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Gene knockdown by intro-thoracic injection of double-stranded RNA in the brown planthopper, *Nilaparvata lugens*

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ABSTRACT

RNA interference (RNAi) is a powerful strategy for gene function study in insects. Here, we described the development of a RNAi technique by microinjection of double-stranded RNA (dsRNA) in the brown planthopper *Nilaparvata lugens*. Based on the mortality and RNAi efficiency criteria, the conjunctive between prothorax and mesothorax was selected as the injection site and 50 nl as injection volume. Three genes with different expression patterns were selected to evaluate the RNAi efficiency. A comparable 40% decrease of gene expression was observed at the 4th day after injection for the ubiquitously expressed calreticulin and the gut specific cathepsin-B genes, but only 25% decrease at the 5th day for the central nervous system specific Nlβ2 gene. Double injection could increase the RNAi efficiency, such as from 25% to 53% for Nlβ2 gene. The gene knockdown technique developed in this study will be an essential post-genomic tool for further investigations in *N. lugens*.

1. Introduction

RNA interference (RNAi) is a form of post-transcriptional gene silencing that has been described in a number of plant, nematode, protozoan, and invertebrate species. RNAi is characterized by a number of features: induction by double-stranded RNA (dsRNA), a high degree of specificity, remarkable potency and spread across cell boundaries, and a sustained down-regulation of the target gene (Caplen et al., 2000). DsRNA mediated RNAi has emerged as one of the most promising tool to study gene function, particularly in organisms for which stable transgenesis is not available, such as insects (Huvenne and Smagghe, 2010). Dicer RNase III type enzymes cleave cytoplasmic dsRNAs into small interfering RNAs (siRNAs) duplexes composed of approximately 21–23 nucleotides. The siRNAs duplexes are incorporated into a multiprotein RNA-inducing silencing complex (RISC) where the antisense strand guide RISC to its homologous target mRNA for endonucleolytic cleavage (Dykxhoorn et al., 2003; Meister and Tuschl, 2004). Exogenous dsRNA can be delivered to insects through different ways, in which artificial feeding and microinjection are usually used (Blandin et al., 2002; Turner et al., 2006; Walshe et al., 2009; Huvenne and Smagghe, 2010). Artificial feeding is a non-invasive technique preserving the integrity of the treated animals. However, the precise amount of the uptaken dsRNA is difficult to monitor. It is also difficult to perform the artificial feeding technique for insect species if the artificial diet is not successfully constructed (Turner et al., 2006). By contrast, microinjection is widely used in multiple insect species because of its precise volume control, such as *Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera* and *Acyrthosiphon pisum* (Dzitoyeva et al., 2001; Blandin et al., 2002; Amdam et al., 2003; Jaubert-Possamai et al., 2007).

The brown planthopper (BPH), *Nilaparvata lugens*, is a major rice pest in many parts of Asia (Liu et al., 2005). In recent years, this pest caused a big loss in rice production in Asian countries (Wang et al., 2008). Wing polymorphism in this species is known to be a common and ecologically important trait, which makes it difficult to control this pest because of the migration (Liu et al., 2008). Many progresses have been achieved in basic and applied aspects of this pest and the recent release of BPH EST Database http://bphest.dna.affrc.go.jp/ by Noda et al. (2008) provides the powerful information for gene function studies in this pest. Functional analyses tools such as inactivation of gene expression will be essential for further investigations in this insect species. Here we report a technology,

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gene knockdown by RNAi, in this important agricultural pest, which will provide powerful tool for gene function study in future. In order to evaluate the universality of RNAi technology, three target genes (calreticulin, cathepsin B-like protease and Nlβ2) with different tissue expression were selected and analysed. Calreticulin is a ubiquitously expressed gene (Jaubert-Possamai et al., 2007), Cathepsin B-like protease is a gut specific gene (Noda et al., 2008) and Nlβ2 is the central nervous system (CNS) specific gene (Liu et al., 2005).

2. Materials and methods

2.1. Experimental insects

The insects used in this study were from a laboratory strain obtained from China National Rice Research Institute in September 2001. Insects were reared on seedlings cultured soilless indoors at 25 (±1) °C, humidity 70–80% and 16/8 h light/dark. In order to synchronize the injected L3 larvae (3rd instar larvae), the 1st larvae were collected every 24 h and placed on fresh rice seedlings. 12 h after ecdysis, L3 larvae were collected for the injection.

