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NlCYP4G76 and *NlCYP4G115* 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;

Otte et al., 2018; Shahandeh et al., 2018; Wang et al., 2019), including ecology, behavior, and biochemistry. Previous researches 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 of CYP4G, there are only one or very few CYP4G genes in most insect species (Feyereisen, 2011, 2012). Based on our transcriptional data and a thorough NCBI database search (Lao et al., 2015), we identified two CYP4G candidate genes: *CYP4G76* (GenBank: KM217045.1) and *CYP4G115* (GenBank: KM217046.1) in the brown planthopper, *Nilaparvata lugens* Stål, both of which may be involved in CHCs biosynthesis of *N. lugens*.

Nilaparvata lugens is a major pest of rice, and it causes huge economic losses worldwide both through direct feeding and as a powerful vector of plant viruses (Zhao et al., 2005). Currently, the primary management strategy against *N. lugens* is frequently used insecticides include pymetrozine, imidacloprid, thiamethoxam, and buprofezin (Smith et al., 2008; He et al., 2011; Butler et al., 2012; Liu et al., 2013). The wax layer and cement layer of the insect cuticle have a blocking effect on the invasion of insecticide, and insects with more wax are less likely to be permeated by insecticides (Sato, 1992). As CHCs are the important constituents of wax layer, inhibiting CHCs biosynthesis in *N. lugens* is likely to increase the penetration rate and to reduce the amount of insecticide used in typical management against the field-resistant population, which would be beneficial for providing an opportunity to develop new strategies with molecular tools to control *N. lugens*.

To achieve the above objectives, we explored the functions of *CYP4G76* and *CYP4G115* in *N. lugens* through RNAi technology and gas chromatography-mass spectrometry (GC-MS), and researched the effects of suppressing these two target genes on the control efficiency of insecticides.

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).

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 was 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 $\mu g \cdot mL^{-1}$) of ds*GFP*, single ds*CYP4G76*, single ds*CYP4G115*, or combined dsRNAs containing ds*CYP4G76* and ds*CYP4G115* (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^{TM} RT reagent Kit (Takara Co., Ltd., 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.,

TABLE 1 | Primers used for PCR amplification, in RT-qPCR analysis and dsRNA synthesis.

Application of primers	Primer name	Sequence of primers (5'-3')	Products (bp)
RT-qPCR analysis	*qCYP4G76-F*	TGTTGTTTGGCGTGGCTGTA	173
	qCYP4G76-R	GTCTCCCTTGTTCACGAT	
	qCYP4G115-F	TCGGTCCAATCCACATCTT	207
	qCYP4G115-R	CTCGGTCCAATCCACATC	
	rp49-F	CTCGTCCGCTCCTTCAATC	197
	rp49-R	TCGGTGACAGTGGGCGTGA	
dsRNA synthesis	ds*CYP4G76-F*	GATCACTAATACGACTCACTATAGGGTTGTTTGGCGTGGCTGTA	435
	ds*CYP4G76-R*	GATCACTAATACGACTCACTATAGGGTGGAAGGTGGGAGCAAT	
	ds*CYP4G115-F*	GATCACTAATACGACTCACTATAGGGTGGCTGAGACCCGACAT	472
	ds*CYP4G115-R*	GATCACTAATACGACTCACTATAGGGTCGGTCCAATCCACATCTT	
	ds*GFP-F*	GATCACTAATACGACTCACTATAGGGGTGGAGAGGTGAAGG	583
	ds*GFP-R*	GATCACTAATACGACTCACTATAGGGGGGCAGATTGTGTGGAC	

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^{TM} qPCR, Hercules, CA, United States). The cDNA for qPCR were obtained with the method described as PrimeScript RT reagent Kit (Takara Co., Ltd., Liaoning, China). Each qPCR date was calculated using five biological replicates, and each biological replicate contained three technical replicates. The qPCR program was as follows: (1) 94°C for 3 min, (2) 40 cycles at 94°C for 10 s, and (3) 56°C for 30 s. The ribosomal protein S3 (*rps3*; GenBank: XM_022328949) in *N. lugens* was used to normalize the transcript levels of the housekeeping gene. The C_t value 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).

