Research Article

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RNA interference in *Nilaparvata lugens* (Homoptera: Delphacidae) based on dsRNA ingestion

Jie Li,[a†] Qiuhong Chen,[b†] Yongjun Lin,[b] Tingru Jiang,[a] Gang Wu[a] and Hongxia Hua[a*]

Abstract

BACKGROUND: An efficient and convenient RNA interference (RNAi) technique involving double-stranded RNA (dsRNA) ingestion is useful for gene function studies of non-model insects.

RESULTS: Three dsRNAs targeting different sites within a gene encoding vacuolar ATP synthase subunit E (V-ATPase-E, *21E01*) were synthesised for RNAi in *Nilaparvata lugens*. dsRNA was found to be stable in 0.1 g mL^{-1} sucrose solution, but unstable in artificial fodder. Therefore, dsRNAs were orally delivered into *N. lugens* in 0.1 g mL^{-1} sucrose solution. RNAi was induced by all three of the dsRNAs at 0.05 μg μL^{-1} in *N. lugens*. Time dynamics analysis of gene silencing indicated that significant suppression of the target gene began as early as 2 days after ingestion of ds2-*21E01* and ds3-*21E01*. However, significant repressive effects were recorded up to 10 days after exposure to ds1-*21E01*. The maximum reduction in target gene mRNA was observed after 10 days of treatment, with suppression ratios induced by ds1-*21E01*, ds2-*21E01* and ds3-*21E01* of 41, 55 and 48% respectively.

CONCLUSION: An efficient and convenient RNAi technique involving dsRNA ingestion has been successfully developed for *N. lugens*. This will be a useful tool for further functional genomic investigation in this organism.

Keywords: *Nilaparvata lugens*; RNA interference; ds*RNA* ingestion; gene silencing

1 INTRODUCTION

RNA interference (RNAi) was first discovered in the nematode *Caenorhabditis elegans*.[1] When exogenous double-stranded RNAs (dsRNAs) are introduced into cells, they are first processed by dicer RNaseIII-type enzymes into many short pieces, each of about 21–23 nucleotides, known as short interfering RNAs (siRNAs).[2,3] Then, siRNA duplexes are incorporated into a multiprotein RNA-inducing silencing complex (RISC) where the antisense strand guides RISC to its homologous target mRNA for endonucleolytic cleavage. dsRNA corresponding to either a portion or the entire coding region of a particular gene can interfere with the expression and function of the endogenous gene.[1,4] Many previous studies have demonstrated that dsRNA-mediated gene silencing is a widespread phenomenon in eukaryotes.[5,6] RNAi has become a powerful tool for gene loss-of-function research, especially in organisms for which stable transgenesis is not available, such as non-model insects.[7–9]

To date, RNAi technology has been successfully applied in many insect species by the methods of injection, soaking and feeding, including Blattaria,[9] Homoptera,[10–12] Hemiptera,[13] Hymenoptera,[14] Diptera,[7] Coleoptera,[15,16] Orthoptera[8] and Lepidoptera.[17,18] The oral delivery of dsRNA is an effective, convenient and economical technique that can maintain insect integrality. According to statistics, RNAi by dsRNA feeding has been successfully established in 15 insects, including important agricultural pests and vector insects.[19]

Brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most destructive insect pests on rice. BPHs mainly damage rice by extracting phloem sap, which act as vectors for rice grassy stunt virus and ragged stunt virus.[20] This important agricultural pest is a long-distance migratory insect, which makes it very difficult to control.[21] Recently there have been many molecular achievements with this organism, in particular a large number of expressed sequence tags (ESTs) of BPH have been released in the public database (http://www.ncbi.nlm.nih.gov).[22] This provides new opportunities for functional genomic investigation of BPH, and the possibility of elucidating the molecular mechanisms of BPH that enable it to adapt to environmental conditions. As a non-model insect, stable transformation of BPH is not yet available, so RNAi represents an important alternative method for gene

* *Correspondence to: Hongxia Hua, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. E-mail: huahongxia@mail.hzau.edu.cn*

† *These authors contributed equally to this work.*

a *Hubei Insect Resources Utilisation and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China*

b *National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China*

function studies in this pest. In this study, three dsRNAs targeting different sites within a gene encoding vacuolar ATP synthase subunit E (V-ATPase-E) were synthesised and orally delivered into *N. lugens* with 0.1 g mL^{-1} sucrose solution. Decreased expression of the target gene was induced by all three of these dsRNAs.

2 MATERIALS AND METHODS

2.1 Insects

BPH wild populations were collected during the summer of 2007 from paddy fields in Wuhan, Hubei Province, China. BPHs were raised on TN1 (Taichuang Native 1, BPH-susceptible rice variety) in a thermostatic chamber. The chamber was maintained at 80% RH, 25 ± 2 °C and 14 : 10 (light : dark).

