Efficient RNA interference for three neuronally-expressed genes in Nilaparvata lugens (Stål) (Hemiptera: Delphacidae)

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A B S T R A C T
In the brown planthopper Nilaparvata lugens, introduction of double-stranded RNA (dsRNA) successfully knocks down genes transcribed in several types of tissues. However, whether RNA interference (RNAi) can effectively reduce the transcripts of neuronally-expressed genes remains undetermined. In the present paper, we selected three neuronally-expressed genes respectively encoding prothoracicotropic hormone (NlPTTH), pigment-dispersing factor (NlPDF) and short neuropeptide F (NlNPF). The transcripts of the three genes were detectable throughout all tested developmental stages, and were mainly restricted to the nervous system of N. lugens. We introduced dsRNA originated from each of the three neuronally-expressed genes at a dose of 300 ng into the nymphal body cavity by microinjection. The target mRNA was successfully knocked down. Moreover, silencing of NlPTTH downregulated the transcripts of two ecdysteroidogenesis genes (NlPHM and NlNIDB), and surprised the expression of an ecdysone-response genes (NlFIZ-F1). Furthermore, RNAi of NlPTTH caused typical 20-hydroxyecdysone deficient phenotypes: nymphal development was delayed and nymphal lethality occurred. In addition, injection of a series of dsNlPDF solutions at the doses of 300, 60, 12, 2.4 and 0.5 ng into the fourth-instar nymphs decreased its target transcript in a dose-dependent manner: the mRNA levels reduced by 94%, 86%, 57%, 33% and 29%, respectively. Therefore, delivery of dsRNA by microinjection can effectively repress the transcripts of the neuronally-expressed genes in N. lugens.

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Introduction

RNA interference (RNAi) is a double-stranded RNA (dsRNA) dependent gene silencing process. It offers great opportunities for insect science investigation, especially to analyze gene function, manage pest populations, and reduce insect-borne infectious diseases (Scott et al., 2013).

Although the success of RNAi has been confirmed in insects from Hemiptera, Diptera, Coleoptera, Lepidoptera, Hemiptera, Dictyoptera, Isopota and Orthoptera, the efficiency varied dramatically among tissues (Jarosch and Moritz, 2011; Scott et al., 2013; Terenius et al., 2011). In the brown planthopper Nilaparvata lugens, microinjection of dsRNA into the nymphal body cavity or ingestion of dsRNA can successfully suppress the expression levels of widely-expressed genes (Lin et al., 2016a; Lin et al., 2016b; Lu et al., 2016a; Xu et al., 2015; Yang et al., 2016; Zhao et al., 2016), genes in the obligatory yeast-like symbiont Entomomyces delphacidicola (Wan et al., 2015b; Wan et al., 2016), corpora allata (Lu et al., 2016b; Lu et al., 2016c), fat body (Liu et al., 2016), and male reproductive tract (Ge et al., 2016). However, whether RNAi can effectively repress the transcripts of neuronally-expressed genes remains undetermined.

In the present paper, we selected three putatively neuronally-expressed genes respectively encoding prothoracicotropic hormone (NlPTTH), pigment-dispersing factor (NlPDF) and short neuropeptide F (NlNPF) in N. lugens. PTTH is present in insect brain. When insect larvae or nymphs have fully grown, PTTH is released and triggers the initiation of metamorphic development through stimulation of ecdysteroid biosynthesis and secretion by the prothoracic glands (PGs) (Mizoguchi et al., 2015). Four cytochrome P450 monoxygenases encoded by the Halloween gene Spook (SPO), Phantom (PHM), Disembodied (DIB) and Shadow (SAD), catalyze the biosynthesis of ecdysone in the PGs. Ecdysone is then released from PG into the hemolymph and transported to peripheral tissues, where it is converted to 20-hydroxyecdysone (20E) under the catalyzation of the product of another Halloween gene Shade (SHD) in the planthoppers (Jia et al., 2015a; Jia et al., 2013a; Jia et al., 2013b, 2015b; Wan et al., 2014a, 2014b, 2015a). Pigment-dispersing factor (PDF) is expressed in insect nervous systems and plays critical roles in the control of behavior and circadian timekeeping (Shafer and Yao, 2013b, 2015b; Wan et al., 2014a, 2014b, 2015a).
The expression of short neuropeptide F (sNPF) family neuropeptides is mainly restricted to insect nervous system. Functional roles of sNPs include the regulation of feeding behavior, locomotion, osmotic homeostasis, sleep, learning and memory (Caers et al., 2016).