2.2. dsRNA synthesis

The full coding sequences of cathepsin B-like protease (Cat; AJ316141), nicotinic acetylcholine receptors (nAChRs) β2 subunit (Nlβ2; AY378703) and the partial coding sequence and 3′-UTR region of calreticulin gene (Cal; Clone ID: C_NLMB3773 from BPH EST Database http://bphest.dna.affrc.go.jp/) were sub-cloned into pGEM-T vector and used as template for the target sequences amplification. The target sequences of three genes were amplified by RT-PCR using specific primers conjugated with 23 bases of the T7 RNA polymerase promoter (Table 1). The PCR products, 675 bp for calreticulin, 585 bp for cathepsin-B and 574 bp for Nlβ2, were used as templates for dsRNA synthesis using the T7 Ribomax Express RNAi System (Promega, Madison, WI, USA). After synthesis, the dsRNA was isopropanol precipitated, resuspended in ultra-pure water, quantified spectrophotometrically at 260 nm and its purity and integrity were determined by agarose gel electrophoresis. It was kept at −80 °C until use. As control, bacterial Lac-Z (AJ308295) dsRNA was also produced as described above.

2.3. Injection of dsRNA

Before injection, 1% agarose plate was made and placed on ice tray. Under carbon dioxide anaesthesia, insects (L3 larvae) were immobilized on the agarose plate with abdomen airward using manual forceps. Different volume (12.5 nl, 25 nl and 50 nl) of water

or purified dsRNA (5 μg/μl) were injected at slow speed using 3.5 Drummond needles and the Nanoinject II nanoinjector (Drummond scientific, Broomall, PA, USA). The injection sites were (I) the conjunctive between prothorax and mesothorax, (II) the conjunctive between the second and third abdominal segments, and (III) the conjunctive between the third and fourth abdominal segments (Fig. 1). For each gene, 300–400 L3 larvae were injected. Injected larvae were placed on fresh rice seedlings to recover and rear at 25 (±1) °C, humidity 70–80% and 16/8 h light/dark for 1–10 days. The dead individuals were recorded every day and mortality was calculated after 3 days following injection. Some injected insects were randomly selected and frozen in liquid nitrogen and kept at −80 °C before use. In order to increase RNAi efficiency, double injection was also tested, in which the second injection was carried out after 24 h following the first injection. The mortality was calculated after 3 days following the second injection.

2.4. Quantitative real-time reverse transcriptase polymerase chain reaction

Nine individuals were collected at each time point from 1 to 7 days after injection. Total RNAs were extracted from 3 pools of 3 injected insects using a Trizol kit (Invitrogen, Carlsbad, CA, USA). DNA contaminations were removed by treating RNA extractions products with RNase-free DNase (Ambion, Austin, TX, USA) and purified by phenol–chloroform. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed in a 25 μl total reaction volume containing 5 ng of total RNA, 0.5 μl primer mix containing 10 μM each of forward and reverse gene specific primers, 0.5 μl of Ex TaqTM HS (5 U/μl), 0.5 μl of PrimeScript RT Enzyme Mix, 12.5 μl of 2× One Step SYBR RT-PCR Buffer and 8.5 μl of H_2O. Two kinds of negative controls were set up: non-template reactions (replacing total RNA by H_2O) and minus reverse transcriptase controls (replacing PrimeScript RT Enzyme Mix by H_2O).

Table 1
Primers used for RT-PCR and Real-time PCR.

Gene	PCR type	Forward	Reverse
Calreticulin	RT-PCR[a]	caagggtccatgggttcac	cacacacacaatagcgcaac
	Real-time PCR[b]	gacaaccctgagtacacagctg	cttcttcctctccacctcgtcc
Cathepsin-B	RT-PCR	cgaatcttgccgatttggac	cagcaacaatcggtccattc
	Real-time PCR	gaatcttgccgatttggacaag	cgttccacttggcattgctcgc
Nlβ2	RT-PCR	ctcagaccaaatcctggac	gatgagtagattgaccgtg
	Real-time PCR	cggatatcgtattgttcaacaac	gccttgaatctggttgtacagcg
Lac-Z	RT-PCR	ggaagatcaggatatgtgg	cttcatcagcaggatatcc
Nlβ1	Real-time PCR	atggagttgtctgacctgcatc	cgaatctgtatgtactggtcttc
β-actin	Real-time PCR	tggacttcgagcaggaaatgg	acgtcgcacttcatgatcgag

[a] The primers for RT-PCR were used in dsRNA synthesis to amplify the target sequences of different genes.
[b] The primers for Real-time PCR were used in qRT-PCR to monitor the mRNA levels of different genes.