2.2 dsRNA synthesis

21E01 (accession number HM243145) was a full-length cDNA from a BPH normalised cDNA library, encoding putative V-ATPase-E. Three dsRNAs (ds1-*21E01*, ds2-*21E01* and ds3-*21E01*) corresponding to different regions of *21E01* were synthesised for the V-ATPase-E gene knockdown. The sequence of *21E01* and the sequence used to synthesise dsRNAs are shown in Fig. 1. ds1-*21E01* corresponded to 276 nucleotides at the 5′-end of the *21E01* coding sequence (CDS). ds2-*21E01* included 195 nucleotides at the 3′-end of CDS, plus 54 nucleotides at the 3′-end of the untranslated regions (UTRs), and ds3-*21E01* targeted 498 nucleotides at the 3′-end of the UTRs. dsRNA was synthesised using the T7 RiboMAX™ Express RNAi System (Promega, USA). The sequence of T7 polymerase promoter was fused with gene-specific primers at the 5′-end, and the resulting PCR products contained the T7 polymerase promoter site at both ends. Then, PCR products were purified using QIAquick™ PCR purification kit (Qiagen, Germany) and used as template *in vitro* transcription. Sense and antisense strands were transcribed from the DNA template in the same reaction. The dsRNAs were extracted with phenol : chloroform and precipitated with isopropanol. The precipitated dsRNAs were dissolved in nuclease-free water and then heated at 75 °C for 10 min and cooled at room temperature. *GFP* dsRNA (ds-*GFP*) served as negative control. The cDNA sequence of *GFP* (accession number U76 561) and the sequence used to synthesise ds-*GFP* are shown in Fig. 2. Primers used for the synthesis of dsRNAs are shown in Table 1.

2.3 Detection stability of dsRNA in different medium solutions

First, 10 µL of DEPC-treated distilled deionised water (ddH_2O), artificial fodder of BPH, 0.1 g mL^{-1} of sucrose solution prepared with ddH_2O and 0.1 g mL^{-1} of sucrose solution prepared with DEPC-treated ddH_2O, each containing 0.5 µg of ds-*GFP*, were incubated at room temperature for 0, 1, 3, 10 and 24 h. Then, the dsRNA stability was checked. Artificial fodder was prepared, and the pH was adjusted to 6.8, according to Fu *et al*.[23] Without any adjustment, the pH value of the two sucrose solutions was 7.5, and the pH value of the DEPC-treated ddH_2O and ddH_2O was 8.0.

To determine whether the pH value of artificial fodder was a factor influencing the stability of ds-*GFP*, it was adjusted with either HCL or NaOH to 5.0, 6.0, 6.8, 7.5 and 8.0. Then, 10 µL of artificial fodder containing 0.5 µg of ds-*GFP* was incubated at room temperature for 0, 1, 3, 10 and 24 h. The stability of the ds-*GFP* was checked after incubation.

2.4 Effect of BPH suction on the integrity of the dsRNA

Fifteen third-instar BPH nymphs were reared with 70 µL of 0.1 g mL^{-1} sucrose solution prepared with ddH_2O, which contained 3.5 µg of ds-*GFP*. The rearing procedure followed that reported by Fu *et al*.[23] Glass cylinders (15 cm length × 2.5 cm diameter) open at both ends were used as feeding chambers for the nymphs. The sucrose solution was held between two layers of stretched Parafilm M (stretched to about 4 times its original area) located at one open end of the chamber. After nymphs were introduced into the chamber, the opposite end of the chamber was enclosed with wet black cotton cloth. All the cylinders were covered with a sheet of wet black cotton cloth, but the end containing the sucrose solution was exposed to light. Nymphs could suck sucrose solution by piercing through the inner Parafilm M of the fodder sachet. This rearing procedure was conducted under conditions of a 14 : 10 h light : dark cycle, a temperature of 28 ± 1 °C and a relative humidity of around 90%. After being fed on by nymphs for 24 h, the sucrose solutions containing ds-*GFP*s were collected and the ds-*GFP*s were precipitated and dissolved in DEPC-treated water. The stability of 0.5 µg dsRNAs in the samples was checked.

2.5 dsRNA ingestion

Second-instar BPH nymphs that had been reared with artificial fodder for 2 days were collected from the feeding chambers. Then, 15 nymphs were fed with 70 µL of 0.1 g mL^{-1} sucrose solution containing dsRNAs or artificial fodder every 24 h. The sucrose solution was prepared with ddH_2O. The final dose of dsRNA in the sucrose solution was 0.05 µg $µL^{-1}$. The survival rate of nymphs was recorded daily. After dsRNA ingestion for 2, 6 and 10 days, insects were collected and immediately frozen in liquid nitrogen for RNA extraction. The artificial fodder preparation and rearing procedure followed those reported by Fu *et al*.[23] and are described in detail in Section 2.4. Thirty nymphs (15 nymphs being reared in one glass cylinder) were used in each replication, and for each treatment three replications were performed (i.e. using a total of 90 BPH nymphs).