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 new fragment was used to amplify the target region, which was the template in dsRNA synthesis. The extra-organismal synthesis and purification of dsRNA was performed *in vitro*, and the reagent was T7 RiboMAX^{TM} Express RNAi Systemt (Takara Co., Ltd., Dalian, Liaoning, China). After quantifying with ultraviolet spectrophotometry at 260 nm (Lee and Schmittgen, 2006), the concentration of dsRNA was diluted to 1000 and 2000 $\mu g \cdot mL^{-1}$ using RNase free water (Scott et al., 2013). The dsRNA was first checked with 1% agarose gel electrophoresis and then stored at −80°C.

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 CO_2, and the prepared dsRNAs were injected into the haemolymph through the thorax ventral using a micro-injector (FemtoJet®4i, Eppendoff 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 ds*CYP4G76* and ds*CYP4G115* (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 purified with ∼300 mg silica gel (70–230 mesh; Sigma-Aldrich, Louis, MO, United States) and poured into a clear chromatogram vial (20 mL), then taken to dryness gently with high-purity N_2. The CHCs were re-suspended into 50 μL hexane for gas chromatography analysis.

The gas chromatograph (GC) used in this study was equipped with an ISQ single quadruple mass spectrometry (MS, Agilent 7010B; Agilent Technologies Co., Ltd., Beijing, China). The carrier gas was helium and the flow was 1 $mL \cdot min^{-1}$. We performed splitless injection of 10 μL into a 30 m × 0.32 mm × 0.25 mm capillary column (Agilent HP-5MS

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 ds*CYP4G76* and ds*CYP4G115* (1:1) dsRNA (40 nL, 2000 $\mu g \cdot mL^{-1}$) 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 dissolved in acetone (40 $mg \cdot L^{-1}$) and 0.5 μL of each insecticide solution was placed on the thorax cuticle using a micro-injector. After 8 h, the residue insecticide on the epidermis was eluted 3 times using 1 mL acetone. The total eluent was collected and dried with high purity N_2. Lastly, buprofezin, imidacloprid, thiamethoxam, and pymetrozine were adusted 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 ml 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-C_{18}-column (150 mm × 46 mm ID; 5 μm particle size). The chart speed was 0.5 $cm \cdot min^{-1}$, 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 $mL \cdot min^{-1}$ (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 $mL \cdot min^{-1}$ (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 $mL \cdot min^{-1}$ (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 $mL \cdot min^{-1}$. The column temperature was programmed as 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:

$$A = \frac{B - C}{B} \times 100\% \quad (1)$$

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, imidaclprid, 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.