Fig. 1. The sites for microinjection in *Nilaparvata lugens* 3rd instar larvae. I: the conjunctive between prothorax and mesothorax; II: the conjunctive between the second and third abdominal segments; III: the conjunctive between the third and fourth abdominal segments.

The PCR was done with the following cycling regime: initial incubation of 42 °C for 5 min and 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s. Standard curves were obtained using decimal dilution series of pooled total RNAs from 20 individuals. mRNA levels were quantified in relation to the expression of β-actin (EU179846; Liu et al., 2008). The primer pair of each gene was designed to amplify about 200 bp PCR products, which were verified by nucleotide sequencing (Table 1). Only data that showed good efficiency (≥85%) and correlation coefficient (≥95%) were included in the analysis. Means and standard errors for each time point were obtained from the average of three independent sample sets. Compared to dsRNA-Lac-Z injected samples, the reduction (%) in expression levels of each gene were calculated in the samples injected with dsRNA-Cal, dsRNA-Cat or dsRNA-Nlβ2 respectively, which was represented as the RNAi efficiency. In order to evaluate the off-target effect, mRNA levels of brown planthopper nAChRs β1 subunit (Nlβ1) were also determined and compared between the dsRNA-Nlβ2 treated insects and controls. The primer pair of Nlβ1 used in qRT-PCR was also included in Table 1.

2.5. Data analysis

Differences in mortality and mRNA levels were analysed by one-way ANOVA with at least three repeats. Differences between values were analysed using an LSD pair wise comparison of means. The level of significance for results was set at $p < 0.05$.

3. Results

3.1. Definition of the injection site and volume

In order to find a suitable injection site, three sites were tested for microinjection (Fig. 1). For L3 larvae of *N. lugens*, the abdominal injection was tested first at the conjunctives between 2nd and 3rd segments (site II in Fig. 1) or 3rd and 4th segments (site III), but very high mortality (49–59%) was observed even when only 25 nl volume was injected. High volume injection gave the higher mortality (data not shown). During injection, the break of needle tips was often observed. In some of the successfully injected larvae, the out-flow of body fluid was observed. These observations showed the intra-abdominal injection in *N. lugens* caused the high wounding effects inevitably. The break of needle tips and out-flow of body fluid were not observed when injection was performed at the conjunctive between prothorax and mesothorax (site I in Fig. 1).

At these three injection sites injected with water or dsRNA, the death of injected individuals was first observed in 6 h after the injection and restricted in the first 3 days (no more death from the 4th day onward). So, here and in the following experiments, mortality was always calculated after 3 days following the injection. At three sites, the microinjection with 25 nl water resulted in big different mortalities, in which the abdominal injection (58.8 ± 10.9% and 49.2 ± 7.1% for sites II and III) caused remarkably higher mortality than the thorax injection (17.5 ± 4.7% for site I; Fig. 2). Based on the lower mortality, the conjunctive between prothorax and mesothorax (site I) was selected for the microinjection.