2.6 qRT-PCR

Total RNA was extracted from 20–30 BPHs from each replication using Trizol reagent (Invitrogen, USA). DNA contamination was removed with RNAse-free DNAse (Ambion, USA). The primers of *21E01* used in quantitative RT-PCR (qRT-PCR) were 5′-cccagccaagtacaaggaagtc-3′ and 5′-caacaatgccctcgatcaga-3′. *Actin1* (accession number EU179 846.1) transcripts were used as an internal control gene. The primers for *actin1* used in qRT-PCR were 5′-ccaaccgtgagaagatgacc-3′ and 5′gatgtcacgcacgatttcac-3′. qRT-PCR reactions were conducted on an ABI Prism 7500 (Applied Biosystems, USA), using SYBR *Premix Ex* Taq™ (Perfect Real Time; Takara Biotechnology Corporation Co. Ltd, Dalian, China) according to the manufacturer's instructions. Triplicate independent reactions were performed for each RNA sample, and the signal intensity of the target gene was represented by the averaged value. The expression level of *21E01* in BPHs fed on ds1-*21E01*, ds2-*21E01* and ds3-*21E01* was quantified relative to that in BPHs fed on ds-*GFP*, according to the method of Livak and Schmittgen.[24]

2.7 Data analysis

All data were analysed using the general linear models procedure.[25] One-way ANOVAs were used to analyse BPH mortality. Differences between means were tested using the least