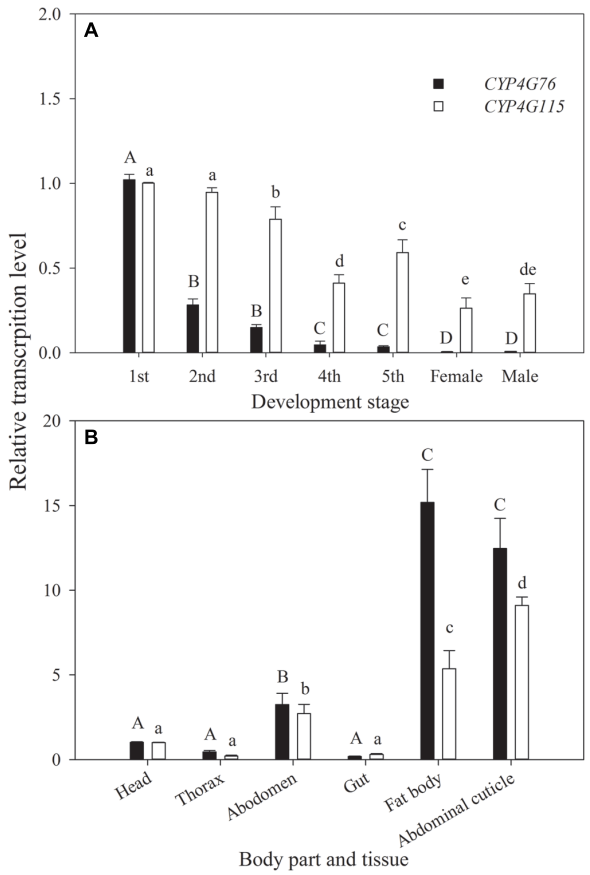

FIGURE 1 | (A) Relative expression levels of *CYP4G76* and *CYP4G115* across developmental stages of *Nilaparvata lugens*, and **(B)** relative expression levels of *CYP4G76* and *CYP4G115* in different body parts or tissues of *N. lugens*. Mean ± SE was calculated from five biological replicates, and each biological replicate contained ten *N. lugens* nymphs or adults. Capital letters indicate significant differences between instar stages of *CYP4G76*, and lowercase letters indicate significant differences in instar stages of *CYP4G115* (ANOVA, LSD, $p < 0.05$).

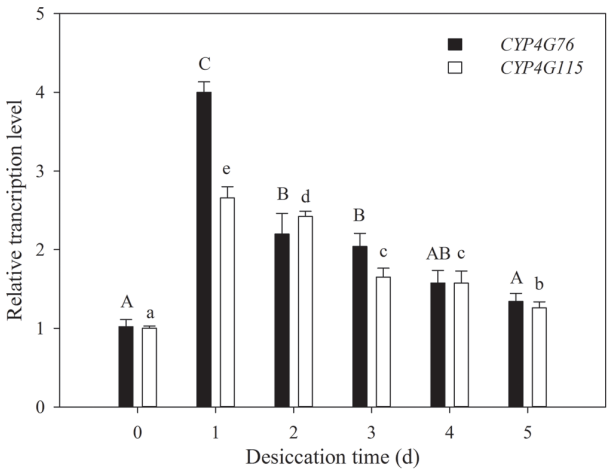

FIGURE 2 | Response of *CYP4G76* and *CYP4G115* transcription levels of third instar *Nilaparvata lugens* nymph to desiccation conditions (RH < 5% for 1–5 days). The control group was at 70% RH. Mean ± SE was calculated from five biological replicates, and each biological replicate contained ten *N. lugens* nymphs. Capital letters indicate significant differences between instar stages of *CYP4G76*, and lowercase letters indicate significant differences in instar stages of *CYP4G115* (ANOVA, LSD, $p < 0.05$).

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 ($F_{6,28} = 1543.723$, $p < 0.001$). 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 ($F_{6,28} = 219.890$, $p < 0.001$). 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 ($F_{5,24} = 178.961$, $p < 0.001$). 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* ($F_{5,24} = 423.923$, $p < 0.001$).

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 1–3 days (RH < 5%) had a significantly higher effect on the expression of *CYP4G76* than *CYP4G115* ($F_{5,24} = 217.593$, $p < 0.001$), though desiccation stress also significantly increased the transcript level of *CYP4G115* ($F_{5,24} = 187.945$, $p < 0.001$).

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 $\mu g \cdot mL^{-1}$) 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 $\mu g \cdot mL^{-1}$) 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 ds*CYP4G76* and ds*CYP4G115* (1:1) of 20, 40, 60, and 80 nL (1000 $\mu g \cdot mL^{-1}$) 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

