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# Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding

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## Abstract

**The migratory locust, *Locusta migratoria*, is one of the most destructive agricultural pests and has been widely used as a model system for insect physiology, neurobiology and behavioural research. In the present study, we investigated the effects of RNA interference (RNAi) using two delivery methods for double-stranded RNA (dsRNA) molecules, namely, injection and feeding, to develop a potential new pest control strategy. Our results showed that locusts have a sensitive and systemic response to the injection of dsRNAs in a dose-dependent manner, but do not respond to the feeding of dsRNAs. Further experiments suggested that the ineffectiveness of dsRNA feeding was attributable to the rapid degradation of dsRNA, which was probably induced by nuclease enzymes in the locust midgut. Moreover, we identified almost all the homologous genes involved in the endocytosis-mediated dsRNA uptake from the locust genome, which provided possible clues regarding the dsRNA uptake mechanisms from the intestine to the midgut epithelium. These findings reveal the differential response models of fourth instar locust nymphs to dsRNA delivery methods, contribute to the current understanding of insect RNAi mechanisms and provide important information for the further application of RNAi as a genetic tool and pest control strategy.**

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**Keywords: *Locusta migratoria*, gene silencing, pest management, RNAi, dsRNA.**

## Introduction

The migratory locust, *Locusta migratoria*, is an important agricultural pest because it feeds on several major grain crops, such as corn, sorghum, rice and wheat. Humans have been challenged by locust pest infestations since the earliest civilizations (Enserink, 2004). Chemical insecticides are widely used for the immediate control of locust outbreaks, but the heavy use of these insecticides has led to a series of environmental issues, such as the loss of biodiversity and environmental pollution (Lomer *et al.*, 2001), therefore, potential new strategies to control locust plagues need to be developed.

The locust has long been an important model for insect physiology, ecology, neurobiology and phenotypic plasticity (Pener & Simpson, 2009; Guo *et al.*, 2011; Ma *et al.*, 2011; Wu *et al.*, 2012), but advanced locust research at the molecular level is generally limited by the lack of advanced molecular resources (Simpson & Sword, 2008). Expressed sequence tags (ESTs) and transcriptome data for the migratory locust have recently become available; these datasets provide abundant sequence information (Kang *et al.*, 2004; Chen *et al.*, 2010). Novel genetic tools are now required to undertake the functional analysis of these genes.

RNA interference (RNAi) causes sequence-specific post-transcriptional gene silencing; this event is typically induced by double-stranded RNAs (dsRNAs) (Fire *et al.*, 1998) and short interfering RNAs (Elbashir *et al.*, 2001), or through the use of hairpin constructs in transgenic insects (Perrimon & Mathey-Prevot, 2007). Given its capacity to suppress genes in a sequence-specific manner, RNAi has been used in functional genomic studies (Arakane *et al.*, 2005; Cronin *et al.*, 2009) and may be a promising insect-specific insecticide to protect plants against insects (Baum *et al.*, 2007; Mao *et al.*,



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2007); however, many recent documents have shown that many factors will affect the efficiency of RNAi, such as gene targets and dsRNA delivery methods (Bellés, 2010; Terenius *et al*., 2011). For example, one study has reported that injection of 15 μg dsRNA reduced gene expression by 75 ± 14% and that feeding 13 μg dsRNA reduced gene expression by 42 ± 10% (Araujo *et al*., 2006). Previous studies have suggested that the injection of dsRNAs can induce gene silencing in the migratory locust (Guo *et al*., 2011; Ma *et al*., 2011; Wu *et al*., 2012), but the feasibility of oral delivery remains unknown in locusts. RNAi induced by oral delivery of dsRNAs is more convenient, less laborious, and may be an effective method for pest control. Some studies have suggested that the efficiencies of oral delivery of RNAi differ among species because of differences in the midgut environment (Huvenne & Smagghe, 2010); therefore, the factors that determine the efficiencies of orally delivered RNAi, such as the pH value, microbes and enzymes in the midgut, also need to be uncovered. Moreover, the systemic RNAi in locusts does not depend on the SID-1-mediated dsRNA transport pathway, which differs from the mechanism of systemic dsRNA uptake and spreading in *Caenorhabditis elegans* and some mammal cells (Luo *et al*., 2012). Alternative dsRNA uptake mechanisms, such as the endocytosis-mediated uptake pathway, have been proposed in other insects (Huvenne & Smagghe, 2010). The study of the possible mechanism(s) and factors that influence the effects of RNAi will help in optimizing its applications. In particular, the study of RNAi induced orally in locusts will provide the basis for developing dsRNA-transgenic crops to control locust plagues.

In the present study, we investigated the effects of RNAi on gene expression and insect phenotype through the injection and oral delivery of dsRNA targeting multiple genes, as well as the possible dsRNA uptake mechanisms involved. The results of this study will help realize the potential applications of RNAi in functional genetics and locust pest management.

## Results

### *RNA intereference induced by the injection of double-stranded RNA*

We first selected nine candidate genes encoding proteins with essential functions in the basic life activities of insects to investigate the RNAi effects in locusts (Table 1). These genes have different functions, including hormone regulation [*krüppel homologue 1* (*Kr-h1*); *Broad*], chitin metabolism [*vermiform* (Verm); *chitin synthase 1* (*CHS1*)], energy metabolism (*V-ATPase subunit A* and *E, V-ATPase A* and *E*), neuromodulation [*G-protein-coupled receptor* (*GPCR*)] and cell structure (*α*- and *β-tublin*). The genes *Kr-h1*, *CHS1* and *α-tublin* are widely expressed whereas the others exhibit tissue-specific expression patterns (Table 1).