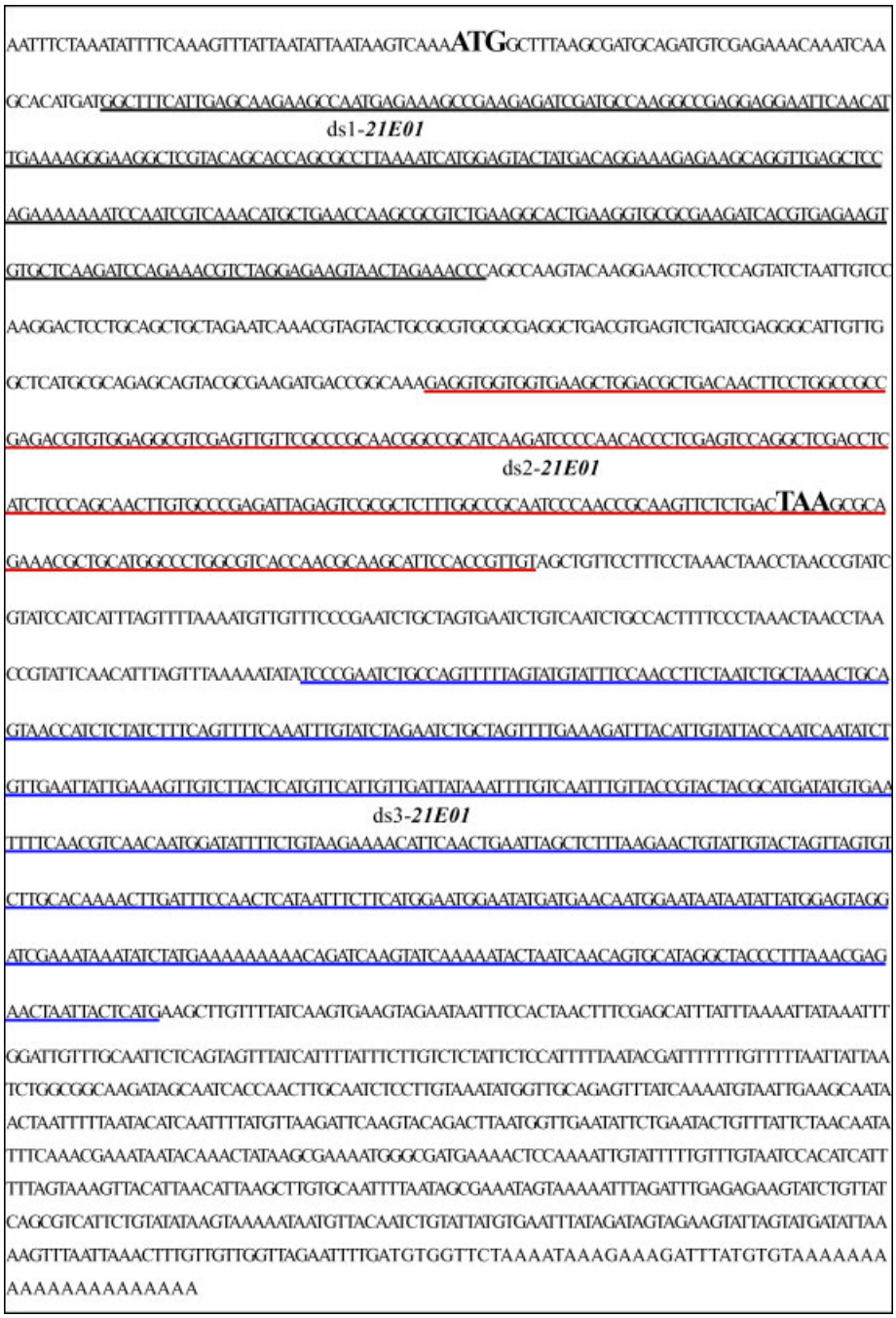

Figure 1. The full-length cDNA sequence of *21E01*. The start codon ATG and stop codon TAA were in bold. The location of the gene sequence used to synthesise ds1-*21E01*, ds2-*21E01* and ds3-*21E01* was underlined in black, red and blue respectively.

significant difference (LSD) test at $P < 0.05$. Percentage values were converted into arcsine before statistical analysis.

3 RESULTS

3.1 dsRNA stability in different media

ds-*GFP* was found to be reasonably stable in DEPC-treated ddH_2O and the two kinds of 0.1 g mL^{-1} sucrose solutions, as shown in Fig. 3. However, ds-*GFP* was unstable in artificial fodder. As soon as ds-*GFPs* were added into artificial fodder (pH = 6.8), their electrophoresis velocity immediately slowed compared with ds-*GFP*s in DEPC-treated ddH_2O and sucrose solution. This indicated that the configuration of ds-*GFP*s was rapidly altered by artificial fodder. After 24 h incubation, ds-*GFP*s were totally degraded in artificial fodder (Fig. 3).

To clarify whether the pH value of artificial fodder was a factor influencing the stability of ds-*GFP*, ds-*GFP*s were added into artificial fodders with different pH values and incubated at room temperature for 0, 1, 3, 10 and 24 h. The results indicated that all the artificial fodder with different pH values had negative effects on stability of ds-*GFP*s, with the instability of ds-*GFP*s increasing with increased pH. After 24 h incubation, ds-*GFP*s

AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAA
AGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTC
GTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTG
CAGGTCGACTCTAGAGGATCCCCGGGTACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCC
CATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG
GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACG
GCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCC
ds-*GFP*
AGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG
AACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAG
CCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGG
CAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCT
GAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCG
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TGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTC
AGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACC
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TTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTG
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ATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTG
ACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAAC
CGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTAT
TAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCT
GCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCAC
TGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACA
GATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAA
CTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCC
ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACA
AAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCA
GAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATAC
CTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTT
ACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAAC
TGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC
AGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCAC
CTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTAC
GGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTT
GAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

Figure 2. The cDNA sequence of *GFP*. The location of the gene sequence used to synthesise ds-*GFP* was underlined.

Table 1. The primer sequence for dsRNA synthesis

DsRNA	Primers for sense RNA synthesis	Primers for antisense RNA synthesis
ds1-*21E01*	5′-ggatcctaatacgactcactatagggatggctttcattgagcaag-3′ 5′-ccttgtacttggctgggttt-3′	5′-gatggctttcattgagcaag-3′ 5′-ggatcctaatacgactcactataggccttgtacttggctgggttt-3′
ds2-*21E01*	5′-ggatcctaatacgactcactatagggaggtggtggtgaagctgga-3′ 5′-acaacggtggaatgcttgcg-3′	5′-gaggtggtggtgaagctgga-3′ 5′-ggatcctaatacgactcactataggacaacggtggaatgcttgcg-3′
ds3-*21E01*	5′-ggatcctaatacgactcactataggtcccgaatctgctagtgaat-3′ 5′-tcatgcgtagtacggtaacaa-3′	5′-tcccgaatctgctagtgaat-3′ 5′-ggatcctaatacgactcactataggtcatgcgtagtacggtaacaa-3′
ds-*GFP*	5′-ggatcctaatacgactcactatagggtaaacggccacaagttcag-3′ 5′-tcggccatgatatagacgtt-3′	5′-gtaaacggccacaagttcag-3′ 5′-ggatcctaatacgactcactataggtcggccatgatatagacgtt-3′