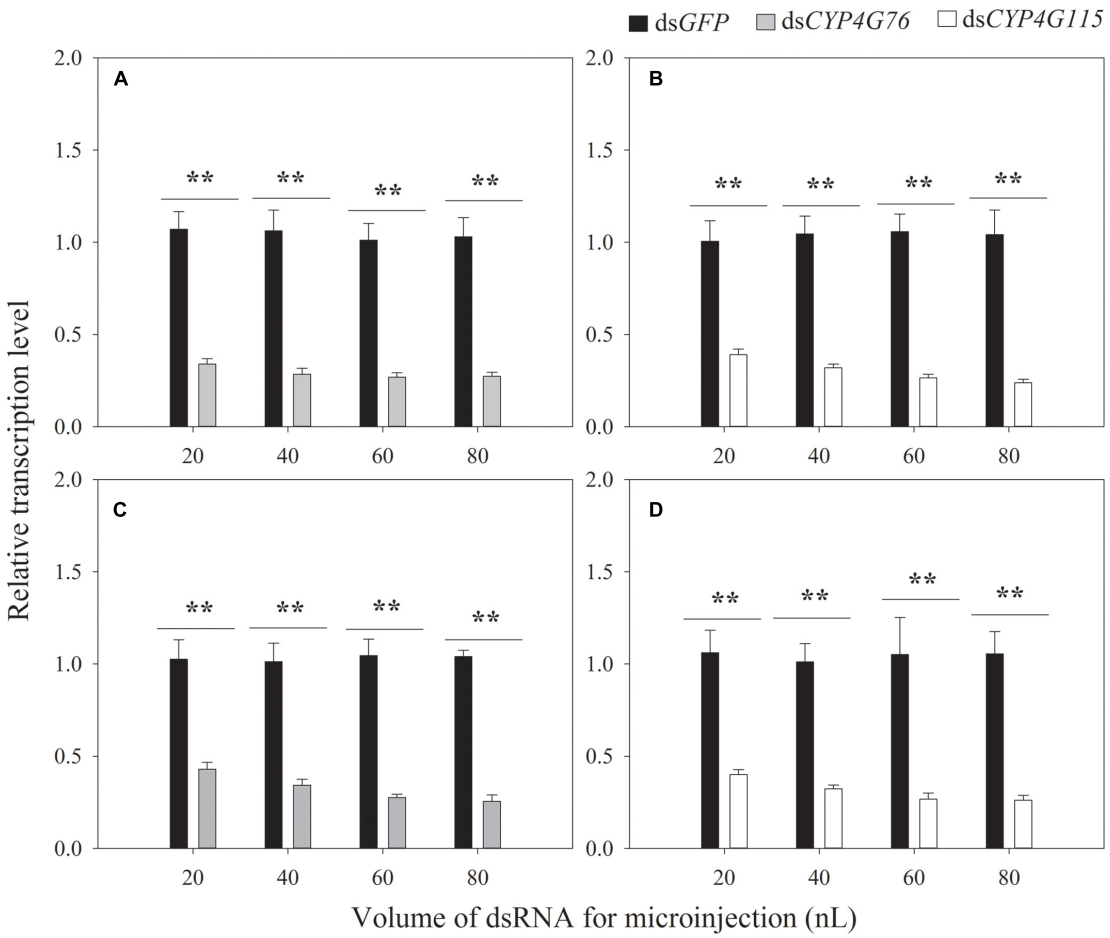

FIGURE 3 | Effect of the volume of dsRNA (1000 $\mu g \cdot mL^{-1}$) on the expression of *CYP4G76* and *CYP4G115* in third instar *Nilaparvata lugens* nymphs 24 h following injection. **(A)** injection of *CYP4G76*, **(B)** injection of *CYP4G115* dsRNA, **(C,D)** the injection of commingled dsRNA containing ds*CYP4G76* and ds*CYP4G115* (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 $\mu g \cdot mL^{-1}$) 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 $\mu g \cdot mL^{-1}$ 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 $\mu g \cdot mL^{-1}$) 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 $C_{16}H_{34}$-$C_{33}H_{68}$ (except for $C_{21}H_{44}$) 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: $C_{29}H_{60}$, $C_{27}H_{56}$, $C_{18}H_{38}$, $C_{31}H_{64}$, $C_{20}H_{42}$, $C_{17}H_{36}$, $C_{28}H_{58}$, $C_{16}H_{34}$, $C_{19}H_{40}$, $C_{22}H_{46}$, $C_{24}H_{50}$, $C_{24}H_{50}$, $C_{23}H_{48}$, $C_{33}H_{68}$, $C_{25}H_{52}$, $C_{32}H_{66}$, and $C_{30}H_{62}$ (**Figure 5**).