Water was then injected with different volumes (12.5 nl, 25 nl and 50 nl). The microinjection with a 50 nl volume resulted in 42.8 ± 7.6% mortality, and the injection with half volume (25 nl) or one fourth volume (12.5 nl) clearly reduced the mortality to less than 20% (Fig. 2). Similar mortality was observed between individuals injected with a same volume of water, the control bacterial dsRNA-Lac-Z or dsRNAs of interest (Fig. 3) indicating that larvae death was not related to gene silencing. These results indicated the higher mortality with 50 nl injection was not only partly due to the disruptive effect of injection itself but also by the high injection
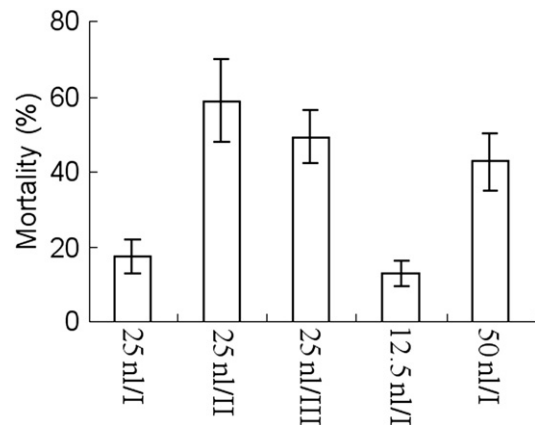

Fig. 2. Mortality induced by microinjection at different sites with different volumes of water. I, II and III in the abscissa were corresponding to three injection sites in Fig. 1. Data points are means of three independent experiments.

volume used to deliver dsRNA. However, in order to optimize the RNAi efficiency, 50 nl was selected as the injection volume, because its RNAi efficiency was much higher than that of the low injection volume 25 nl (Fig. 4).

3.2. Silencing of calreticulin and cathepsin-B

When water or dsRNA-Lac-Z was injected at same volumes, there was not significant difference in mRNA levels of three target genes (calreticulin, cathepsin-B and Nlβ2; data not shown). Compared to water, the component of dsRNA-Lac-Z solution is close to target gene dsRNA solution, so dsRNA-Lac-Z injection was used as negative control instead of water in further experiments.

Two volumes (25 nl and 50 nl) of dsRNA-Cal or dsRNA-Lac-Z were injected into L3 larvae and the RNAi efficiency on calreticulin expression in injected larvae was investigated by qRT-PCR over seven days after the injection. Fig. 4 showed the RNAi efficiency of dsRNA-Cal on calreticulin in injected samples. Calreticulin expression was reduced by 16.5 ± 4.7% in one day after injection with 50 nl dsRNA-Cal. The maximum reduction of 43.8 ± 10.3% occurred at the fourth day and no effect was observed from the eighth day onward. Although the maximum reduction induced by both 25 nl and 50 nl injections occurred at the fourth day, 25 nl injection only caused 13.9 ± 4.7% reduction, which was only one third of that of 50 nl injection (43.8 ± 10.3%).
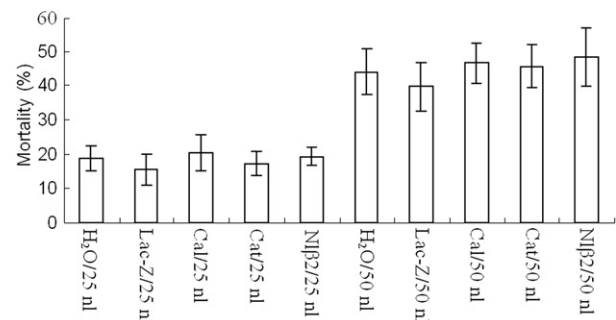

Fig. 3. Mortality induced by microinjection with different volumes. dsRNAs for calreticulin, cathepsin-B, Nlβ2 and bacterial Lac-Z were indicated by Cal, Cat, Nlβ1 and Lac-Z in the figure. The conjunctive between prothorax and mesothorax (site I) was injected here and in further experiments. Two different volumes were injected (25 nl and 50 nl). Data points are means of three independent experiments.
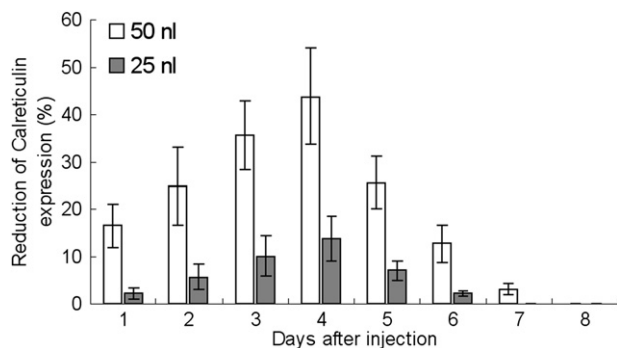
Fig. 4. Knockdown of calreticulin expression. Calreticulin mRNA level in dsRNA-Lac-Z and dsRNA-Cal injected larvae were monitored by qRT-PCR over eight days after injection. Each kinetic point was performed in triplicate on 3 pooled larvae.

