**NICYP4G76 and NICYP4G115 Modulate Susceptibility to Desiccation and Insecticide Penetration Through Affecting Cuticular Hydrocarbon Biosynthesis in *Nilaparvata lugens* (Hemiptera: Delphacidae)**

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The functions of cuticular hydrocarbons (CHCs) are varied in insects, but one example is to reduce water loss. Previous work has suggested that biosynthesis of CHCs is strongly related to the CYP4G sub-family. Targeting these genes in the brown planthopper, *Nilaparvata lugens* Stål, might be a new application for integrated pest management. Therefore, we explored the functions of CYP4G76 (GenBank: KM217045.1) and CYP4G115 (GenBank: KM217046.1) genes in this study. The desiccation treatment (RH < 5%) for the duration of 1–3 days significantly increased the transcription level of CYP4G76 and CYP4G115. RNAi through the injection of CYP4G76 and CYP4G115 dsRNA could significantly decrease their expression, respectively, and further reduced the biosynthesis of CHCs, i.e., saturated and straight-chain alkanes. When CYP4G76 and CYP4G115 were suppressed, the susceptibility of *N. lugens* nymphs to desiccation increased, due to the deficiency of the CHCs in the insect’s cuticle. When the expression of CYP4G76 and CYP4G115 was decreased, this resulted in an increased rate of penetration of the four insecticides: pymetrozine, imidacloprid, thiamethoxam and buprofezin. Therefore, CYP4G76 and CYP4G115 appear to regulate the biosynthesis of CHCs in *N. lugens* nymphs, which play a major role in protecting insects from water loss and the penetration of insecticides. CYP4G76 and CYP4G115 might be used as a novel target in integrated pest management to *N. lugens*.

**Keywords**: *Nilaparvata lugens*, CYP4G, hydrocarbons, waterproofing, insecticide penetration

**INTRODUCTION**

Insect cuticular hydrocarbons (CHCs) are a mixture that contains many straight and branched saturated alkanes and unsaturated alkenes from C_{21} to C_{37} (Blomquist et al., 1987; Lockey, 1988). Many previous researches showed that the functions of CHCs were various (Châline et al., 2005; Blomquist and Bagneres, 2010; Balabanidou et al., 2016; Chen et al., 2016; Yu et al., 2016;
including ecology, behavior, and biochemistry. Previous research also indicated that the CHCs are formed with long-chain fatty alcohol or aldehyde by the insect-specific CYP4G, which encodes an oxidative decarboxylase belonging to the cytochrome P450 gene family (Qiu et al., 2012; Balabanidou et al., 2016; Chen et al., 2016; Yu et al., 2016; Otte et al., 2018). Modulating CHCs biosynthesis to regulate water loss through the cuticle might be the primary role of the CYP4G subfamily (Qiu et al., 2012; Chen et al., 2016; Yu et al., 2016). Furthermore, metabolizing the hazardous materials such as insecticides is also an important function of CYP4G members (Guo et al., 2010; Martínez-Paz et al., 2012). In the subfamily insecticides is also an important function of CYP4G members (Qiu et al., 2012; Chen et al., 2016; Yu et al., 2016). Furthermore, metabolizing the hazardous materials such as insecticides is also an important function of CYP4G members (Qiu et al., 2012; Martínez-Paz et al., 2012). In the subfamily (Qiu et al., 2012; Balabanidou et al., 2016; Chen et al., 2016; Yu et al., 2016).

In order to explore the mortality and phenotype of N. lugens nymphs under a desiccation condition, the third instar nymphs were injected with 40 nL (2000 μg·mL⁻¹) of dsGFP, single dsCYP4G76, single dsCYP4G115, or combined dsRNAs containing dsCYP4G76 and dsCYP4G115 (1:1), these injected insects were raised for 4 days to produce a new cuticle and then were kept at a desiccation condition (RH < 5%) for 1 day.

### Tissue Dissection, RNA Isolation, cDNA Synthesis, and Cloning

Before extracting the total RNA, all treated N. lugens nymphs were stored at −80°C in an ultra-low temperature freezer. The method and reagents for extracting the total RNA followed those outlined by Dalian Takara Co., Ltd. (Liaoning, China). The first strand of cDNA was synthesized with 500 ng total RNA and PrimeScript™ RT reagent Kit (Takara Co., Ltd., Dalian, Liaoning, China). Paired primers (10 nM) were designed and used to clone two 383 and 420 bp fragments of the target genes CYP4G76 and CYP4G115, respectively. The green fluorescent protein (GFP, GenBank: AF372525.1) with an 864 bp fragment was used as a control. All primers were shown in Table 1.