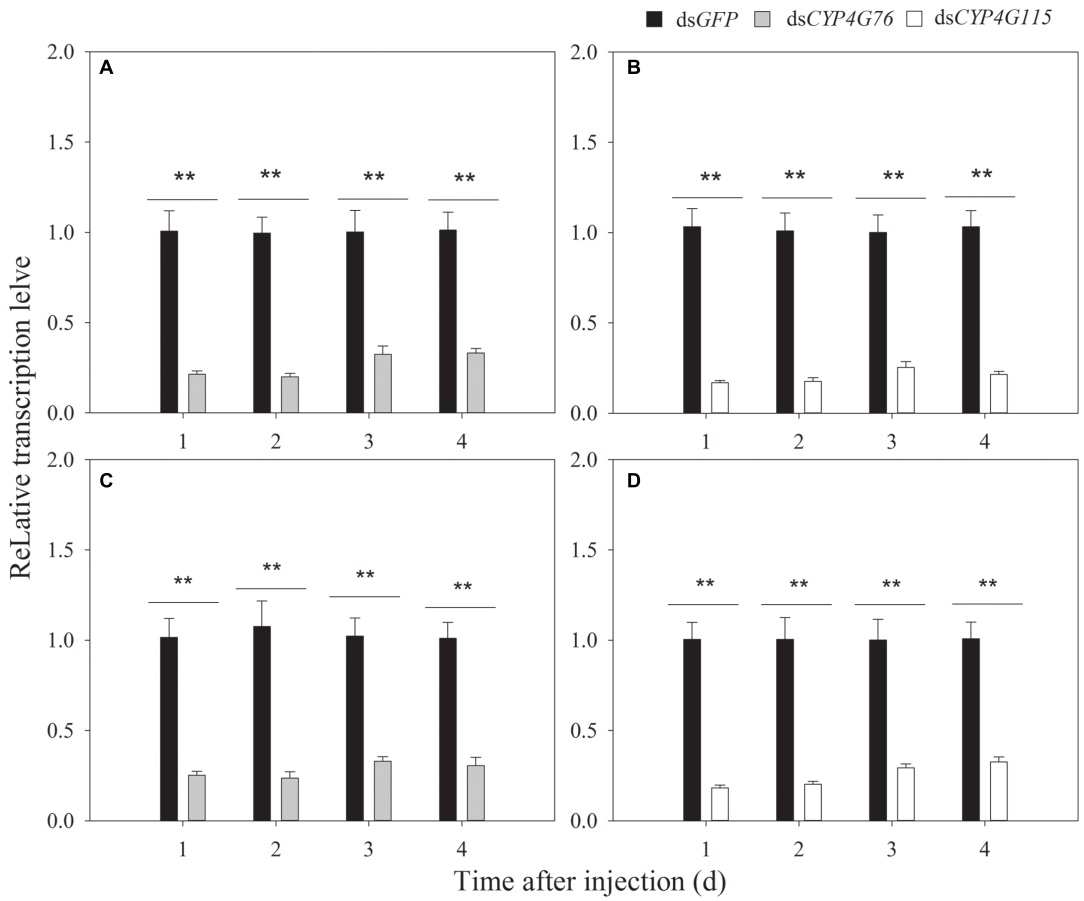

FIGURE 4 | The expression of *CYP4G76* and *CYP4G115* in third and fourth instar *Nilaparvata lugens* nymphs 1–4 days after injection of single or commingled dsRNA (40 nL, 2000 $\mu g \cdot mL^{-1}$). **(A)** Injection of *CYP4G76* dsRNA, **(B)** Injection of *CYP4G115* dsRNA, **(C,D)** Injection of commingled dsRNA with ds*CYP4G76* and ds*CYP4G115* (1:1). The *CYP4G76* and *CYP4G115* transcript levels 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 at each time point (Student's *t*-test, $p < 0.001$).

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 $C_{17}H_{36}$, $C_{18}H_{38}$, $C_{20}H_{42}$, $C_{22}H_{46}$, $C_{23}H_{48}$, $C_{24}H_{50}$, $C_{25}H_{52}$ ($F_{3,8} = 114.969$, $p < 0.001$), $C_{26}H_{54}$, $C_{27}H_{56}$ ($F_{3,8} = 6.377$, $p = 0.016$), $C_{28}H_{58}$, $C_{29}H_{60}$, $C_{30}H_{62}$, $C_{31}H_{64}$, $C_{32}H_{66}$, 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 ds*CYP4G76* and ds*CYP4G1115* 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 were 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 ds*GFP*, ds*CYP4G76*, ds*CYP4G115,* and commingled dsRNA treatments. Compared to the ds*GFP* 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 ds*CYP4G76*, ds*CYP4G115,* and commingled dsRNA, the survival rate of the third instar nymphs under desiccation conditions (RH < 5%) was less than those in control group