Similar to the silencing of calreticulin, the injection of dsRNA-Cat reduced cathepsin-B expression by $19.2 \pm 6.3\%$ at the first day after the injection and induced a maximum inhibition of $36.4 \pm 3.9\%$ at the fourth day. There was not any effect from the eighth day onward (Fig. 5).

3.3. Silencing of Nlβ2

A similar experiment was performed to inhibit nicotinic acetyl-choline receptor (nAChR) β2 subunit Nlβ2. At the first day after injection, the injection of dsRNA-Nlβ2 only caused the negligible RNAi (Fig. 5), compared to over 15% reduction of calreticulin and cathepsin-B expression. The maximum reduction of $25.3 \pm 5.2\%$ of the transcript level occurred at the fifth day, but not at the fourth day.

Fig. 5 also showed that the RNAi efficiency on Nlβ2 was lower than that on cathepsin-B. The maximum reduction in Nlβ2 transcript level was $25.3 \pm 5.2\%$ at the fifth day, which was significantly lower ($p < 0.05$, one-way ANOVA test) than $36.4 \pm 3.9\%$ at the fourth day for cathepsin-B. 25% RNAi efficiency might not be enough to study nAChR subunits function, so the second injection was performed after 24 h following the first injection. The double injection resulted in a $53.8 \pm 6.1\%$ reduction in Nlβ2 transcript level at the sixth day after the first injection (Fig. 6). Although the cumulative mortality of the double injection was high ($67.4 \pm 11.2\%$), the high RNAi efficiency is favorable to study on insect nAChR function.

3.4. Off-target effect on Nlβ1

In order to evaluate the off-target effects of the present RNAi technique, mRNA levels of another brown planthopper nAChRs β subunit Nlβ1 were determined and compared between the
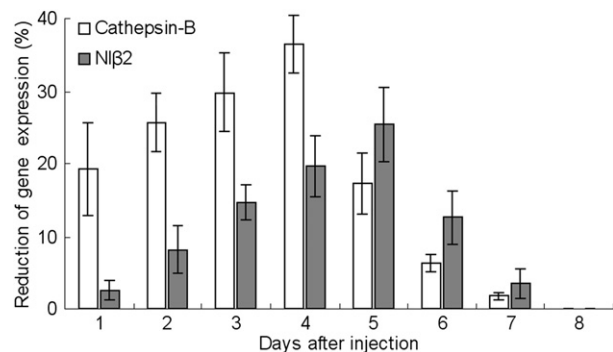
dsRNA-Nlβ2 treated insects and controls. As shown in Fig. 7, both single injection and double injection with dsRNA-Nlβ2 did not induce a significant reduction of Nlβ1 transcript, which indicated there were not the off-target effects of dsRNA-Nlβ2 on Nlβ1.

Fig. 6. Knockdown of Nlβ2 expression by double injection. The second injection was performed after 24 h following the first injection. Nlβ2 mRNA level in dsRNA-Lac-Z and dsRNA-Nlβ2 injected larvae were monitored by qRT-PCR. Each kinetic point was performed in triplicate on 3 pooled larvae.

4. Discussion

Delivery of dsRNA into an organism can lead to the post-transcriptional knockdown of genes sharing specific sequence with the introduced dsRNA. Finding a suitable delivery system for dsRNA can be a major limitation of the RNAi system in insects. In most cases, microinjection is the widely suitable method to deliver dsRNA, although the high mortality often occurs when the big volume is injected in order to achieve the high RNAi efficiency (Jaubert-Possamai et al., 2007). Some alternatives to microinjection have been used, such as feeding dsRNA (Turner et al., 2006; Walshe et al., 2009), soaking of embryos in dsRNA (Eaton et al., 2002) and delivering dsRNA by recombinant viruses (Travanty et al., 2004), in which dsRNA feeding is one of the promising ways developed recently (for a review, see Huvenne and Smagghe, 2010). Huvenne and Smagghe (2010) summarized the RNAi methods through feeding in 15 insect species belonging to 7 different orders. Through feeding, RNAi with dsRNA showed high efficiency in target genes from gut (Huvenne and Smagghe, 2010). For target genes from other tissues, RNAi efficiency through feeding varied among target genes and insect species. For example on genes from fat body, dsRNA feeding induced 90% reduction of vitellogenin expression in *A. mellifera* (Nunes and Simões, 2009) and 50–70% reduction of caste regulatory hexamerin storage protein in *Reticulitermes flavipes* (Zhou et al., 2008), but showed no effect on transferrin in *Glossina morsitans* (Walshe et al., 2009).