After injection of a high dose of dsRNA (18 μg) to the fourth instar nymph locusts, we found that the expression of seven target genes was reduced, as compared with the green fluorescent protein (GFP)-injection used as a control, but expression of *GPCR* and *β-tubulin* was not reduced (Student's *t*-test, $P < 0.05$; Fig. 1A, *top*, and C, *left*). Mortality was significantly increased by the silencing of those seven genes (Student's *t*-test, $P < 0.05$; Fig. 1C, *right*). The injection of dsRNAs targeting *Verm, CHS1* and *Kr-h1* genes led to the abnormal development of hind limbs (78.7%; Fig. 1B, *top*) and an incomplete, thin pronotum and soft head (93.4%; Fig 1B, *middle*), with averted wings after moulting (57%; Fig. 1B, *bottom*), respectively.

### *Dose–response relationships of RNA interference after double-stranded RNA injection*

To further investigate the dose-dependent effects of RNAi, we injected different doses of dsRNAs targeting the five genes that were significantly silenced in the aforementioned experiments, including *Kr-h1*, *Verm*, *CHS1*, as well as the *V-ATPase A* and *E* genes. A negative correlation between the dsRNA dose and the mRNA level was generally observed. A clear dose-dependent relationship was observed for *Kr-h1, CHS1* and *V-ATPase E* genes

**Table 1.** List of target genes for the RNA interference experiments in *Locusta migratoria*

| Gene ID | Annotation | Double-stranded RNA length | Location |
|---|---|---|---|
| JN676089 | krüppel homologue 1 | 347 | Broadly expressed |
| JN676094 | Broad, isoform A | 401 | Highly expressed in OV and T |
| JN676088 | vermiform | 366 | Not expressed in M |
| JN676090 | chitin synthase 1 | 597 | Broadly expressed |
| JN676096 | V-ATPase subunit A | 473 | Highly expressed in M |
| JN676085 | V-ATPase subunit E | 439 | Highly expressed in M |
| JN676092 | G protein coupled receptor | 383 | Highly expressed in B |
| JN676086 | β-tubulin | 365 | Highly expressed in M, FB, B |
| JN676091 | α-Tubulin | 340 | Broadly expressed |

B, brain; M, midgut; OV, ovary; T, testis; FB, fat body. The agarose gel electrophoresis was used for gene's location analysis (data not shown).

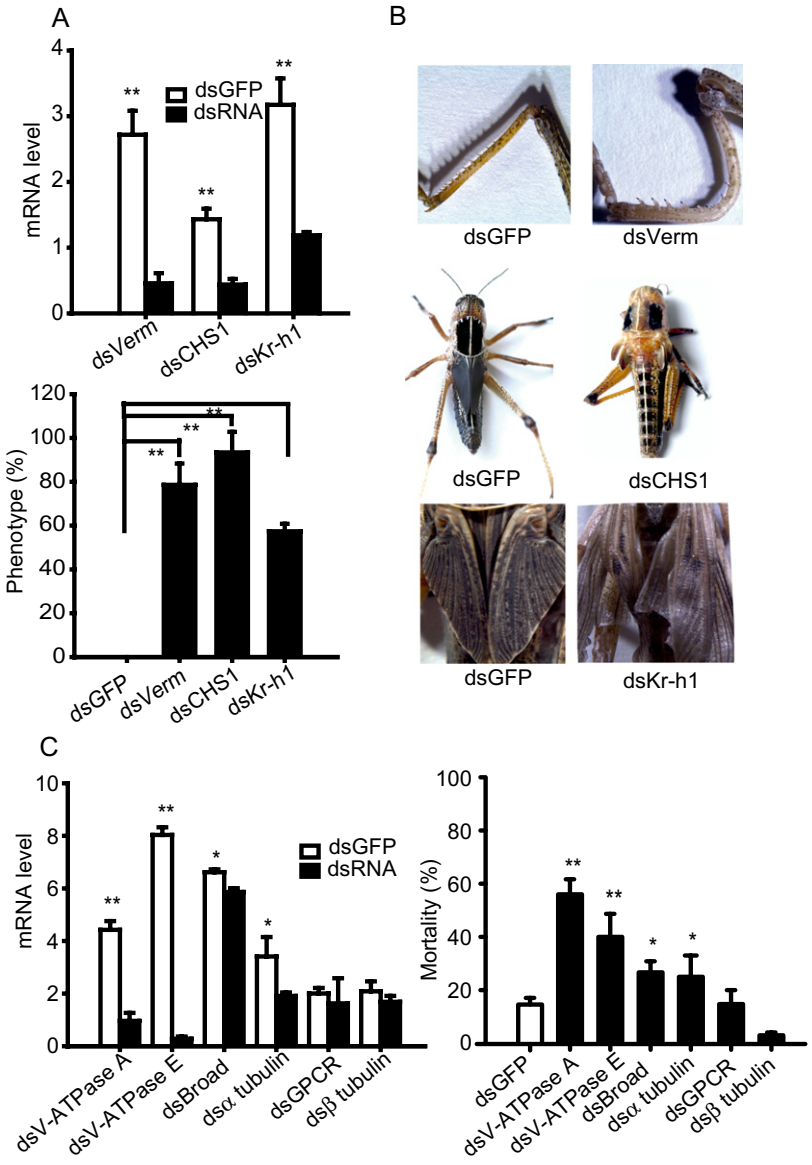


**Figure 1.** Effects of RNA interference on mRNA levels and phenotypes of fourth instar nymphs of *Locusta migratoria*, as induced by the injection of 18 μg double-stranded (ds)RNAs. (A) mRNA levels of three genes, *Verm*, *CHS1* and *Kr-h1*, after the injection of respective dsRNAs (*top*), and the percentage of animals with defects (*bottom*). (B) Specific phenotype after injection of ds*Verm* (*top*), ds*CHS1* (*middle*), and ds*Kr-h1* (*bottom*). (C) mRNA levels (*left*) and mortality (*right*) of locusts injected with the other six dsRNAs, targeting the following genes: *Broad*, *V-ATPase A*, *V-ATPase E*, *GPCR*, and *α*- and *β-tublin*. Each gene contained five replicates, and 15 nymphs were included in each replicate. Statistical analysis was performed using Student *t*-test (mean ± SE; *, $P < 0.05$; **, $P < 0.01$).