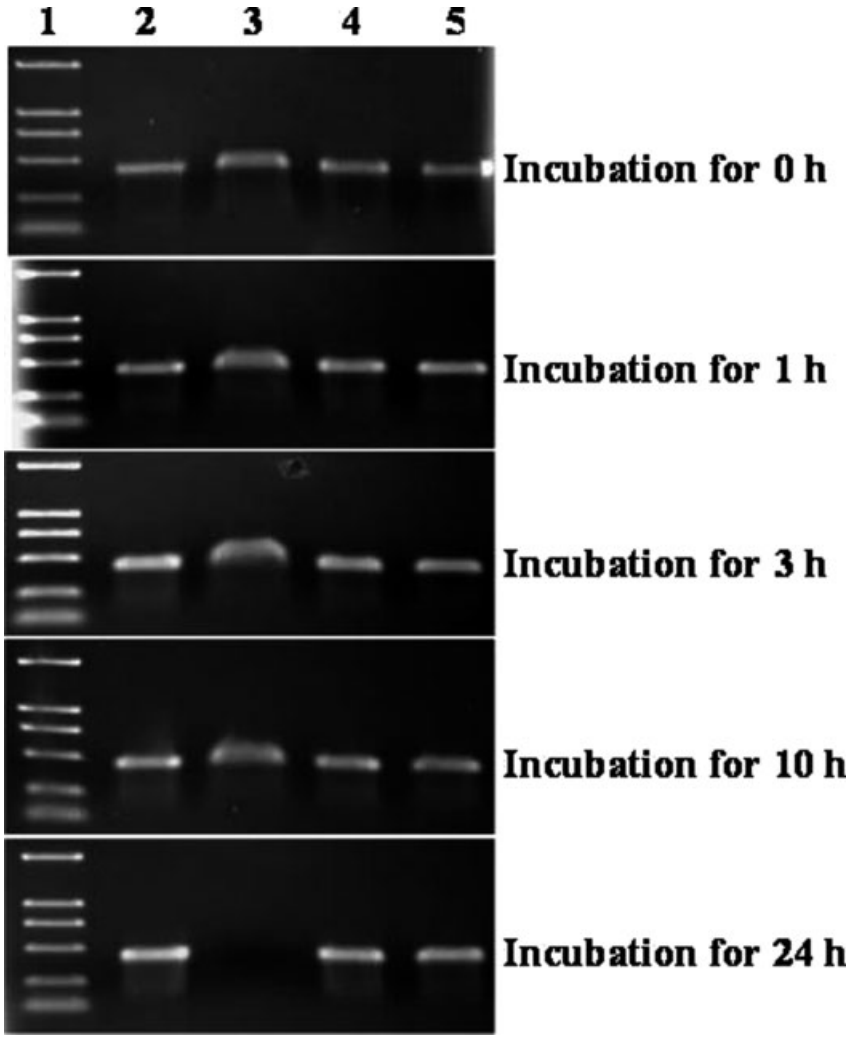

Figure 3. ds-*GFP* (0.5 μg) stability after incubation with different medium solutions at room temperature for 0, 1, 3, 10 and 24 h. Lane 1: 2 kb ladder; lane 2: ds-*GFP* in DEPC-treated water (pH = 8.0); lane 3: ds-*GFP* in artificial fodder (pH = 6.8); lane 4: ds-*GFP* in 0.1 g mL^{-1} sucrose solution prepared with DEPC-treated ddH_2O (pH = 7.5); lane 5: ds-*GFP* in 0.1 g mL^{-1} sucrose solution prepared with ddH_2O (pH = 7.5).

in artificial fodder with a pH value of 6.8, 7.5 and 8.0 were completely degraded, whereas ds-*GFP*s in artificial fodder with a pH value of 5.0 or 6.0 were partially degraded (Fig. 4). By contrast, when a pH of 8.0 was maintained, ds-*GFP*s in DEPC-treated ddH_2O were reasonably stable, while those in artificial fodder were unstable and easily degraded. Similarly, at the same pH of 7.5, ds-*GFP*s in sucrose solution were reasonably stable, while those in artificial fodder were unstable and easily degraded.

These data suggested that the pH value of artificial fodder could influence the stability of dsRNAs, but this was not the only factor resulting in the instability of dsRNAs. Other factors, such as certain ingredients or ions in the artificial fodder, also affected the stability of dsRNAs. Therefore, sucrose solution prepared from