The PCR thermocycler parameter was as follows: (1) 94°C for 3 min; (2) 34 cycles at: 94°C for 30 s, 55°C for 30 s, 72°C for 20 s; and (3) 72°C for 5 min. All reagents were supplied by Takara Co., Ltd. (Dalian, Liaoning, China). The target fragments were retrieved from 1.0% agarose gel with Gel Extraction Kit (OMEGA Bio-tek, Norcross City, Georgia, United States), and were then cloned in pGEM-T Easy Vector (Takara Co., Ltd.,

### Bioassay of Desiccation Resistance

To explore the influence of desiccation on the transcript levels of CYP4G76 and CYP4G115, thirty instar N. lugens nymphs were exposed to the desiccation conditions. According to the method depicted by Gibbs et al. (1997), 200 g of arid allochroic silica gel (2.0–5.6 mesh, Qingdao, Shandong, China) were placed into a 2-l sealed box to decrease the relative humidity by 5% during the course of 1 h. Each biological replicate contained thirty N. lugens nymphs in a glass tube (30 mL) that had been sealed with nylon gauze (20 meshes). Five biological replicates and 200 g arid allochroic silica gel were put into a sealed box. The desiccation treatment lasted for 1, 2, 3 and 4 days, resulting in four allochroic silica gel treatments. As a control, N. lugens nymphs were placed in a glass tube and then placed in a climate chamber set at ~70% RH. After the treatments, ten living nymphs were randomly selected from each biological replicate, the total RNA was extracted, the cDNA was synthetized, and the relative transcript level of the four allochroic silica gel treatments was compared with the control using relative quantitative PCR.

In order to explore the mortality and phenotype of N. lugens nymphs under a desiccation condition, the third instar nymphs were injected with 40 nL (2000 μg·mL⁻¹) of dsGFP, single dsCYP4G76, single dsCYP4G115, or combined dsRNAs containing dsCYP4G76 and dsCYP4G115 (1:1), these injected insects were raised for 4 days to produce a new cuticle and then were kept at a desiccation condition (RH < 5%) for 1 day.

### MATERIALS AND METHODS

#### Nilaparvata lugens Populations and Insecticides

Rice (var. ‘TN1’) plants were grown in a glass culture dish (12 cm diameter) and enclosed in nylon cages (60 cm × 60 cm × 60 cm), which were watered and fertilized as needed (Compost, COMPO Expert GmbH, Germany). An N. lugens was released by the College of Agriculture and Biotechnology at Zhejiang University in 2013, and was reared on the rice plants (10 cm height) at Lab of Insect Physiology, Zhejiang A&F University. Both of rice plants and N. lugens were maintained at the same greenhouse at 25 ± 0.5°C, RH 70 ± 5%, and with a photoperiod of 14/10 h (light/dark).

Technical grade buprofezin (98.0% pure; CAS: 69327-76-0), imidacloprid (99.9% pure; CAS: 138261-41-3), and thiamethoxam (99.0% pure; CAS: 153719-23-4) obtained from Biaozheng Chemical Company, Inc. (Xi’an, Shaanxi, China) and pymetrozine (98.6% pure; CAS: 123312-89-0) was purchased from Longdeng Chemicals Pty Ltd. (Kunshan, Jiangsu, China).
Dalian, Liaoning, China), according to product manual. All positive clones for CYP4G76 and CYP4G115 were corroborated by DNA sequencing (Biosune Co., Ltd., Shanghai, China).

**Quantitative PCR of CYP4G76 and CYP4G115 mRNA**

The relative expression levels of CYP4G76 and CYP4G115 in N. lugens at all life stages, and among different tissues, were measured by Bio-Rad Quantitative PCR (CFX96Touch™ qPCR, Hercules, CA, United States). The cDNA for qPCR were obtained with the method described as PrimeScript RT reagent Kit (Takara Co., Ltd., Dalian, Liaoning, China). Each qPCR date was calculated with the method described as PrimeScript RT reagent Kit (Takara Co., Ltd., Dalian, Liaoning, China). The injected nymphs were reared on rice plants in glass bottles (8 cm diameter, 12 cm height), and were collected at each suitable time point. The method for extracting total RNA and synthesizing cDNA was the same as described. The relative transcript levels of CYP4G76 and CYP4G115 were measured by qPCR.

**Synthesis of dsRNA**

Two fragments were selected from CYP4G76 and CYP4G115 as RNAi target regions, and were 383 and 420 bp, respectively (Table 1). The GFP gene was used as control in RNAi experiment. The promoter for the RNAi target regions and the T7 RNA polymerase promoter sequence were bound together by pGEM-T vector, and the lengths were 435 and 472 bp, respectively. The PCR product was verified using 1% agarose gel electrophoresis and then stored at −80°C.