(Fig. 2A), whereas the RNAi effects for *Verm* and *V-ATPase A* decreased after they reached the maximum (Fig. 2B). Less than 1 μg of dsRNA induced significant RNAi effects for most of the genes (Student's *t*-test, $P < 0.05$; Fig. 2).

### *RNA interference elicited by double-stranded RNA oral delivery*

The two midgut-specific genes (*V-ATPase A*/*E*) and three broadly expressed genes (*CHS1*, *Kr-h1*, *Verm*) were significantly suppressed by dsRNA injection in the above experiment. These five genes were selected to test the RNAi effects of oral dsRNA delivery. After continuous feeding of dsRNA for 8 days, no significant RNAi effects were observed at the mRNA level and the mortality for all five genes was similar to those of dsGFP-feeding controls (Student's *t*-test, $P > 0.05$; Fig. 3A, and B). A separate group of locusts was fed with dsRNA encapsulated into liposomes, which would facilitate dsRNA uptake from the gut lumen in *Drosophila* (Whyard *et al.*, 2009); however, this treatment did

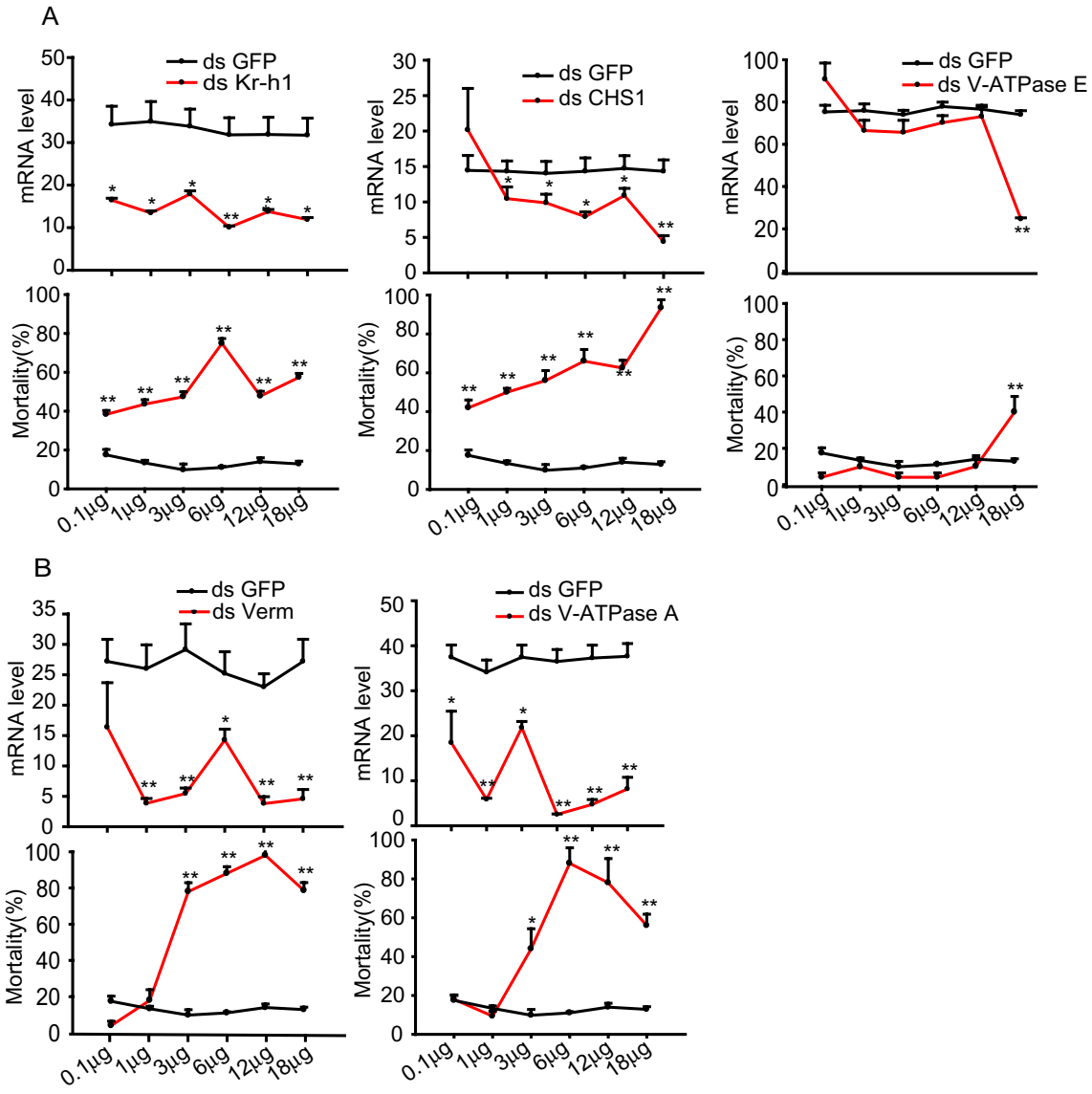


**Figure 2.** Dose–response relationships of RNA interference in the fourth instar nymphs of *Locusta migratoria*, as induced by the injection of various doses of double-stranded (ds)RNAs. (A) mRNA levels and mortality after the injection of various doses of dsRNAs targeting *Kr-h1*, *CHS1* and *V-ATPase E* genes. (B) mRNA levels and mortality after the injection of various doses of dsRNAs targeting *Verm* and *V-ATPase A* genes. Each dosage contained five replicates, and 15 nymphs were included in each replicate. Statistical analysis was performed with Student's *t*-test (mean ± SE; *, $P < 0.05$; **, $P < 0.01$).

not improve the RNAi efficiency (ANOVA, $P > 0.05$; Fig. 3C, D).