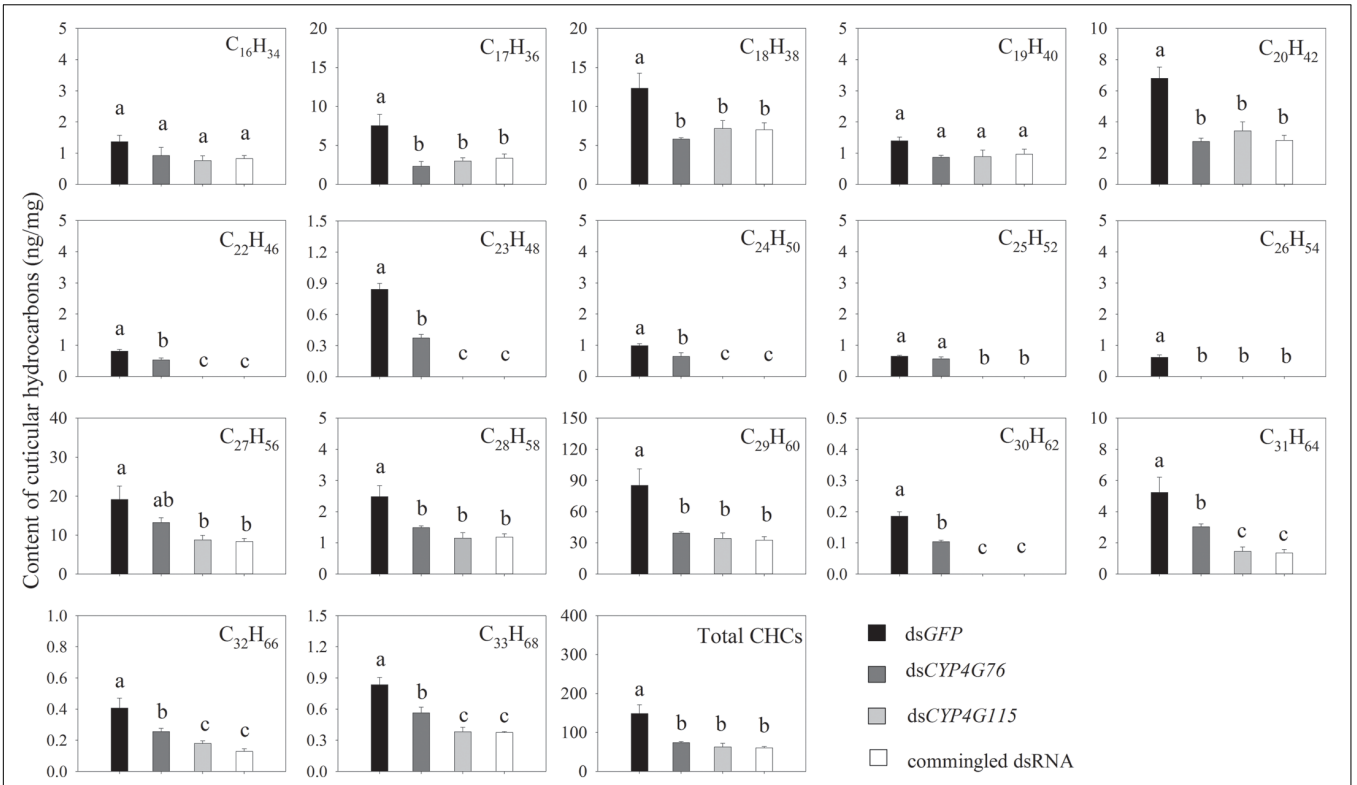

FIGURE 5 | CHCs content measured from the external surface of *Nilaparvata lugens* nymphs. Commingled dsRNA contained *CYP4G76* and *CYP4G115* (1:1). Mean ± SE was calculated from three biological replicates, and each biological replicate contained fifty fourth instar *N. lugens* nymphs. Different lowercase letters in each figure showed the significant difference (ANOVA, LSD, $p < 0.05$).