**Delivery of dsRNA and Detection of RNAi Efficiency**

The RNAi treatment of N. lugens nymphs was performed by using microinjection methods. Third instar nymphs were anesthetized with CO2, and the prepared dsRNAs were injected into the haemolymph through the thorax ventral using a micro-injector (FemtoJet®4i, Eppendorf international trade Co., Ltd., Shanghai, China). The injected nymphs were reared on rice plants in glass bottles (8 cm diameter, 12 cm height), and were collected at each suitable time point. The method for extracting total RNA and synthesizing cDNA was the same as described. The relative transcript levels of CYP4G76 and CYP4G115 were measured by qPCR.

**Extraction and Quantification of CHCs**

In RNAi treatment, the GFP, CYP4G76, CYP4G115, and commingled dsRNA containing dsCYP4G76 and dsCYP4G115 (1:1) were injected into third instar N. lugens nymphs, and hydrocarbons on newly molted N. lugens nymph cuticles were extracted using the procedure outlined by Young and Schal (1997).

Fifty fourth-instar nymphs and 5 mL n-hexane were put into a clear glass bottle (20 mL), and 200 ng of n-heneicosane was added as an internal standard. The bottle was agitated gently for 3 min to dissolve CHCs. The solution was drawn into a new chromatogram vial (20 mL) using a glass pipette. The glass bottle was rinsed twice with 3 mL n-hexane, and the three solutions were combined together. The combined solution (9 mL) was diluted to 1000 and 2000 µg·mL−1 using RNase free water (Scott et al., 2013). The dsRNA was first checked with 1% agarose gel electrophoresis and then stored at −80°C.

| Application of primers | Primer name | Sequence of primers (5’-3’) | Products (bp) |
|------------------------|-------------|-----------------------------|--------------|
| RT-qPCR analysis       | qCYP4G76-F  | TGTTGTTTGCGGTGCGTGTA        | 173          |
|                        | qCYP4G76-R  | GTCCTCCCTTGTCACGAT          |              |
|                        | qCYP4G115-F | TCGGTCCTCCTGTCACGAT         | 207          |
|                        | qCYP4G115-R | CTCGCTCCTGTCACGAT           |              |
| dsRNA synthesis        | dsCYP4G76-F | GATCACTAATACGACTACATATAGGA | 436          |
|                        | dsCYP4G76-R | GATCACTAATACGACTACATATAGGA |              |
|                        | dsCYP4G115-F| GATCACTAATACGACTACATATAGGA | 472          |
|                        | dsCYP4G115-R| GATCACTAATACGACTACATATAGGA |              |
|                        | dsGFP-F     | GATCACTAATACGACTACATATAGGA | 583          |
|                        | dsGFP-R     | GATCACTAATACGACTACATATAGGA |              |

**Note:** The gDNA was first normalized with rps3 standard values and then was used to calculate the quantitative variation of target genes using the method proposed by Pfaffl (2001).

**TABLE 1 | Primers used for PCR amplification, in RT-qPCR analysis and dsRNA synthesis.**
UI, Santa Clara, CA, United States), operated at 60°C for 2 min, then increased 5°C min⁻¹ up to 320°C, where it was kept for 10 min. The injector and detector temperatures were set at 300 and 280°C, respectively. Mass detection was operated under an EI mode with a 70 eV ionization potential and a 45–650 m/z scan range at a 5 scan/s scan rate.

**Cuticular Penetration Rate of Insecticide**

The *GFP, CYP4G76, CYP4G115*, and commingled dsRNA containing dsCYP4G76 and dsCYP4G115 (1:1) dsRNA (40 nL, 2000 μg·mL⁻¹) were injected into the third instar *N. lugens* nymphs. The fourth instar newly molted *N. lugens* nymph were used to measure the cuticular penetrating rate of insecticides using the micro-spot method (Liu et al., 2013). Buprofezin, imidacloprid, thiamethoxam, and pymetrozine were adjusted to a volume of 100 μL using acetonitrile-water (3:7 [V/V]), methanol-dichloromethane (5:95), methanol, and n-hexane, respectively (Obana et al., 2002; Singh et al., 2004; Campbell et al., 2005; Zhang, 2007; dos Santos et al., 2008). All liquids were stored in the dark at room temperature before testing.

Imidacloprid, thiamethoxam, and pymetrozine were measured using high efficiency liquid chromatography (Segura et al., 2000). A liquid chromatographic system (Waters model 990; Milford, MA, United States) was used for the quantification and confirmation of imidacloprid, thiamethoxam, and pymetrozine and was equipped with a Model 600E constant-flow pump, a Rheodyne six-port injection valve with a 20 μL sample loop, and a Model 990 photodiode-array detector. The spectral resolution was 1.4 nm per diode in the range 200–290 nm. HPLC separations were carried out using a Hypersyl Shandon Green Environ-C18 column (150 mm × 46 mm ID; 5 μm particle size). The chart speed was 0.5 cm·min⁻¹, and the detector sensitivity was 0.02 a.u.f.s.