Fig. 5. Knockdown of cathepsin-B and Nlβ2 expression. mRNA level in dsRNA-Lac-Z, dsRNA-Cat and dsRNA-Nlβ2 injected larvae were monitored by qRT-PCR over eight days after injection. Each kinetic point was performed in triplicate on 3 pooled larvae.
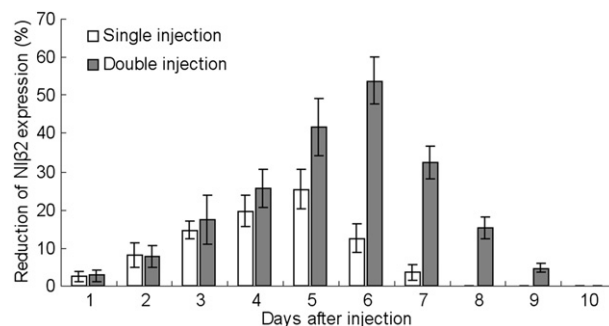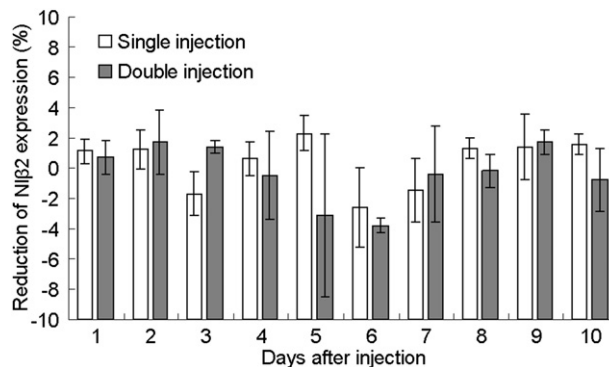

Fig. 7. Off-target effects of Nlβ2 dsRNA on Nlβ1 expression. Nlβ2 mRNA level in dsRNA-Lac-Z and dsRNA-Nlβ2 injected larvae were monitored by qRT-PCR. Each kinetic point was performed in triplicate on 3 pooled larvae.

The RNAi efficiency through feeding is also influenced by insect developmental stage. For example, on nitroporin 2 from saliva glands in *Rhodnius prolixus*, artificial diet feeding induced $42 \pm 10\%$ reduction of gene transcript in 2nd instars, but showed no effect in 4th instars (Araujo et al., 2006). Although dsRNA feeding is thought as a non-invasive and promising way, the precise amount of the uptaken dsRNA by feeding is difficult to monitor and RNAi efficiency among individuals is of high variability (Turner et al., 2006). In contrast, the delivered amount of dsRNA through microinjection is under control exactly.

In most cases of microinjection, the abdomen is the first choice (Dzitoyeva et al., 2001; Amdam et al., 2006; Jaubert-Possamai et al., 2007). Because of high mortality in microinjection at site II and site III from brown planthopper abdomen, the choice for injection site was changed to the thorax, in which the conjunctive between prothorax and mesothorax is the pliable membrane (site I). At this site, injection with a 25 nl volume only resulted in 18% mortality, significantly lower than that of intra-abdominal injections (49–59%). At site I, increase of injection volume caused high mortality, which was similar to other insects with the intra-abdominal injection (Dzitoyeva et al., 2001; Amdam et al., 2006; Jaubert-Possamai et al., 2007). Because the increase of injection volume also provided higher RNAi efficiency, in order to optimize the RNAi, the appropriately high volume was often chosen (Bettencourt et al., 2002; Amdam et al., 2006; Jaubert-Possamai et al., 2007). In the present study, 50 nl volume was selected, because the mortality and RNAi efficiency were acceptable.