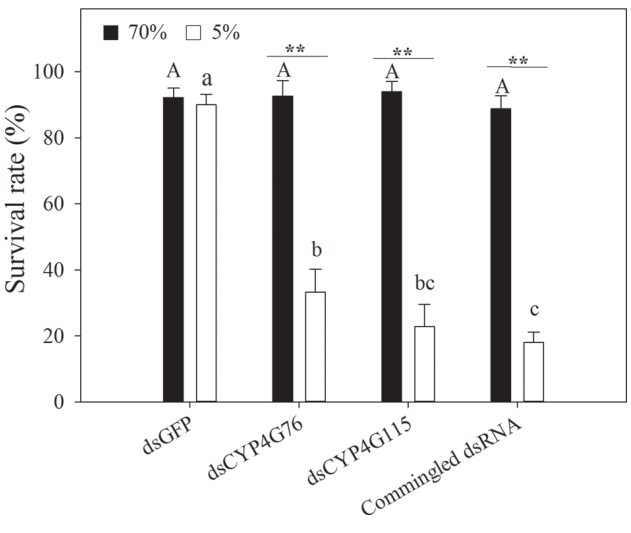

FIGURE 6 | Effect of RNAi suppression of *CYP4G76* and *CYP4G115* on the survival rate of *Nilaparvata lugens* nymph following desiccation treatment (RH < 5% for 24 h). Commingled dsRNA contained *CYP4G76* and *CYP4G115* (1:1). Mean ± SE was calculated from five biological replicates, and each biological 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, $p < 0.001$).

(ds*CYP4G76*: $p < 0.001$; ds*CYP4G115*: $p < 0.001$; 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 ds*CYP4G115*. 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 ($F_{3,8} = 9.867$, $p < 0.001$).

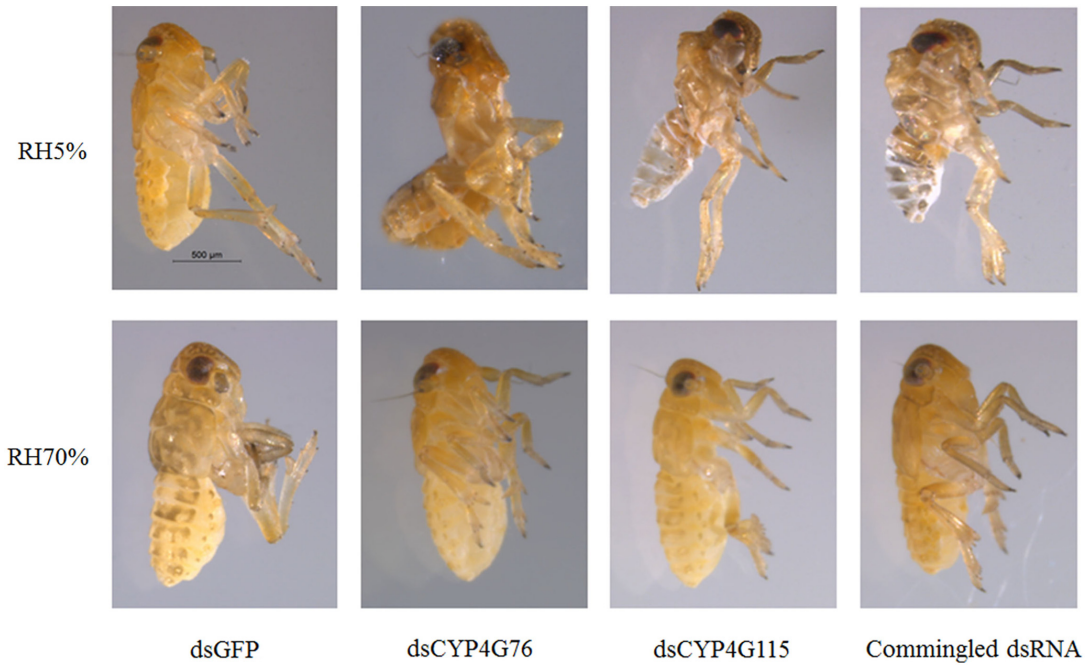

FIGURE 7 | Effect of RNAi suppression of *CYP4G76* and *CYP4G115* on the phenotype of *Nilaparvata lugens* nymph following desiccation treatment (RH < 5% for 24 h). Commingled dsRNA contained *CYP4G76* and *CYP4G115* (1:1).