For pymetrozine, a carbinol-phosphate buffer (35:65 [v/v]) was used for the mobile phase, with a flow rate of 0.08 mL·min⁻¹ (Li et al., 2011). The photometric detection was performed at 298 nm and the column temperature was 25°C. The analytical methods for imidacloprid followed a mobile phase of acetonitrile-water (20:80 [v/v]) at a flow rate of 1 mL·min⁻¹ (Segura et al., 2000). The photometric detection was performed at 270 nm and the column temperature was 35°C. The mobile phase for thiamethoxam was carbinol-water (18:82 [v/v]), at a flow rate of 1 mL·min⁻¹ (Rancan et al., 2006). The photometric detection was performed at 250 nm and the column temperature was 25°C.

The residual quantity of buprofezin on the cuticle of *N. lugens* nymph was measured by Shimadzu GC-17A gas chromatograph–mass spectrometry (dos Santos et al., 2008). A fused-silica column DB-5MS (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, Folsom, CA, United States), was used in conjunction with helium (purity 99.999%) as carrier gas, and at a flow-rate of 1.8 mL·min⁻¹. The column temperature was programmed at 60°C for 1 min, 270°C for 10°C·min⁻¹, followed by 3 min of holding time at 270°C. The solvent delay was 5 min. The injector port was maintained at 250°C and 1 μL was injected during splitless mode (0.7 min). The eluent from the GC column was transferred (via a transfer line) at 280°C and fed into a 70-e Velectron-impact ionization source. Data were acquired and processed by Shimadzu class 5000 software (Shimadzu Co., Shanghai, China). The penetration rate of insecticide was calculated as follow:

\[
A = \frac{B - C}{B} \times 100\%
\]

Where, A = penetration rate; B = the total weight of insecticide (10 ng), and C = the residue of insecticide on *N. lugens* nymph cuticle.

**Bioassay of Four Insecticides to Nilaparvata lugens Nymph Silenced Target Genes**

The third instar *CYP4G76* and *CYP4G115* silenced *N. lugens* nymphs were obtained using microinjection methods and were used as test sample in this study. Under carbon dioxide anesthesia, a droplet (0.5 μL) of acetone insecticide solution was applied topically to the prothorax notum using a single channel adjustable range micro applicator (Eppendorf Scientific, Inc., Hamburg, Germany). Only acetone was used for the control nymphs. Each bioassay included 5 to 6 concentrations, and 23 third instar *N. lugens* nymphs were treated in each concentration. Each treatment was repeated 3 times. The treated nymphs in each concentration were reared on three rice plants (10 days) in a three plastic cups, and maintained at 27 ± 1°C at a photoperiod of 16:8 h (L:D). The mortality caused by the pymetrozine, imidacloprid, and buprofezin treatments was recorded after 4 days, and thiamethoxam after 3 days. Nymphs were considered dead if they did not move after gentle prodding with a fine brush.

**Statistical Analyses**

All data are presented as the mean ± Standard Error (SE) on the basis of independent biological replicates. Statistically analyses were performed using the Statistical Package for the Social Sciences 19.0 software (SPSS Inc., Chicago, IL, United States). Significant differences between two samples and among multi-samples were determined with Student’s t-test and one-way ANOVA followed by the least significant difference test (LSD), respectively, and means were separated at the level p < 0.05. The raw data of the toxicity of four insecticides were corrected for mortality observed in the control and analyzed using the program POLO Plus 1.0 for Probit analysis.
RESULTS

Spatio-Temporal Expression of CYP4G76 and CYP4G115

The relative transcript levels of CYP4G76 and CYP4G115 at different developmental stages and tissues are presented in Figure 1. CYP4G76 expression in the first instar was significantly higher than all later developmental stages (F6,28 = 1543.723, p < 0.001). The relative transcript level of CYP4G115 in most of the nymph stages was significantly higher than those in adult stages, and the transcript level in first instar larva was highest (F6,28 = 219.890, p < 0.001). In terms of the effect on a specific body part, the CYP4G76 transcript level decreased in the order of fat body, abdominal cuticle, abdomen, head, thorax, and gut (F5,24 = 178.961, p < 0.001). However, the expressing level of CYP4G115 in the abdominal cuticle was significantly higher than that in the fat body, and the transcript level in other body parts and tissues decreased gradually with the same order for CYP4G76 (F5,24 = 423.923, p < 0.001).