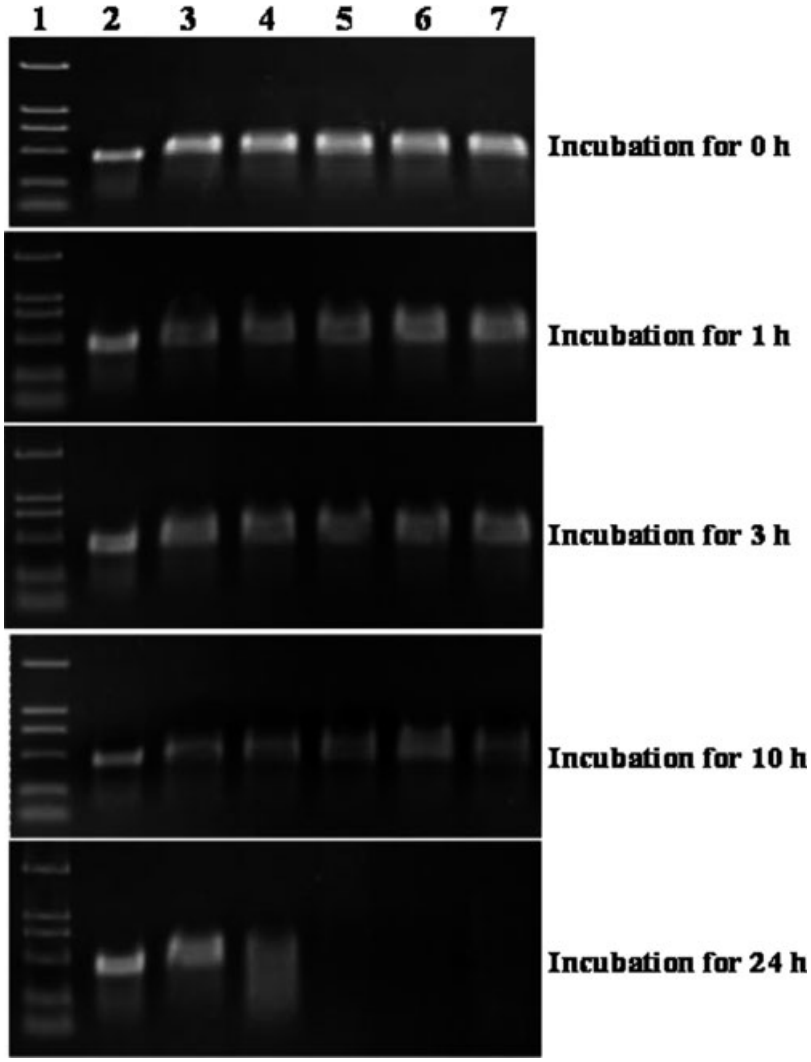

Figure 4. ds-*GFP* (0.5 μg) stability after incubation with artificial fodder with different pH values at room temperature for 0, 1, 3, 10 and 24 h. Lane 1: 2 kb ladder; lane 2: ds-*GFP* incubation with DEPC-treated ddH_2O with a pH value of 8.0; lane 3: ds-*GFP* incubation with artificial fodder with a pH value of 5.0; lane 4: ds-*GFP* incubation with artificial fodder with a pH value of 6.0; lane 5: ds-*GFP* incubation with artificial fodder with a pH value of 6.8; lane 6: ds-*GFP* incubation with artificial fodder with a pH value of 7.5; lane 7: ds-*GFP* incubation with artificial fodder with a pH value of 8.0.

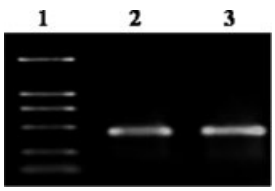

Figure 5. Effects of BPH suction on the stability of ds-*GFP* (0.5 μg). Lane 1: 2 kb ladder; lane 2: ds-*GFP* in 0.1 g mL^{-1} sucrose solution prepared with ddH_2O, not fed on by BPH nymphs; lane 3: ds-*GFP* in 0.1 g mL^{-1} sucrose solution prepared with ddH_2O, sucked by BPH nymphs for 24 h.

ddH_2O instead of artificial fodder was used as the medium with which dsRNAs were delivered into BPH guts.

3.2 Effects of BPH suction on dsRNA stability

The suction of BPH nymphs had little negative effect on the ds-*GFP* configuration. After being fed on by nymphs for 24 h, ds-*GFP*s remained stable. This indicated that, under these conditions, dsRNAs could be integrally delivered into BPH guts (Fig. 5).

3.3 Effects of ds1-*21E01*, ds2-*21E01* and ds3-*21E01* on BPH mortality

At a concentration of 0.05 μg μL^{-1} there was no significant difference in nymph mortality between BPHs fed on ds-*GFP* and ds1-*21E01* at the three kinetic points (Table 2). The same was true with ds2-*21E01* and ds3-*21E01* (Table 2). Unspecific mortality was controlled below 20%.