In the work present here, we performed bioassays with dsRNAs targeting these three genes to test whether microinjection of dsRNA can effectively silence neurally-expressed genes in *N. lugens*.

**Materials and methods**

**Insect culture**

An *N. lugens* colony used in this work was established from a field collection >20 years and maintained on rice (*Oryza sativa*) variety Tai-chung Native 1 (TN1, a susceptible rice variety to *N. lugens*), in an insectary under controlled conditions of 28 ± 1 °C, 80 ± 10% relative humidity and a 16 h light/8 h dark photoperiod.

**dsRNA preparation and bioassay**

*NlPDF-1, NlPDF-2, NlsNPF-1, NlsNPF-2, NlsPTTH* and enhanced green fluorescent protein (Gfp) fragments were amplified by PCR using specific primers conjugated with the T7 RNA polymerase promoter (primers listed in Table 1). The dsRNA originated from each of the above-mentioned sequences was synthesized using the MEGAscript T7 High Yield Transcription Kit (Ambion, Austin, USA) according to the manufacturer's instructions. Subsequently, the synthesized dsRNA was determined by agarose gel electrophoresis and the Nanodrop 1000 manufacturer's instructions. Subsequently, the synthesized dsRNA was digested by agarose gel electrophoresis and the Nanodrop 1000 spectrophotometer and kept at −70 °C until use.

**Bioassay**

The in *vivo* dsRNA injection bioassay bioassays were performed to test the RNAi efficiency. An aliquot of 50 nl of dsNIPTH, dsNIPDF, or dsNlsNPF at the concentrations of 6.0 ng/ml was injected into 90 (for dsNIPDF and dsNlsNPF) or 180 (for dsNIPTH) fourth-instar nymphs (1-day old) at the thorax between the mesocoxa and the hind coxa according to a described method (Liu et al., 2010). Negative control insects were injected with 6.0 ng/ml of dsGfp. Planthoppers without dsRNA injection were used as blank control (CK). Another in *vivo* dsRNA injection bioassay was used to test the influence of dsNlsNPF at the doses of 0.5, 2.4, 12, 60, and 300 ng on the target gene expression.

The bioassays were also carried out with newly-emerged female adults. For each dsRNA, 90 planthoppers in 3 replicates were individually injected with dsNIPTH, dsNIPDF, or dsNlsNPF at the concentration of 6.0 ng/ml (in 50 nl). Negative control and blank control insects were described as above.

One day after injection, dead individuals (assumed to be caused by physical damage) were discarded. The remaining treated individuals were grouped into 3 (for dsNIPDF and dsNlsNPF) or 6 (for dsNIPTH) replicates (25 individuals each). Out of these replicates, three that is for 4 days after injection were used to extract total RNA for qRT-PCR 4 days after injection. Another three replicates (for dsNIPTH) were used to observe survival rate, duration of nymphs, and defective phenotypes daily. The experiment was repeated three times as independent biological replicates.

**qRT-PCR analysis**

Total RNA samples were isolated from whole bodies of first- through fifth-instar nymphs at mid instar stages, mature male and female adults, and from brain-corpora cardiaca-corpora allata complex, fat body, thorax muscle, gut, epidermis and Malpighian tubules of the fifth-instar nymphs. Samples from the nymphs of the bioassays were also prepared. Each sample contained 5–10 individuals and repeated in biological triplicate. The RNA was extracted using the SV Total RNA Isolation System Kit (Promega). The transcript abundance of selected genes in each sample was estimated using *Nlactin* and *Nlps15* as internal reference genes (Yuan et al., 2014). All qRT-PCR primers were listed in Table 1. A RT-negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. Each sample was repeated in technical triplicates. Data were analyzed by the 2ΔΔCt method, using the geometric mean of internal control genes for normalization. All methods and data were confirmed to follow the MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin et al., 2009).