CYP4G76 and CYP4G115 Expression Under Desiccation Stress

The influences of desiccation on the expression of CYP4G76 and CYP4G115 were investigated in third instar larvae (Figure 2). Desiccation stress for 1–5 days (RH < 5%) had a significantly higher effect on the expression of CYP4G76 than CYP4G115 (F5,24 = 217.593, p < 0.001), though desiccation stress also significantly increased the transcript level of CYP4G115 (F5,24 = 187.945, p < 0.001).

Silencing of CYP4G76 and CYP4G115 With dsRNA

The optimization of the volume of dsRNA is shown in Figure 3. The volumes of 20, 40, 60, and 80 nL of CYP4G76 dsRNA (1000 µg·mL⁻¹) significantly decreased the transcript level to 34.0% (p < 0.001), 28.5% (p < 0.001), 26.9% (p < 0.001) and 17.4% (p < 0.001), respectively. The volumes of single CYP4G115 dsRNA in 20, 40, 60, and 80 nL (1000 µg·mL⁻¹) were efficient for silencing of expression and resulted in a significant reduction to 39.1% (p < 0.001), 31.9% (p < 0.001), 26.5% (p < 0.001) and 15.9% (p < 0.001) in mature larva, respectively. The commingled dsRNA containing dsCYP4G76 and dsCYP4G115 (1:1) of 20, 40, 60, and 80 nL (1000 µg·mL⁻¹) resulted significantly reduced the expression of CYP4G76 to 42.9% (p < 0.001), 34.2% (p < 0.001), 27.5% (p < 0.001) and 17.6% (p < 0.001), respectively. The same commingled dsRNA silenced CYP4G115 by 40.0% (p < 0.001), 32.2% (p < 0.001), 26.7% (p < 0.001), and 24.3% (p < 0.001), for the same
FIGURE 3 | Effect of the volume of dsRNA (1000 µg·mL⁻¹) on the expression of CYP4G76 and CYP4G115 in third instar Nilaparvata lugens nymphs 24 h following injection. (A) injection of CYP4G76, (B) injection of CYP4G115 dsRNA, (C,D) the injection of commingled dsRNA containing dsCYP4G76 and dsCYP4G115 (1:1). The nymph injected dsRNA was collected at 24 h and the transcript levels of CYP4G76 and CYP4G115 were analyzed by qRT-PCR. Mean ± SE was calculated from five biological replicates, and each biological replicate contained ten third instar N. lugens nymphs. ** in each figure showed significant difference between treatments and control (Student's t-test, p < 0.001).

20, 40, 60 and 80 nL (1000 µg·mL⁻¹) samples, respectively. We also observed that when the volume of dsRNA was 60 and 80 µL, it was possible for the dsRNA to overflow the injection pinhole.

Due to our initial findings, we increased the concentration of dsRNA to 2000 µg·mL⁻¹ and adjusted the injected volume to 40 nL. The RNAi efficiency of single and commingled dsRNA on the transcript expression level at 1, 2, 3, and 4 days after the microinjection is shown in Figure 4. The single injection of dsRNA reduced CYP4G76 expression to 21.5% (p < 0.001), 20.1% (p < 0.001), 32.6% (p < 0.001), and 33.3% (p < 0.001) at 1, 2, 3, and 4 days after the injection, respectively. In addition, the similar single injection of dsRNA reduced CYP4G115 expression to 17.8% (p < 0.001), 19.4% (p < 0.001), 26.1% (p < 0.001), and 21.0% (p < 0.001) at 1, 2, 3, and 4 days after the injection, respectively. The volume of 40 nL (2000 µg·mL⁻¹) commingled dsRNA containing CYP4G76 and CYP4G115 (1:1) significantly reduced the transcript of CYP4G76 to 20.6% (p < 0.001), 22.8% (p < 0.001), 22.4% (p < 0.001), and 28.9% (p < 0.001) at 1, 2, 3, and 4 days, respectively. The same commingled dsRNA reduced the expression of CYP4G115 to 19.5% (p < 0.001), 21.4% (p < 0.001), 26.0% (p < 0.001), and 28.9% (p < 0.001) at 1, 2, 3, and 4 days, respectively.