We examined the following reasons that could explain why the feeding of dsRNA did not induce RNAi in locusts: the rapid degradation of dsRNA in the locust midgut and the lack of dsRNA uptake and spreading mechanisms in the epithelial cells of the locust midgut. First, we found that dsRNA was quickly degraded after incubation in fresh locust midgut fluid (pH = 6.8, Fig. 4A). To test the influence of pH values on the degradation of dsRNA in the midgut, we modified the pH values of the locust midgut fluid to different pH values. The results show that dsRNA was quickly degraded in the locust midgut fluid, except in extremely acidic (pH = 4.2, 5.5) and alkaline (pH = 10.7) environments (Fig. 4A); however, the dsRNA remained intact after incubation in freshly collected midgut fluid from the Lepidopteran insect, *Spodoptera exigua* (pH = 8.8). *S. exigua* has been reported to be sensitive to dsRNA feeding (Tian *et al.*, 2009). When the pH value of the *S. exigua* gut fluid was adjusted so that it was similar to the locusts' (pH = 6.8), the dsRNA remained intact (Fig. 4B). The encapsulation by liposome did not protect the dsRNA from degradation by the locust gut fluid (Fig. 4B).

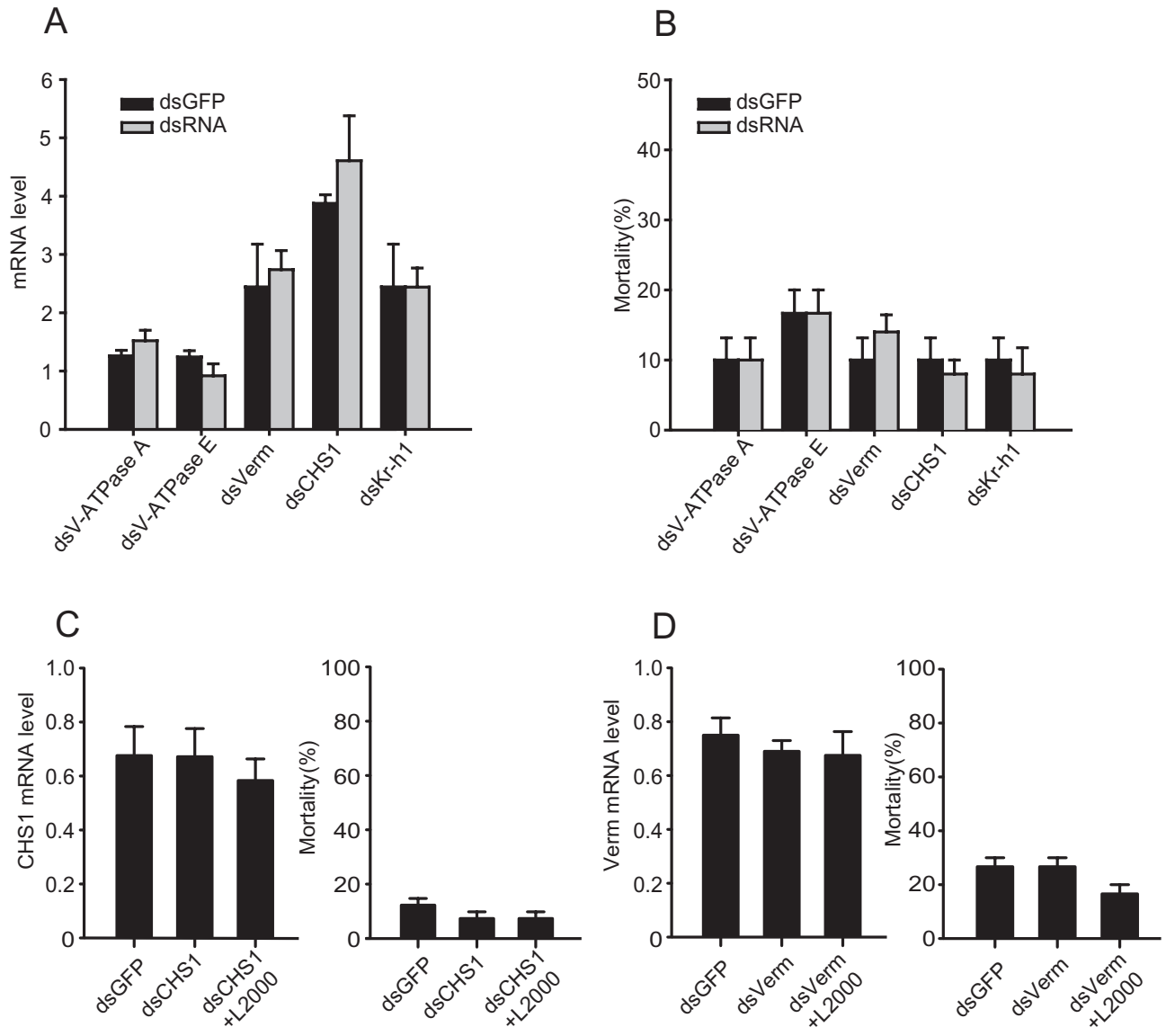


**Figure 3.** RNA interference effects induced by double-stranded (ds)RNAs and L2000-encapsulated dsRNAs via oral delivery in the fourth instar nymphs of *Locusta migratoria*. (A) mRNA levels of *V-ATPase A*, *V-ATPase E*, *Verm*, *CHS1* and *Kr-h1* genes after oral administration of dsRNAs. (B) Nymph mortality after feeding with dsRNAs targeting *V-ATPase A*, *V-ATPase E*, *Verm*, *CHS1* and *Kr-h1*. (C) mRNA levels of *CHS1* gene and nymph mortality after the feeding of green fluorescent protein (*GFP*)-*dsRNA*, *CHS1*-dsRNA and L2000-encapsulated *CHS1*-dsRNA. (D) mRNA levels of *Verm* gene and nymph mortality after feeding with *GFP*-dsRNA, *Verm*-dsRNA, and L2000-encapsulated *Verm*-dsRNA, using GFP-dsRNA as the control. Each treatment contained four replicates, and 15 nymphs were included in each replicate. Statistical analysis was performed with Student's *t*-test and one-way ANOVA.