3.4 Silencing of *21E01* in BPH

The inhibition effect against the target gene caused by ds1-*21E01*, ds2-*21E01* and ds3-*21E01* was investigated by qRT-PCR (Fig. 6). The results showed that all three dsRNAs corresponding to different regions of *21E01* decreased the amount of endogenous *21E01* mRNA. However, the time required for significant downregulation of the target gene was different among the three dsRNAs. ds2-*21E01* and ds3-*21E01* exhibited significant suppression of the endogenous *21E01* mRNA as early as at 2 days of ingestion, the expression level of *21E01* decreased continuously at 6 days of ingestion and the maximum reduction in the transcript level of *21E01* occurred at 10 days of ingestion, while significant

Table 2. Mean ± SE of BPH nymph mortality fed on dsRNA at different kinetic points

	Mortality (%)[a]		
Ingested material	2 days	6 days	10 days
ds-*GFP*	1.11 ± 1.11 a	4.44 ± 1.11 a	13.33 ± 0.00 a
ds1-*21E01*	1.11 ± 1.11 a	5.56 ± 1.11 a	12.22 ± 1.11 a
ds2-*21E01*	4.44 ± 2.22 a	5.56 ± 1.11 a	13.33 ± 1.92 a
ds3-*21E01*	4.44 ± 2.22 a	6.67 ± 0.00 a	16.67 ± 1.92 a

[a] Within a column, means followed by different lower-case letters are significantly different at the $P < 0.05$ level (LSD test).

suppression of *21E01* triggered by ds1-*21E01* occurred as late as at 10 days of ingestion.

RNAi efficiency was found to differ among the three dsRNAs. The highest RNAi efficiency was triggered by ds2-*21E01*, followed by ds3-*21E01*, and then ds1-*21E01*. After exposure to ds2-*21E01* for 2, 6 and 10 days, the relative expression level of the target gene in BPH was reduced by 26, 48 and 55% respectively (Fig. 6B). After ingestion of ds3-*21E01* for 2, 6 and 10 days, the relative expression level of *21E01* in BPH was significantly decreased by 21, 38 and 48% respectively (Fig. 6C). There was no significant reduction in the relative expression level of *21E01* in BPH after feeding on ds1-*21E01* for 2 and 6 days ($P > 0.05$), a significantly lower relative expression level of *21E01* was observed in BPH until 10 days of ingestion of ds1-*21E01* ($P < 0.05$) and the expression level of the target gene was reduced by 41% (Fig. 6A).

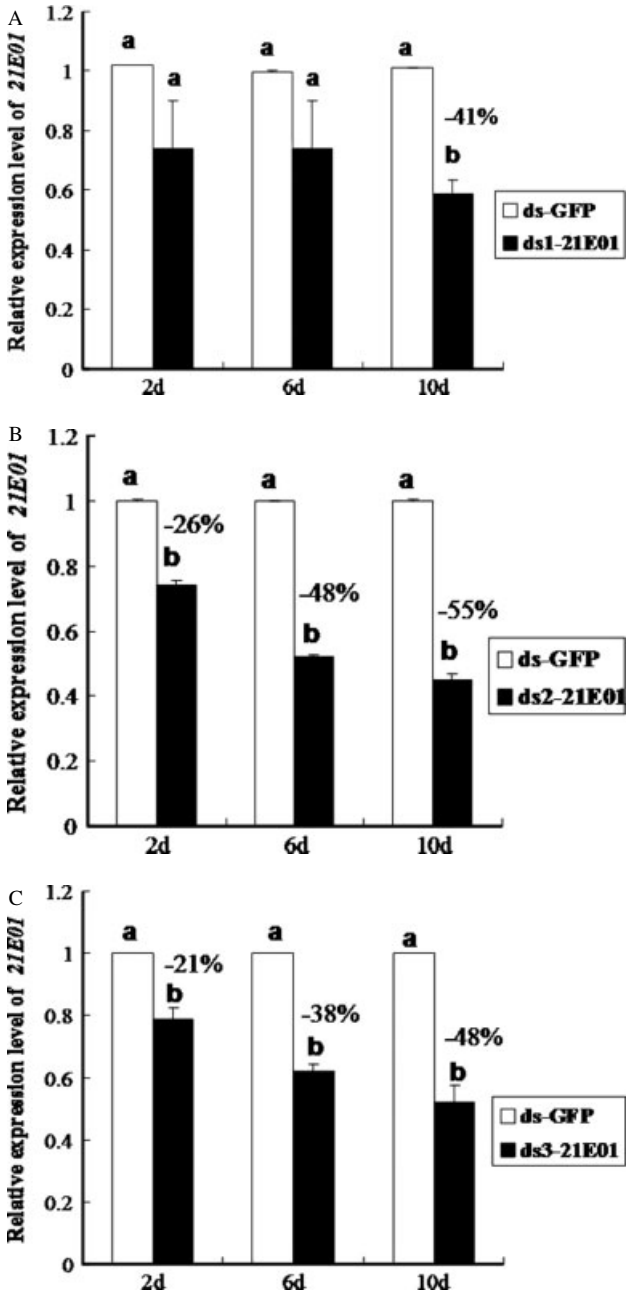

Figure 6. The relative expression levels of *21E01* in BPH after feeding on 0.05 μg μL^{-1} ds1-*21E01* (A), ds2-*21E01* (B) and ds3-*21E01* (C) for 2, 6 and 10 days. At each kinetic point, triple biological replications were performed for each treatment on 20–30 pooled BPH nymphs. BPH *actin1* was used as the internal control. The expression level of *21E01* in BPHs fed on ds1-*21E01*, ds2-*21E01* and ds3-*21E01* was quantified relative to that in BPHs fed on ds-*GFP*, according to the method of Livak and Schmittgen.[24] The error bars indicate the standard errors. Bars labelled with different letters differed significantly ($P < 0.05$).