Effect of CYP4G76 and CYP4G115 Knockdown on CHCs Biosynthesis

The GC-MS test results indicated that the CHCs of the N. lugens nymph and pupa in the control treatment were a series of n-alkanes of C₁₆H₃₄-C₃₃H₆₈ (except for C₂₁H₄₄) that were saturated and without methyl branched CHCs. In the control treatment, the content of these alkane groups in the CHC decreased in the following order: C₂₉H₆₀, C₂₇H₅₆, C₁₈H₃₈, C₁₇H₃₆, C₂₁H₄₄, C₂₀H₄₂, C₁₇H₃₆, C₂₃H₅₈, C₁₆H₃₄, C₁₉H₄₀, C₂₂H₄₆, C₂₄H₅₀, C₂₄H₅₀, C₂₃H₄₈, C₂₃H₆₈, C₂₅H₅₂, C₃₂H₆₆, and C₃₀H₆₂ (Figure 5).
CHCs in the RNAi treatment are shown in Figure 5. The injection with single dsCYP4G76 significantly decreased the level of the external alkanes for all alkanes: C_{17}H_{36} (F_{3,8} = 7.798, p = 0.009), C_{18}H_{38} (F_{3,8} = 6.026, p = 0.019), C_{20}H_{42} (F_{3,8} = 14.633, p = 0.001), C_{22}H_{46} (F_{3,8} = 93.096, p < 0.001), C_{23}H_{48} (F_{3,8} = 145.517, p < 0.001), C_{24}H_{50} (F_{3,8} = 52.087, p < 0.001), C_{26}H_{54} (F_{3,8} = 75.672, p < 0.001), C_{28}H_{58} (F_{3,8} = 9.036, p = 0.006), C_{29}H_{60} (F_{3,8} = 8.564, p = 0.007), C_{30}H_{62} (F_{3,8} = 141.144, p < 0.001), C_{31}H_{64} (F_{3,8} = 11.926, p = 0.003), C_{32}H_{66} (F_{3,8} = 11.635, p = 0.003) and C_{33}H_{68} (F_{3,8} = 18.501, p = 0.001). Except for C_{16}H_{34} and C_{19}H_{40}, RNAi by silencing CYP4G115 also significantly decreased the content of HCs, including C_{17}H_{36}, C_{18}H_{38}, C_{20}H_{42}, C_{22}H_{46}, C_{23}H_{48}, C_{24}H_{50}, C_{25}H_{52}, C_{30}H_{62} (F_{3,8} = 114.969, p < 0.001), C_{26}H_{54}, C_{27}H_{58} (F_{3,8} = 6.377, p = 0.016), C_{28}H_{58}, C_{29}H_{60}, C_{30}H_{62}, C_{31}H_{64}, C_{32}H_{66}, and C_{33}H_{68}, and C_{22}H_{46}, C_{23}H_{48}, C_{24}H_{50}, C_{25}H_{52}, C_{26}H_{54}, and C_{30}H_{62} disappeared. It is interesting that the injection with commingled dsRNA containing dsCYP4G76 and dsCYP4G115 triggered a significant reduction in all external alkanes except for C_{16}H_{34} and C_{19}H_{40}. The amount of CHCs in three silencing treatments was significantly less than that in GFP-silenced control (F_{3,8} = 11.399, p = 0.003).

Effect of dsRNA on the Susceptibility of N. lugens Larva From Desiccation

CYP4G76 and CYP4G115-suppressed N. lugens larva were investigated under desiccation (RH < 5%) and control (RH = 70%) conditions (Figure 6). When the RH was at 70%, there was no significant difference in the percentage of weight loss among dsGFP, dsCYP4G76, dsCYP4G115, and commingled dsRNA treatments. Compared to the dsGFP control, the knockout of CYP4G76 and/or CYP4G115 significantly increased the susceptibility of the third instar to desiccation (F_{3,8} = 196.594, p < 0.001). After injecting dsCYP4G76, dsCYP4G115, and commingled dsRNA, the survival rate of the third instar nymphs under desiccation conditions (RH < 5%) was less than those in control group
FIGURE 5 | CHCs content measured from the external surface of Nilaparvata lugens nymphs. Commingled dsRNA contained CYP4G76 and CYP4G115 (1:1). Mean ± SE was calculated from three biological replicates, and each biological replicate contained fifty fourth instar N. lugens nymphs. Different lowercase letters in each figure showed the significant difference (ANOVA, LSD, \( p < 0.05 \)).

FIGURE 6 | Effect of RNAi suppression of CYP4G76 and CYP4G115 on the survival rate of Nilaparvata lugens nymph following desiccation treatment (RH < 5% for 24 h). Commingled dsRNA contained CYP4G76 and CYP4G115 (1:1). Mean ± SE was calculated from five biological replicates, and each biological replicate contained thirty fourth instar N. lugens nymphs. Different capital and lowercase letters in each figure showed the significant difference (ANOVA, LSD, \( p < 0.05 \)). ** in each figure showed significant difference (Student’s t-test, \( p < 0.001 \)).

(dsCYP4G76: \( p < 0.001 \); dsCYP4G115: \( p < 0.001 \); commingled dsRNA: \( p < 0.001 \)).

