madison, WI, USA) in 20 μL reaction mixtures as described by the manufacturer. Primers for RT-qPCR were listed in Table 1. β-actin was used as the house-keeping gene in the analysis\(^{32,33}\). The specificity of

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**Figure 10.** Schematic representation of the aphid sodium channel (A) and cloning strategies for the *R. padi* voltage-gate sodium channel gene (B). (A) The aphid sodium channel contains H1 and H2 subunits, each of which contains two homologous domains, which consist of six transmembrane regions (4 × 6 TM). The approximate position of the fast inactivation particle ‘MFM’ motif and ion (Na\(^{+}\)) selectivity filter consists of one loop from each domain (located between S5 and S6 of the pore region), which collectively form a ‘DENS’ (D1-aspartate; DII-glutamate; DIII-asparagine; DIV-serine) amino acid sequence motif at the entry to the pore. (B) The locations and lengths of the cDNA fragments amplified in this study are shown.
primer pairs (QH1 and QH2 for target gene amplification and β-actin as the endogenous control) was tested and confirmed by sequencing based on preliminary experiment (data not shown). qPCR reactions were performed in a 20μL total reaction volume including 10μL of 2 × SYBR® EX Taq™ (Takara) master mix, 0.8μL each of gene-specific primers (Table 1), 2μL of cDNA template, and 6.4μL of ddH2O. Samples without reverse transcriptase enzyme or cDNA template were used as negative controls. Three technical replicates were run for each sample. Reactions were carried out using an iQ5™ (Bio-Rad, Hercules, CA, USA) according to the operation manual. The PCR parameters were as follows: 3 min at 95 ºC; 40 cycles of 10 s at 95 ºC, of 20 s at 58 ºC, and of 20 s at 72 ºC; followed by 15 s at 55 ºC (gradient rising to 95 ºC at 0.5 ºC intervals) to generate a melt curve. At least three individual samples were prepared, and each sample was analyzed in triplicate.

**Rpvgsc expression after induction with beta-cypermethrin.** According to assays of the toxicity of beta-cypermethrin (96%, Yancheng Nongbo Biotechnology Co., Ltd., Jiangsu China) to *R. padi* (data not shown), we used sublethal LC10 (0.3987 mg/L) and LC30 (0.9280 mg/L) concentrations of beta-cypermethrin and assessed the response of *Rpvgsc* genes. Beta-cypermethrin was dissolved in acetone as a stock solution (10g/L). Then, the stock solution was further diluted to the LC10, LC30 and LC50 beta-cypermethrin solution using 0.1% Triton X-100 solution, with 0.0040% (v/v) acetone in LC10 solution, 0.0093% (v/v) acetone in LC30 solution, and 0.0167% (v/v) acetone in LC50 solution, respectively. The leaf-dipping method was adopted for the reagent 15,33. Wheat leaves with apterous adult aphids were dipped in the insecticide solutions for 10 s. The leaves were then removed from the solution, and residual droplets on the leaf were adsorbed using clean, dry filter paper. Then, the leaves were transferred to plastic Petri dishes lined with moistened filter paper. Each insecticide concentration was assayed in triplicate, each using 100 apterous adult aphids. Water was used as a control. Live aphids treated with insecticide or water were collected at 12, 24, and 36 h for determination of mRNA expression levels. qPCR was performed to assess insecticide-induced *Rpvgsc* expression. The qPCR method was identical to that described above.

**Double-stranded RNA synthesis of H1 and H2 subunits.** Sequence-specific primers of H1 and H2 subunits (Table 1) were synthesized to amplify target sequences. The PCR fragments were gel-purified (Promega, Madison, WI), cloned into the pGEM-Teasy Vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM109. The recombinant plasmids were sequenced by Sangon Biotech (Shanghai, China) to ensure the reliability of these primers. To synthesize H1-dsRNA, two separate PCR reactions were performed using a sequence-specific primer and a primer by attaching T7 promoter sequences at 5’ terminal (Table 1). The same method was used to synthesize H2-dsRNA. Then, the PCR products were purified and utilized to synthesize dsRNA using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The dsRNAs were dissolved in nuclease-free water. The purity and concentration of dsRNAs were examined the biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany). The dsRNAs were stored at −80 ºC until use. The dsGFP was synthesized and used as a control.

**RNAi of H1 and H2 subunit genes and insecticide bioassays.** A volume of 41.4 μL dsRNA (4.1 μg/μL) was injected into body cavity of each one-day-old apterous adult aphid by a micro glass needle using automatic nanoliter injector (Märzhäuser, Wetzlar, Germany). Injection location on the aphids were at the suture joining the ventral mesothorax and metathorax34. The mechanical injuries caused about 20% mortality to the aphids. After injection, each aphid alive was reared on a wheat leaf in a plastic petri dish (9 cm in diameter) under condition of 24 ºC, 70% relative humidity and a 16:8 h L: D photoperiod.

To determine the reduction of transcription levels of H1 and H2 subunit genes and to determine the time of highest interference efficiency, ten survival adults were randomly collected at 24 h, 48 h, 72 h, 96 h or 120 h after injection. qPCR was used to analyze the transcription levels of H1 and H2 subunit genes of the survival aphid collected. The qPCR primers were from a separate region of H1 and H2 subunit genes to those used for RNAi. Three replications were carried out, with at least 200 aphids per treatment.

After the time of the highest RNAi efficiency for each of the two genes were determined, about 200 one-day-old apterous adult aphids were randomly collected and divided into two groups, and H1-dsRNA and dsGFP were injected into the aphids of two groups, respectively. Then, the LC30 (1.667 mg/L) concentrations of beta-cypermethrin was applied on each survival aphid from the two groups at time of highest interference efficiency using the aforementioned leaf-dipping method, and around 80 naive aphids were used as control. The mortality of the aphids in the three treatments was assessed 24 h after exposure to the chemical. Three replicates were carried out. The same method was used to examine the effect of H2 subunit gene RNAi on the sensitivity of *R. padi* to beta-cypermethrin.

**Data analysis.** DNA sequences were analyzed using ClustalX (http://www.clustal.org/). A BLAST search was performed at the NCBI Website http://blast.ncbi.nlm.nih.gov/Blast.cgi. qPCR results were analyzed using the `2−ΔΔCt` method35. Bioassay data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A two tailed t test at the significance level 0.05 was used to test whether the expression level of *Rpvgsc1* and *Rpvgsc2* was significant different between aphids with and without exposure to beta-cypermethrin. Mortality rate data (percentage) were transformed using arcsine square-root transformation, and then the transformed data were subjected to ANOVA. Data of relative expression was also subjected to ANOVA. All ANOVA was followed by Tukey’s honest significant different (HSD) multiple comparisonst using ProStat software (Poly Software International, Pearl River, NY).

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Author Contributions
Y.Z., X.P. and M.C. designed the research, interpreted the data and wrote the paper. Y.Z., X.P., K.W., Y.L. and F.L. performed experiments.

Additional Information

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