We speculated that the nuclease enzymes in the locust midgut fluid led to the degradation of dsRNA, or that nuclease enzyme inhibitors in the *S. exigua* gut fluid protect against the degradation of dsRNA. To test these hypotheses, we heated the locust midgut fluid to 80 °C for 10 min to inactivate the potential nuclease enzymes. We found that this treatment significantly reduced the degradation of dsRNAs (Fig. 4C). In addition, our results suggested that the mixture of the fresh midgut fluid from the locust (pH = 6.8) and that of *S. exigua* (pH = 8.8) induced the degradation of dsRNA through a dilution effect rather than through the existence of inhibitors in the *S. exigua* midgut fluid (Fig. 4D).

Meanwhile, to test the hypothesis of the lack of dsRNA uptake and spreading mechanisms in epithelial cells in the locust midgut, we screened for homologous genes related to the endocytosis-mediated uptake mechanism in the whole genome sequences of *L. migratoria* [based on unpublished *L.migratoria* genome data from the laboratory of one of the present authors (L.K.)]. Homologues for

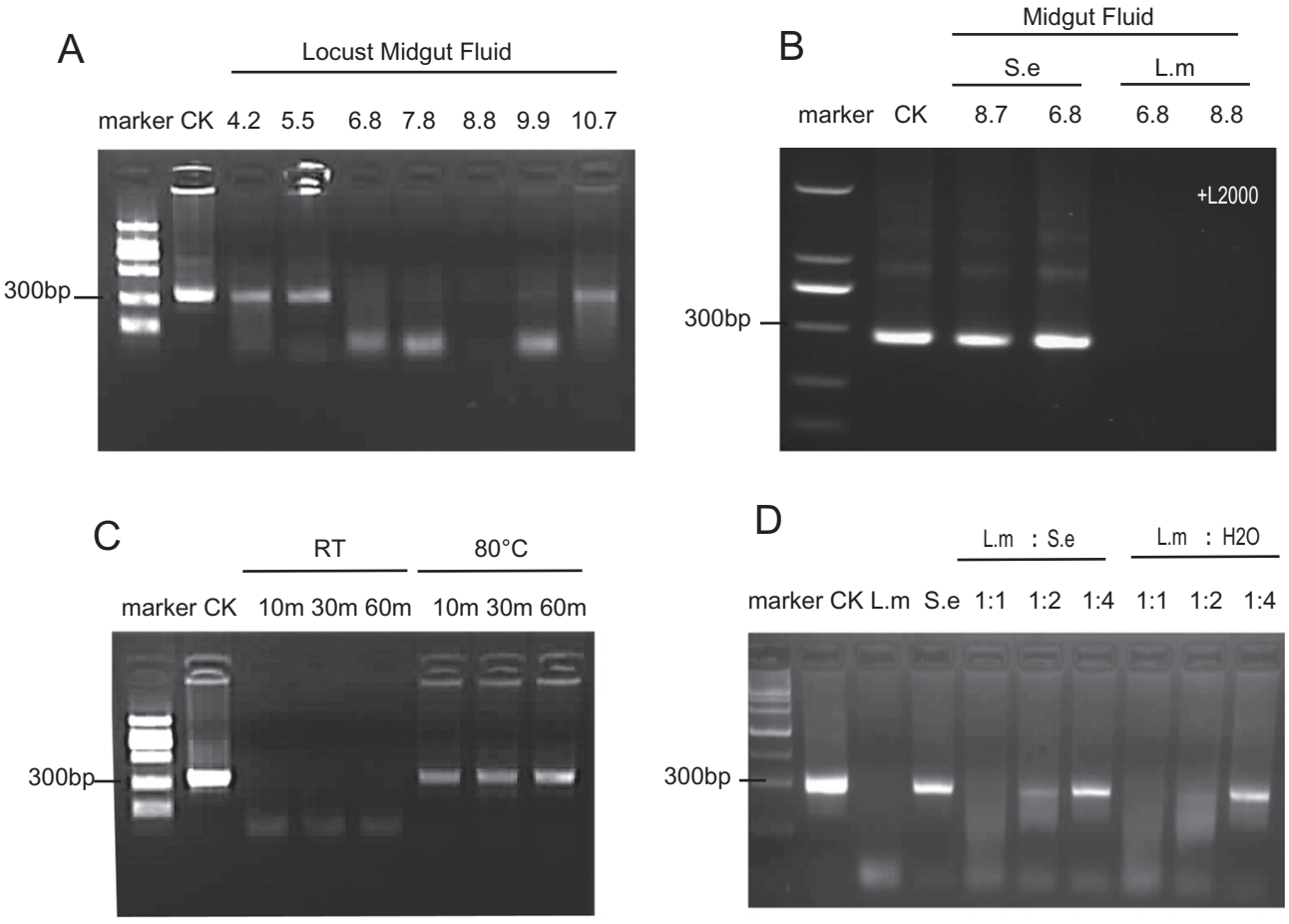


**Figure 4.** Incubation of double-stranded (ds)RNAs in midgut fluids of different species. (A) dsRNA in fresh midgut fluid from *Locusta migratoria* at various pH values for 10 min. (B) dsRNAs and L2000-encapsulated *Verm*-dsRNA in fresh fluid from *L. migratoria* midgut (pH = 6.8), and dsRNAs in *Spodoptera exigua* midgut (pH = 8.7) or at pH = 6.8 for 10 min. (C) dsRNAs in the 80 °C heated inactivated *L. migratoria* midgut fluid for 10, 30 and 60 min. (D) Incubation of dsRNAs in the mixed gut fluids of *L. migratoria* and *S. exigua* at ratios of 1:1, 1:2 and 1:4 for 10 min and in the mixed gut fluids of *L. migratoria* with nuclease-free water at 1:1, 1:2, and 1:4 for 10 min. CK: incubation of dsRNAs in nuclease-free $H_2O$. L.m: *L. migratoria*; S.e: *S. exigua*. RT: room temperature.

almost all genes involved in dsRNA uptake in *Drosophila* (Saleh *et al*., 2006) were identified in the locust genome (Table 2). Most of these genes had a clear one-to-one orthology; several genes had more than one copy in the locust genome.