**Statistical analyses**

The data were given as means ± SE, and analyzed by ANOVAs followed by the Tukey-Kramer test, using SPSS for Windows (SPSS, Chicago, IL, USA). Since no significant differences between dsRNAs targeting two different regions of each of the two genes (*ds NIPDF-1* and *ds NIPDF-2*, *dsNlsNPF-1* and *dsNlsNPF-2*) were found, the data of each gene were then combined.

**Table 1**

<table>
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<th>Fragment name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
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<td>GTGGGATCAAGAAGACG</td>
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</table>
Results

The temporal expression patterns of the three neuronally-expressed genes

To test RNAi efficiency to neuronally-expressed genes, we selected NIPPTH (AB817276), NIPDF (AB817273) and NIsNPF (AB817279). The correctness of these sequences was substantiated by RT-PCR. Their transcription levels were determined at the nymphal and adult stages by qRT-PCR (Fig. 1). All the three genes were transcribed throughout tested developmental stages. In nymphal stage, the highest and the second highest levels of NIPPTH were at the third and fourth instar stages respectively. Similarly, the peaks of NIPDF were seen at the second and fourth instar stages, and those of NIsNPF were found at the second and fifth instar stages. In the adult stage, the mRNA levels of the three neuronally-expressed genes were lower in the females than those in the males (Fig. 1).

The tissue expression profiles of the three neuronally-expressed genes

The tissue expression profiles of the three neuronally-expressed genes in the fifth-instar nymphs were also tested (Fig. 2). Their transcripts were mainly restricted to N. lugens brain-corpora cardiaca-corpora allata complex, although trace levels were also found in the fat body, thorax muscle, gut, epidermis and Malpighian tubules (Fig. 2).

RNAi-aided knockdown of the three neuronally-expressed genes

To investigate RNAi efficacy for the three neuronally-expressed genes in N. lugens, dsNIPPTH, dsNIPDF, dsNIsNPF and dsGfp were respectively injected into the newly-molted fourth-instar nymphs, and the mRNA levels were determined on day 4 after injection. qRT-PCR revealed that introduction of dsNIPPTH decreased 91.8% of its target transcript; injection of dsNIPDF reduced 57.4% of its mRNA level; delivery of dsNIsNPF diminished 98.7% of its transcript, compared with negative control injected with dsGfp. ANOVA analysis revealed that the target transcripts were also significantly repressed on day 4 after injection (Fig. 3D–F).
Knockdown of NlPTTH inhibits ecdysone biosynthesis and affects nymph development

Since knockdown of NlPDF and NlsNPF did not result in obvious negative phenotypes, we focused on knockdown of NlPTTH in the present paper. PTTH stimulates ecdysteroidogenesis by the PGs (Mizoguchi et al., 2015). Therefore, we measured the transcription levels of two Halloween genes, NlPHM and NlDIB, that are involved in ecdysone biosynthesis in the planthoppers. Knockdown of NlPTTH reduced 73.8% and 84.3% of the expression levels of NlPHM and NlDIB, compared with negative control. ANOVA analysis demonstrated that the target transcripts of the two Halloween genes were significantly lower than negative control (Fig. 4A, B).

Knockdown of NlPTTH caused nymphal lethality. Four and six days after injection, the nymphal mortality of the dsNlPTTH-treated planthoppers reached approximately 25%, significantly higher than those of negative and blank controls (Fig. 5A).

Moreover, the average nymph durations from the fourth instar to adults were 6.0 and 5.5 days in control females and males. The periods were lengthened to 7.1 and 6.6 days in the dsNlPTTH-treated females and males, respectively (Fig. 5B, C).

Dose-dependent effect of dsNlsDPF

A series of dsNlsDPF solutions at the doses of 0.5, 2.4, 12, 60, and 300 ng were individually injected to the fourth-instar nymphs. The mRNA levels were decreased 94%, 86%, 57%, 33% and 29% respectively. Thus, NlsDPF mRNA abundance was reduced in a dose-dependent manner (Fig. 6).

Discussion

In the present study, we determined the RNAi efficacy for three putatively neuronally-expressed genes in N. lugens. At first, we tested their temporal expression patterns. Our results showed that all the three genes were transcribed throughout all tested developmental stages. This indicated that the selected genes might act in various development stages to regulate important physiological processes in N. lugens, as they...
acted in other insect species (Caers et al., 2016; Mizoguchi et al., 2015; Shafer and Yao, 2014).