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 $C_{21}H_{44}$ and $C_{23}H_{48}$, we identified thirteen compounds of saturated $C_{16}H_{34}$-$C_{31}H_{64}$ straight chain n-alkanes and determined there was no methyl branched HC on the cuticular surface. We also showed that just five compounds ($C_{17}H_{36}$, $C_{18}H_{38}$, $C_{20}H_{42}$, $C_{27}H_{56}$ and $C_{29}H_{60}$) 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

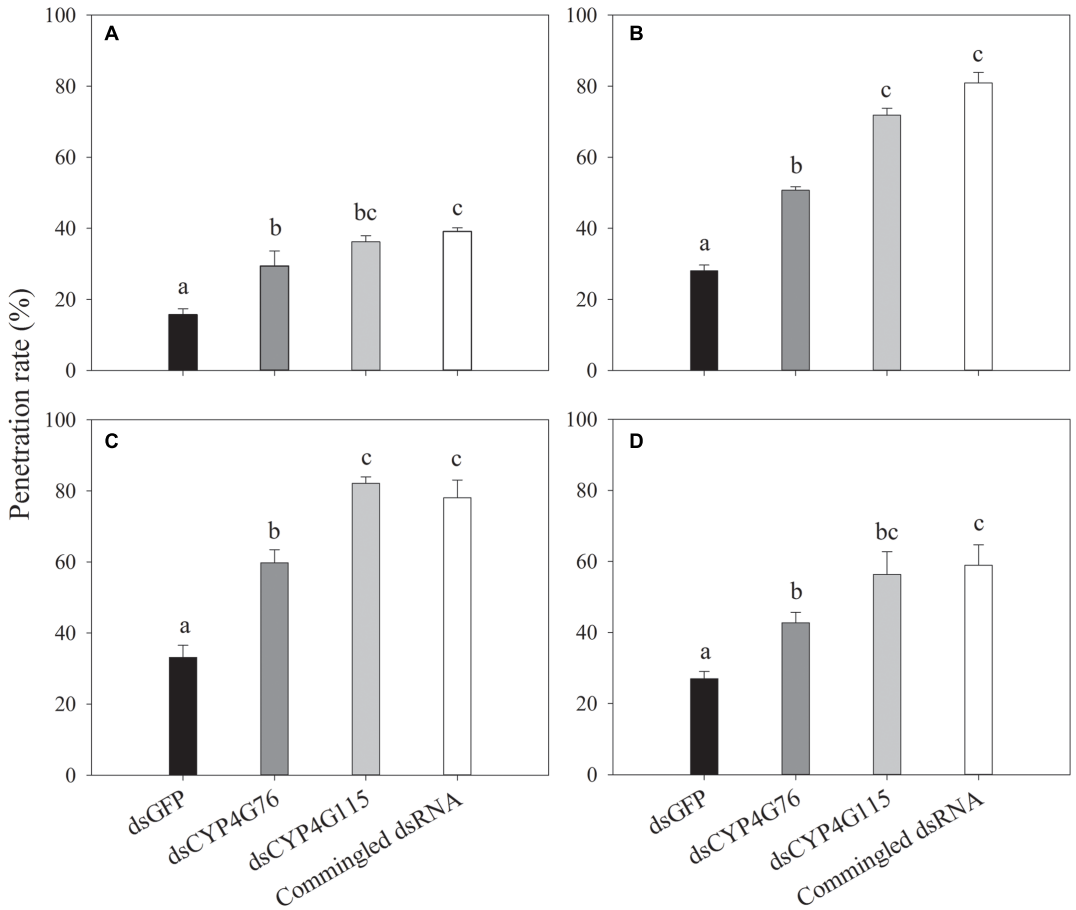

FIGURE 8 | Effect of RNAi suppression of *CYP4G76* and *CYP4G115* on the penetration rate of four insecticides: **(A)** buprofezin, **(B)** imidaclprid, **(C)** thiamethoxam, and **(D)** pymetrozine, in *Nilaparvata lugens* nymphs after 8 h of exposure. Mean ± SE was calculated from three biological 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	LC_{50} (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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