## Discussion

### *Factors influencing RNA interference efficiency of double-stranded RNA injection*

Our results suggest that the RNAi effects induced by dsRNA injection are influenced by several factors, including the functions and tissue-specific expression patterns of target genes, as well as the dosage of dsRNA that was used. Numerous studies showed that the effect of RNAi was influenced by the original functions of the target genes (Terenius *et al*., 2011). The genes with essential functions were more vulnerable to silencing because a slight decrease in the expression of these genes led to more serious consequences, thereby allowing the easy detection of their silencing effects. Two genes that were highly expressed in the neural system were difficult to knockdown (Fig. 1, Table 1). This result is consistent with the observations from *C. elegans*. For example, in *C. elegans*, the genes expressed in the neural cells and gonads were difficult to knockdown (Kennedy *et al*., 2004). Meanwhile, the different tissue-specific expression levels of the core machinery for RNAi, such as the cleaving enzymes Dicer and Argonaute, intrinsically influence the RNAi potency (Bellés, 2010). Furthermore, we found that the expression level of the target genes affected the efficiency of RNAi. For instance, both *V-ATPase A* and *E* genes are expressed in the midgut, although *V-ATPase A* was more sensitive to RNAi than *V-ATPase E* (Fig. 2). The high expression levels of the target genes require more time and/or more dsRNA to achieve efficient gene knockdown (Miller *et al*., 2008).

**Table 2.** List of locust homologues of the candidate genes of *Drosophila melanogaster* (Saleh *et al.*, 2006) involved in endocytosis-mediated double-stranded (ds)RNA uptake

| Gene name | Dm gene ID | Lm gene ID | biological fuction |
|---|---|---|---|
| Saposin r | CG12070 | LM10157264 | Lipid metabolism |
| VhaSFD | CG17332 | LM10177851,LM10177861 | ATP synthase/ATPase |
| Light | CG18028 | LM10138702 | Lysosomal transport |
| Vha16 | CG3161 | LM10165536,LM10099526 | ATP synthase/ATPase |
| Sr-CI | CG4099 | LM10169967 | Inate immune response/phagocytosis |
| Sr-CII | CG8856 | LM10169967 | Inate immune response/phagocytosis |
| Sr-CIII | CG31962 | LM10169967 | Inate immune response/phagocytosis |
| Sr-CIV | CG3212 | LM10169967 | Inate immune response/phagocytosis |
| P13K59F | CG5373 | LM10082784,LM10063961 | Lipid metabolism |
| Nina C | CG54125 | LM10173500,LM10136778 | Rhodopsin mediated signaling |
| Rab 7 | CG5915 | LM10140018 | Endosome transport |
| Arf72A | CG6025 | LM10182089 | Endosome transport |
| AP 50 | CG7057 | LM10009504 | Endocytosis |
| Egghead | CG9659 | LM10117037 | Oogenesis |
| Clathrin hc | CG9012 | LM10043824 | Endocytosis |
| IdICP | CG6177 | LM10107265 | Endocytosis |
| Eater | CG6124 | | Inate immune response/phagocytosis |
| Gmer | CG3495 | | Metabolism |
| | CG5434 | LM10143541 | Translation regulation |
| | CG8773 | LM10153647,LM10165874,LM10106983 | Peptidase |
| | CG5053 | LM10145665 | Signal transduction |
| | CG8184 | LM10181957,LM10181959 | Ubiquitin ligase |
| | CG5382 | | Zinc finger transcription factor |
| | CG8671 | LM10123833 | Unknown |
| | CG3248 | LM10139862 | Unknown |
| | CG3911 | LM10065251 | Unknown |
| | CG4572 | LM10163418 | Peptidase |
| | CG5161 | LM10090167,LM10005643 | Unknown |

Dm, Drosophila melanogaster; Lm, Locusta migratoria.

The dose of injected dsRNA likewise affects the RNAi effects. Our results showed evident dose–response relationship patterns, which are consistent with those reported in other RNAi studies (Saleh *et al.*, 2006; Baum *et al.*, 2007; Terenius *et al.*, 2011). Moreover, we found that the RNAi effects were again decreased after reaching the maximum in some cases (Fig. 2B). Although the accurate mechanisms still need to be identified, this phenomenon indicated that the dose of dsRNA needs to be optimized to achieve the highest potential and to avoid off-target effects in practical applications. Previous studies have suggested that the range of the amounts of dsRNA injected to induce RNAi varied between 1 μg and 100 μg; the dose–response relationship was rarely reported in lower concentrations (Terenius *et al.*, 2011). Our study used lower doses of dsRNAs to establish the dose–response relationship; this method may be beneficial because the maximum effects can be achieved with fewer off-target effects (Ma *et al.*, 2006).

### *Possible reasons for the failure of RNA interference by oral double-stranded RNA delivery*

Our results suggest that RNAi by oral dsRNA delivery did not work in locusts, although successful RNAi by dsRNA feeding has been reported in Coleoptera, Hymenptera, Hemiptera, Isoptera, Lipedoptera and Orthoptera species (Huvenne & Smagghe, 2010). In the most successful cases of RNAi by oral dsRNA delivery, the application of high doses of dsRNA has been reported to be effective for improving the dsRNA uptake in the midgut of insects (Whyard *et al.*, 2009; Rodríguez–Cabrera *et al.*, 2010); however, in the present study, feeding dsRNA did not initiate RNAi, even when several extra strategies were used, such as starvation before feeding, encapsulation of dsRNA by liposomes and long-term high doses of dsRNA feeding (Figs 3, 4A, B). Recently, several studies have shown that the RNAi efficiencies induced by different delivery methods of dsRNA are species-specific in insects. For example, the injection of dsRNA can induce highly efficient RNAi silencing in *Spodoptera frugiperda*, whereas the feeding of dsRNA in other lepidopteran species, such as *Ostrinia nubilalis* and *Manduca sexta*, is more effective (Terenius *et al.*, 2011).