The transcription of the three neuronally-expressed genes was mainly restricted to the nervous system. However, trace levels of the three genes were also found in the fat body, thorax muscle, gut, epidermis and Malpighian tubules. It may be that these tissue samples are contaminated by neurons. Similarly, D. melanogaster PTTH signaling pathway acts on two light sensors in the brain, the Bolwig’s organ and the peripheral class IV dendritic arborization neurons, to regulate light avoidance at the end of larval life (Yamanaka et al., 2013). Moreover, in Glossina morsitans morsitans adult females GmsNPF genes are mainly restricted to the nervous system (Caers et al., 2016). In D. melanogaster, a large number of neurons of the central nervous system in both larvae and adults express snPF (Carlsson et al., 2013). These results are compatible with the common idea that the three genes function in the nervous system for the regulation of various physiological processes.

In the present paper, we provided three lines of evidence to support that RNAi can effectively silence neuronally-expressed genes in N. lugens nymphs. Firstly, our results revealed that injection of dsRNAs originated from the three neuronally-expressed genes successfully lowered their mRNA level. It has been documented that injection or ingestion of dsRNA can knock down target genes transcribed in planthoppers tissues including obligatory yeast-like symbiont Entomomyces delphacidicola, corpora allata, fat body and male reproductive tract (Ge et al., 2016; Lin et al., 2016a; Lin et al., 2016b; Liu et al., 2016; Lu et al., 2016a; Lu et al., 2016b; Lu et al., 2016c; Wan et al., 2015b; Wan et al., 2015c;
Aedes aegypti results, dsRNA dose dependent RNAi effect concentrations into the fourth-instar nymphs respectively decreased gene expression. Different letters indicate significant difference at P < 0.05.  

Wan et al., 2016; Xu et al., 2015; Yang et al., 2016; Zhao et al., 2016). To the best of our knowledge, we are the first to confirm that RNAi can successfully knock down neuronally-expressed gene in L. aerugineum. In agreement with our results, ingestion of dsRNA silenced brain-transcribed gene in L. decemlineata (Meng et al., 2015).

Secondly, several Halloween genes are involved in ecdysteroidogenesis in planthoppers (Jia et al., 2015a; Jia et al., 2013a; Jia et al., 2013b, 2015b; Wan et al., 2014a, 2014b, 2015a). In the present paper, we found that knockdown of NIPTH downregulated the transcription of two ecdysteroidogenesis genes (NIPHM and NIDIB). Similarly, two Halloween genes LdPHM and LdDIB were decreased and 20E titer was reduced in the LdTorso depleted L. decemlineata (Zhu et al., 2015).

It is well known that 20E signal, acting through its EcR/USP heterodimeric receptor, induces transcription of a series of 20E-response genes such as H3 and FITZ-F1 (Guo et al., 2015b; Guo et al., 2016; Liu et al., 2014). In the present paper, we discovered that RNAi of NIPTH repressed the expression of a 20E-response gene (FITZ-F1). Similarly, the expression of several 20E signaling genes (LdECR-A, LdECR-B, LdUSP, LdH3R, and LdFITZ-F1) was downregulated in the LdTorso depleted L. decemlineata (Zhu et al., 2015).

Moreover, silencing of NIPTH caused typical 20E deficient phenotypes (Jia et al, 2015a; Jia et al., 2015b; Wan et al., 2015a): nymphal development was delayed and nymphal lethality occurred. Similar phenotypes were seen in the LdTorso depleted L. decemlineata (Zhu et al., 2015).

Thirdly, injection of a series of dsNlNPF solutions differing in the concentrations into the fourth-instar nymphs respectively decreased target mRNA levels in a dose-dependent manner. Consistent with our results, dsRNA dose dependent RNAi efficiency has been documented in Aedes aegypti (Singh et al., 2013), Sogatella furcifera, Laodelphax striatellus (Wan et al., 2014b) and L. decemlineata (Guo et al., 2015a).

In a word, the results in this study reveal that injection of dsRNA can effectively silence neuronally-expressed genes in N. lugens. This offers great opportunities to analyze the function and to develop potential management strategies for neuronally-expressed genes.

Acknowledgments

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