The nymphs that were injected with CYP4G76 and CYP4G115 dsRNA appeared shriveled and brittle under desiccation conditions (Figure 7). In addition, the color of the epidermis turned white and hyaline when the nymphs were injected with dsRNA containing dsCYP4G115. This phenomenon suggests that moisture-holding and mechanical properties of the cuticle may depend on CYP4G76 and CYP4G115 function.

**Effect of RNAi on the Cuticular Penetration Rate of Insecticides**

The penetration rates of four insecticides in the fourth instar N. lugens nymph are shown in Figure 8. Buprofexin has significantly greater penetration rate in CYP4G76 and CYP4G115-suppressed nymphs than the GFP control \( (F_{3,8} = 17.776, p < 0.001) \). The penetration rate of imidacloprid in CYP4G76 and CYP4G115 suppressed fourth instar nymphs was significantly greater \( (F_{3,8} = 136.686, p < 0.001) \). Decreasing the expressing level of CYP4G76 and CYP4G115 significantly increased the penetrating rate of thiamethoxam in fourth instar nymphs \( (F_{3,8} = 196.594, p < 0.001) \). The penetration rate of pymetrozine and the expressing level of the two target genes were negatively related \( (F_{3,8} = 9.867, p < 0.001) \).
Effect of RNAi on the Toxicity of Four Insecticides

The synergistic effects of RNAi treatment on pymetrozine, imidaclprid, thiamethoxam, and buprofezin were tested with the susceptible strains of *N. lugens* (Table 2). Results showed an increase in the synergistic effect of the treatments when CYP4G115 was silenced, but greater synergism was found only with thiamethoxam when silencing CYP4G76.

DISCUSSION

In this present study, we have successfully repressed two cytochrome P450 genes, CYP4G76 and CYP4G115, using RNAi technique to determine the lethal phenotype of *N. lugens* nymphs under desiccation conditions. Functional studies have revealed that, CYP4G76 and CYP4G115 are critically related to CHCs biosynthesis. Reducing the CYP4G76 and CYP4G115 expression level through RNAi technology resulted in a higher mortality rate of *N. lugens* nymphs under desiccation conditions, and a greater penetration rate of insecticides.

We have represented that the CHCs profile of *N. lugens* is unique. Except for C21H44 and C23H48, we identified thirteen compounds of saturated C16H34-C21H64 straight chain n-alkanes and determined there was no methyl branched HC on the cuticular surface. We also showed that just five compounds (C17H36, C18H38, C20H42, C27H58 and C29H60) comprise more than 90% of the straight chain n-alkanes on the CHCs. The influence of desiccation on the mortality rate of CYP4G76- and CYP4G115-silenced *N. lugens* provided solid evidence that saturated and straight-chain CHCs are responsible for reducing the water loss from *N. lugens* cuticle by evaporation.

We found that CYP4G76 and CYP4G115 were highly expressed in the fat body and abdominal cuticle, which suggests that oenocytes might be precisely located in the fat body and the abdominal cuticle. Our results are different from previous studies on the fruit fly (*Drosophila melanogaster*), however, wherein CYP4G15 was highly expressed in the larval brain and central nervous system (Maïbeche-Coisne et al., 2000; Chung et al., 2014). Therefore, future research should investigate the precise location of oenocytes by CYP4G members in *N. lugens*.

Together with previous studies (Blomquist and Bagneres, 2010; Qiu et al., 2012; Chen et al., 2016; Yu et al., 2016; Shahandeh et al., 2018), we suggested that CYP4G might have multiple functions in many insect species. Although we have found that CYP4G76 and CYP4G115 are related to CHCs biosynthesis, the specific enzymatic activity and precursor to CHCs are still unclear. To illuminate the functions of CYP4G76 and CYP4G115 and other genes belonging to the CYP4G subfamily in the *Insecta* class, more extensive and intensive research needs to be conducted.

Basically, the mechanisms of insect resistance consist of reducing the penetration rate of insecticide, enhancing the activity of detoxification, and target mutation (Oppenoorth, 1985; Balabanidou et al., 2016; Garrood et al., 2016, 2017). Whether insecticides penetrate the thorax cuticle into the insect body depends on the structure of the insect cuticle and the physical and chemical characteristics of the insecticides (Yang et al., 2011). Since four tested insecticides are lipophilic, the penetration rate of insecticide was mainly affected by the
FIGURE 8 | Effect of RNAi suppression of CYP4G76 and CYP4G115 on the penetration rate of four insecticides: (A) buprofezin, (B) imidaclprid, (C) thiamethoxam, and (D) pymetrozine, in Nilaparvata lugens nymphs after 8 h of exposure. Mean ± SE was calculated from three biological replicates, and each biological replicate contained ten fourth instar N. lugens nymphs. Different lowercase letters in each figure showed the significant difference (ANOVA, LSD, p < 0.05).