A successful RNAi induced by oral dsRNA delivery usually requires the following steps. First, a sufficient amount of intact dsRNAs is taken up by the intestinal cells. The dsRNAs are then exported and transmitted from the intestinal cells to other tissues (e.g. neurons, fat bodies and germline cells). Finally, the target gene is suppressed via the cell autonomous RNAi machinery (Whangbo & Hunter, 2008). The last two steps should exist in locusts in

support of the sensitive responses of midgut-specific expressed genes (e.g. the V-ATPase subunit-encoding genes) to RNAi triggered by dsRNA injection; thus, the rapid degradation of dsRNAs in the gut lumen or the lack of the machinery for activating the uptake of intact dsRNA from the intestine to the midgut epithelium are possible reasons for the failure of RNAi induced by dsRNA oral delivery. The first possibility is supported by our observation from the incubation of dsRNA in fresh midgut digestion fluid. The dsRNA was quickly degraded after incubation in fresh midgut fluid from the locust, but not from *S. exigua*, which was reported to be sensitive to dsRNA feeding (Fig. 4A, B). Furthermore, our results suggested that the dsRNA-degrading nuclease (dsRNase) may be the main reason for the different capabilities in dsRNA degradation between these two insect species, rather than the changes in the pH of the gut (Fig. 4C). dsRNase has been proven to be specific for dsRNA degradation in the *Bombyx mori* midgut (Arimatsu *et al.*, 2007). Additional evidence is likewise provided by the degradation of dsRNAs when treated with a mixture of the midgut fluid from *S. exigua* and *L. migratoria* (Fig. 4D).

We also investigated whether or not the midgut epithelium of locusts lacked the required machinery for the active uptake of intact dsRNA from the intestine. Based on current knowledge, two mechanisms for dsRNA uptake have been proposed; namely, the transmembrane channel-mediated uptake mechanism (Winston *et al.*, 2002; Feinberg & Hunter, 2003) and the endocytosis-mediated uptake mechanism (Saleh *et al.*, 2006). The evidence from our previous study (Luo *et al.*, 2012) had refuted the transmembrane channel-based uptake mechanism mediated by SID-1 proteins and proposed the possible vital roles of the endocytosis-mediated uptake mechanism in the dsRNA uptake from the environment in locusts. Endocytosis in *D. melanogaster* involves a number of genes, such as scavenger receptors, vesicle-mediated transport, the conserved oligomeric Golgi complex family, cytoskeleton organization and protein transport (Saleh *et al.*, 2006). We found that the locust genome possesses homologues of almost all the *D. melanogaster* genes involved in endocytosis-mediated uptake process (Table 2), thereby further supporting our hypothesis. Further functional analysis of these genes and the development of cell lines from the locust midgut microvillar epithelial cells would be helpful in answering the fundamental questions on dsRNA uptake mechanisms.

## Conclusion

The results described in the present paper show that RNAi elicited by dsRNA injection is a useful tool for studying gene function in migratory locusts because of its extremely sensitive response to systemic RNAi in most tissues; however, RNAi by oral dsRNA delivery does not work in locusts because of the rapid degradation of dsRNA in the midgut, thereby making it difficult to develop RNAi-based locust-insecticides or to exploit transgenic plants engineered to express dsRNA against the outbreaks of locust plagues. Possible genetic and technical methods to protect dsRNA from degradation should be considered for the successful application of RNAi through feeding in locusts, such as in the remodelling of dsRNA structures or the use of dsRNA nuclease inhibitors. Our findings provide very useful information for the potential application of the RNAi approach in genetic manipulation and pest control.

## Experimental procedures

### *Locusts*

The locusts used in the experiments originated from migratory locust (*L. migrato*ria) colonies maintained in the Institute of Zoology, Chinese Academy of Sciences. Locust nymphs were cultured in boxes (25 cm × 25 cm × 25 cm) at population densities of 200–300 insects per cage. This colony was reared under 14 h light/10 h dark conditions at 30 ± 2 °C. Fresh wheat seedlings and wheat bran were used to feed the locusts.

### *cDNA cloning of the target genes for RNA interference screening*

We obtained the cDNA sequences of the target genes using a BLAST search against the EST library (Kang *et al.*, 2004; Ma *et al.*, 2006) and the whole-genome database of *L. migratoria* (unpublished data). Specific primers were designed to obtain the fragment of the target genes by PCR. The following primers were used (F, forward primer; R, reverse primer): *Kr-h1*F: 5′-TCG CCTTCCAGTGGTCCTT-3′; R: 5′-CGAGTGGCTCTTGATGTG-3′; *Broad*F: 5′-CTCTGACTTGACGCCATCTATC-3′; R: 5′-CACC AGTACATTCGACACCC-3′; *Verm*F: 5′-AAGCAAATCAGTTGT CCCTCCG-3′; R: 5′-TGGCACCTGGTTGGGTTCTAT-3′; *CHS1*F: 5′-ATGGAGGACGTCTTGTTTGG-3′; R: 5′-TTCAGCCTTTGA GTCCAT-3′; *V-ATPase A*F: 5′-GAAGCTGCTCAAAGTCTGC-3′; R: 5′-CATCAACTGGCTCATCTCGT-3′; *V-ATPase E*F: 5′-TTATG GAATTGCTGGTTT-3′; R: 5′-TTGCTTGAGATTACTCATCTTTA-3′; *GPCR*F: 5′-GGTGAACTGCCAGGGTGA-3′; R: 5′-CTTGA GCTGCTGCCATTA-3′; *β-tublin*F: 5′-AAGCCAGGCATGAAGAA GTG-3′; R: 5′-AAGAATACCCAGACCGCATC-3′; *α-tublin*F: 5′-ACTGGTTCAGGCTTTACTTCA-3′; R: 5′-TGGATACGAGG GTAGGGA-3′. The obtained cDNA for using as the template was prepared from whole-body or specific tissues that were harvested from fourth instar nymphs. The total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was reverse-transcribed from 2 μg of total RNA using MMLV reverse transcriptase (Promega, Madison, WI, USA).