4 DISCUSSION

A recent study reported that gene knockdown was achieved by introthoracic injection of dsRNA in *N. lugens*.[26] Here, it is demonstrated that delivery of long dsRNA into BPH intestines could also silence expression of a target gene. RNAi based on dsRNA ingestion was efficient and convenient, and represents a powerful tool for functional genomic studies of BPH.

To ensure that intact dsRNA could be orally delivered into BPH guts, the effects of medium solution and BPH suction on dsRNA stability were investigated in this study. In general, dsRNAs were reasonably unstable and easily degraded in artificial fodder, but showed good stability in DEPC-treated ddH_2O and 0.1 g mL^{-1} sucrose solution. The pH value of the artificial fodder could influence the stability of dsRNAs, but this was not the only factor resulting in instability of dsRNAs. Other factors such as certain ingredients or ions in the artificial fodder also affected the stability of dsRNAs. BPH suction had little negative effect on dsRNA stability; after being fed on by BPH nymphs for 24 h, the band of dsRNAs on agarose gel remained sharp. In the present experiments, BPHs were fed with 0.1 g mL^{-1} sucrose solution containing dsRNAs or artificial fodder alternatively at 24 h intervals, ensuring that dsRNA could be integrally delivered into BPH guts. This also minimised the malnourished effects of sucrose solution on BPHs. Through the use of these optimised measures, dsRNAs successfully induced the inhibition of target gene expression, and unspecific mortality was maintained below 20% in BPH nymphs fed on ds-*GFP*.

21E01 is a full-length cDNA encoding V-ATPase E of BPH. V-ATPase E like membrane transporter binding protein, locates gastrointestinal epithelial cells of insect and is the energy resource of the plasma membrane for alkaline and amino acids absorption in several insects.[27] Some reports have documented that ingestion of dsRNAs targeting V-ATPase E caused significant mortality in Coleopteran beetles, *Diabrotica undecimpunct* and *Leptinotarsa decemlineata*.[16] However, no significant mortality was observed in V-ATPase E gene-silenced BPHs. The absence of significant mortality needs to be studied further.

In general, the efficiency of RNAi techniques is affected by various factors, such as insect species, dosage and the dsRNA

sequence corresponding to the target gene. In the present study, the maximum depletion of the target gene in BPH was about 55%, which was lower than that previously reported in lepidopteran chewing insects.[28,29] In the present study, a difference in gene silencing efficiency was observed among the three dsRNAs corresponding to different parts of the CDS of the target gene, which was different from the results obtained with *Acyrthosiphon pisum*.[11] After 10 days of treatment, the highest RNAi efficiency was triggered by ds2-*21E01* (55%), which included 195 nucleotides at the 3′-end of CDS, plus 54 nucleotides at the 3′-end of the UTRs. The lowest RNAi efficiency was induced by ds1-*21E01* (41%), which corresponded to 276 nucleotides at the 5′-end of the *21E01* CDS. The RNAi efficiency of ds3-*21E01* (48%), which targeted 498 nucleotides at the 3′-end of the UTRs, lay between ds2-*21E01* and ds1-*21E01*. The time required for significant downregulation of the target gene caused by ds1-*21E01* was 10 days, longer than that of ds2-*21E01* and ds3-*21E01*. Considering the higher error bars of the gene expression level at 2 and 6 days in Fig. 6A compared with those in Figs 6B and 6C, it could be inferred that the lack of significant downregulation of the target gene by ds1-*21E01* was probably due to test error between biological replicates. It has previously been reported that, when dsRNA was synthesised and delivered orally at 1 $\mu g\ \mu L^{-1}$ into *A. pisum* and *Rhodnius prolixus*, the expression level of the target gene decreased in *A. pisum* by more than twofold within 24 h, and by 42% in *R. prolixus* within 48 h.[12,13] In the present study, the dosage of dsRNA was 0.05 $\mu g\ \mu L^{-1}$, which was lower than that used in *A. pisum* and *R. prolixus*. After feeding on dsRNA for 48 h, gene inhibition in BPH caused by ds2-*21E01* and ds3-*21E01* was 26 and 21% respectively, lower than that seen in *A. pisum* and *R. prolixus*. Whether the lower RNAi efficiency observed in this study was caused by the lower dosage of dsRNA needs to be studied further.

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