TABLE 2 | Effect of RNAi suppression of CYP4G76 and CYP4G115 on the synergistic ratios of four insecticides buprofezin, imidaclprid, thiamethoxam, and pymetrozine in third instar Nilaparvata lugens nymphs.

| Insecticide | Treatment           | Slope ± SE | LC50 (95% FL)       | Synergistic ratio |
|-------------|---------------------|------------|---------------------|------------------|
| Buprofezin  | dsGFP               | 1.960 ± 0.213 | 1.167 (0.910 — 1.437) | 1.000            |
|             | dsCYP4G76           | 1.835 ± 0.166 | 1.094 (0.864 — 1.368) | 1.067            |
|             | dsCYP4G115          | 1.603 ± 0.155 | 0.431 (0.348 — 0.527) | 2.708*           |
|             | Commingled dsRNA    | 1.931 ± 0.203 | 0.436 (0.339 — 0.547) | 2.677*           |
| Imidaclprid | dsGFP               | 1.474 ± 0.149 | 20.662 (15.762 — 26.509) | 1.000            |
|             | dsCYP4G76           | 1.321 ± 0.143 | 13.290 (10.452 — 16.850) | 1.555*           |
|             | dsCYP4G115          | 1.949 ± 0.206 | 6.531 (4.945 — 8.272)  | 3.164*           |
|             | Commingled dsRNA    | 1.602 ± 0.155 | 7.262 (5.846 — 8.881)  | 2.845*           |
| Thiamethoxam| dsGFP               | 1.950 ± 0.203 | 3.141 (2.527 — 3.835)  | 1.000            |
|             | dsCYP4G76           | 1.655 ± 0.156 | 0.812 (0.643 — 1.009)  | 3.868*           |
|             | dsCYP4G115          | 1.818 ± 0.202 | 0.541 (0.413 — 0.684)  | 5.806*           |
|             | Commingled dsRNA    | 1.966 ± 0.171 | 0.299 (0.249 — 0.355)  | 10.505*          |
| Pymetrozine | dsGFP               | 1.563 ± 0.132 | 17.725 (14.951 — 21.667) | 1.000            |
|             | dsCYP4G76           | 1.522 ± 0.128 | 13.756 (11.178 — 16.876) | 1.289            |
|             | dsCYP4G115          | 1.852 ± 0.171 | 5.332 (4.250 — 6.613)  | 3.324*           |
|             | Commingled dsRNA    | 1.607 ± 0.193 | 6.124 (4.837 — 7.589)  | 2.894*           |

FL, fiducial limits. * in each figure showed the significant difference (ANOVA, LSD, p < 0.05).
structure of cuticular layer. The blocking effect of insect cuticle decreased the invasion of insecticide and insects with more wax are less likely to be permeated (Sato, 1992; Balabanidou et al., 2016). In this research, we found that silencing two target genes (CYP4G75 and CYP4G115) could significantly reduce the content of CHCs, further significantly increase the penetration ratio of four tested insecticides, which suggests that, reducing CHCs biosynthesis is beneficial for increasing the penetration ratio of insecticides. Furthermore, we found that suppressing CYP4G75 and CYP4G115 increased the synergistic ratio, and the synergistic effect of thiamethoxam was greatest, which suggested that suppressing the biosynthesis of CHCs could increase the control efficacy of insecticide on N. lugens. Thus, when the RNAi technique and traditional pest management strategies are combined, the field control efficiency of thiamethoxam may be the best.

Our findings confirm that CYP4G76 and CYP4G115 are involved in desiccation resistance of N. lugens by modulating CHCs production. Based on the higher mortality rate in CYP4G76- and CYP4G115-silenced N. lugens nymphs following the desiccation treatment, we propose that, depletion of CHCs may enhance the permeability of the cuticle, causing increased water loss, further resulting in death at last. In addition, silencing CYP4G76 and CYP4G115 in N. lugens nymphs increased penetration rates and synergistic effect of thiamethoxam was best, which suggested that it would be interesting to investigate the field control efficiency of thiamethoxam. Furthermore, CYP4G76 and CYP4G115 might be a promising RNAi candidate for providing an environmentally responsible approach to managing N. lugens populations. Since it was only found in insects (Qiu et al., 2012), targeting the insect-specific CYP4G gene might develop into a completely new application for integrated pest management.

**AUTHOR CONTRIBUTIONS**

SW and DZ designed the experiments and wrote the manuscript. SW and BL conducted the experiments. SW, BL, and DZ conducted the data analysis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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