RNAi efficiency might depend on the targeted gene and its expression profile. Three target genes (calreticulin, cathepsin-B and Nlβ2) with different tissue expression patterns were analysed in this study. Microinjection with dsRNA induced the similar RNAi efficiency on the ubiquitously expressed gene calreticulin and gut specific gene cathepsin-B, which is close to RNAi efficiency on the orthologues of these two target genes in pea aphid *A. pisum* by the abdominal injection (Jaubert-Possamai et al., 2007). Different from calreticulin and cathepsin-B, RNAi efficiency on Nlβ2 was lower and the maximum inhibition of Nlβ2 transcript occurred later. These results demonstrate that different tissues or genes seem to be unequally sensitive to gene knockdown by dsRNA injection.

The maximum depletion of approximately 40–50% (25% for Nlβ2) of mRNAs obtained is not a high efficiency for RNAi compared to what was previously described in *Spodoptera litura* (95% of inhibition for aminopeptidase gene) and in *D. melanogaster* (62% for Drs gene) (Rajagopal et al., 2002; Goto et al., 2003). However, similar efficiency has been reported in *Schistocerca americana* (Dong and Friedrich, 2005), *R. prolixus* (Araujo et al., 2006) and *A. pisum* (Jaubert-Possamai et al., 2007). Various parameters can be modified to increase RNAi efficiency. The most obvious parameter is to increase the injection volume. Actually, high injection volume gave high RNAi efficiency, but was also with high mortality. For example, 100 nl injection gained 60% RNAi efficiency for calreticulin gene and 43% for Nlβ2 gene, but also caused 85% mortality (data not shown), which indicated the increase of injection volume was not a suitable way to increase RNAi efficiency in the brown planthopper. Another choice is to increase RNAi efficiency by multi-injection. In *R. prolixus*, two successive injections of dsRNA resulted in an increase of RNAi efficiency from 38 to 75% (Araujo et al., 2006). Because the single injection only gave 25% RNAi efficiency for Nlβ2 gene, double injection was performed on Nlβ2 gene after 24 h following the first injection. In double injection, the maximum inhibition of Nlβ2 expression reached 54%, which was two times of single injection efficiency (25%), although the higher mortality was also observed (67%). Comparing double injection (50 nl/injection) and single injection with high volume (100 nl), it was found double injection achieved higher RNAi efficiency and lower mortality, which demonstrated that the double injection was the suitable way to increase RNAi efficiency in *N. lugens* larvae.

Beside the important parameters (a suitable injection site, optimal injection volume and double injection) discussed above to optimize the microinjection RNAi technique in the brown planthopper, more factors could be modified to improve such RNAi method. First, insect developmental stages should be carefully selected. As mentioned above, RNAi efficiency of the same RNAi technique on the same target gene was much different in different stages of one insect species (Araujo et al., 2006; Griebler et al., 2008). Second, microinjection with high dsRNA concentration might induce high RNAi efficiency and be applied instead of high volume injection or double injection. However, high concentration may give a high concentration pressure and cause a big different physiological condition from insect itself when injection. Actually, for every target gene, the optimal concentration should be different and has to be determined respectively (Huvenne and Smagghe, 2010). The exceeding concentration might not result in more silencing and cause further side-effect, such as high mortality (Meyering-Vos and Muller, 2007; Shakesby et al., 2009). Third, the nucleotide sequence and length of dsRNA fragment should be chosen based on each target gene itself. These two factors are important to avoid off-target effects (Huvenne and Smagghe, 2010). In the present study, the nucleotide sequence (574 bp for dsRNA fragment) selection of Nlβ2 was based the amino acid sequence conservation of insect nAChRs subunits, in which the large cytoplasmic loop (between transmembranes III and IV) are poorly conserved between subunits in both length and amino acid sequence (Gundelfinger, 1992). No off-target effects of Nlβ2 dsRNA on Nlβ1 transcript were found. Fourth, some parameters for every target gene should be optimized independently. These parameters include the selected nucleotide sequence, dsRNA fragment length, dsRNA concentration, injection volume and so on. The technique present in this study is only a general description. For a special target gene, the modifications should be made according to the target gene properties, such as the expression pattern and the main role in insect life-cycle.

Acknowledgments

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