### *RNA interference treatment by double-stranded RNA injection*

The dsRNA was generated by *in vitro* transcription using the T7 RiboMAX system (Promega) according to the manufacturer's

protocol. Templates for *in vitro* transcription reactions were prepared by PCR amplification from plasmid DNA of the cDNA clone of target genes using the primer pairs with T7 polymerase promoter sequence at 5′-end. For primary screening, 2 μl of dsRNAs (9 μg/μl) for the target genes or GFP controls were injected into the ventral part of the second abdomen segment of the fourth instar nymphs. For each gene, 75 nymphs were injected and divided into five groups. The effects of RNAi on the mRNA levels were investigated by quantitative real-time (qRT)-PCR at 72 h after injection. To monitor the transcript levels of the target genes, total RNA was extracted from whole bodies for broadly expressed genes and from individual tissues for tissue-specific expressed genes. For each target gene, three individuals from each group were used for RNA extraction. The phenotypes/mortality were observed until the nymphs moulted into adults for the remaining nymphs. For the dose–response tests, a series of dosages, 100 ng, as well as 1, 3, 6, 12 and 18 μg were used. For each dose, 2 μl of dsRNA was injected as described above. Each treatment included 75 nymphs that were injected and divided into five groups. The effect of RNAi on the mRNA levels and phenotypes or mortality was monitored as described above.

*RNA interference treatment by double-stranded RNA feeding*

Freshly moulted fourth instar nymphs of *L. migrato*ria were starved for 24 h before feeding. The dsRNA was diluted to 1 ml in RNase-free water, and then mixed with 0.5 g wheat bran to feed 60 nymphs in the morning. After the bran was consumed (within 1 h), fresh wheat seedlings were added in the afternoon. For each gene, locust nymphs were continuously fed with dsRNA for 8 days (6 μg/individual/day). GFP-dsRNA was used as the control. Each treatment contained four replicates, and 15 nymphs were included in each replicate. The Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA) was used to encapsulate dsRNA before feeding according to the manufacturers' instructions. The effects of RNAi on the mRNA levels were investigated by qRT-PCR at 72 h after feeding, and the phenotypes and mortality were examined until the locusts moulted into adults.

*Incubation of double-stranded RNA in the fresh midgut fluid*

The excrement from five individual midguts of the fourth instar locust nymphs was collected and placed into one centrifuge tube. The tissues were ground using a glass rod and were diluted in 1 ml of nuclease-free $H_2O$. After centrifugation at 10,000 x g for 30 min, the supernatant was collected and used for future experiments. To test whether dsRNA was degraded in the locust midgut and to verify the effects of the pH of the digestive fluids on the degradation of dsRNA, fresh digestive fluids from the midgut were adjusted to different pH values. To adjust the pH, 1 ml of the supernatant was collected and mixed with 2 ml of nuclease-free $H_2O$; the pH value was then tested and adjusted using HCl or NaOH. Approximately 3 μg of dsGFP was incubated for 10 min in 20 μl of the digestive fluid with the respective pH. The dsRNA was incubated in nuclease-free $H_2O$ as control. To reveal whether there were dsRNases in the locust midgut leading to the degradation of dsRNA, fresh digestive fluid from the locust midgut was heated to 80 °C for 10 min to inactivate dsRNases. Then 3 μg of dsGFP was incubated in 20 μl inactivated digestive fluids for 10, 30 and 60 min. The dsRNA was likewise incubated in locust midgut fluids at room temperature for 10, 30 and 60 min as controls. To determine whether dsRNase inhibitors exist in the midgut digestive fluid of *S. exigua*, we collected the midgut digestive fluid from *S. exigua* using the same method as described above. Midgut fluids from *L. migratoria* were mixed with digestive fluids from *S. exigua* or with nuclease-free water at ratios of 1:1, 1:2, and 1:4 the mixtures were then used to incubate dsGFP for 10 min. The integrity of dsRNA was tested by agarose gel electrophoresis.

*Quantitative PCR*

The same methods of RNA extraction and reverse-transcription were used as described above. To quantify the gene expression, a standard curve method (Larionov *et al*., 2005) was used, with β-actin as the positive control and for normalizing the data. The following primers were used for q-PCR (F, forward primer; R, reverse primer): *Kr-h1*F: 5′-CCCCGGTGGCTCTTGATG-3′; R: 5′-GGCAAGTCGTTCGGCTACAA-3′; *Broad*F: 5′-GCGACGGTTTACCAGAAGG-3′; R: 5′-CGGGCGATCAATCACAGA-3′; *Verm*F: 5′-TACTGGACCCAGGGCTCCTAC-3′; R: 5′-CCACCGACACGCAAATACG-3′; *CHS1*F: 5′-ATGGAGGACGTCTTGTTTGG-3′; R: 5′-ATCACTGCTTTTCGGTCCAC-3′; *V-ATPase A*F: 5′-CAGTTTGTCCCACTGCGTA-3′; R: 5′-CTAATAGTTTGGCAACCTCA-3′; *V-ATPase E*F: 5′-AAGCCTTCTTGAACCCAG-3′; R: 5′-TGCGATTTGGGTTACGAC-3′; *GPCR*F: 5′-GCCTTCGCACAAGTCAAA-3′; R:5′-CTGGGAACCTGAACAGAAGC-3′; *β-tublin*F: 5′-GGCTTTCCTTCACTGGTA-3′; R: 5′-TCATCAAACTCGGCATCT-3′; *α-tublin*F: 5′-AATAAACTACCAGCCTCCTACT-3′; R:5′-CTTGGCATACATCAAATCG-3′; PCR amplification was conducted using Roche 480 spectrofluorometric thermal cycler and RealMaster-Mix (SYBR Green) kit (Tiangen), with a 2 min initial denaturation at 95 °C, followed by 40 cycles of 95 °C, 20 s; 58 °C, 20 s; 68 °C, 20 s. Melting curve analysis was performed to confirm the specificity of amplification.

*Statistical analysis*

The differences between treatments were compared either by Student's *t*-test or by one-way ANOVA followed by a Tukey's test for multiple comparisons. Differences were considered significant at $P < 0.05$. Values were reported as mean ± SE. Data were analysed using the SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA).

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