A nuclease specific to lepidopteran insects suppresses RNAi

Ruo-Bing Guan1, Hai-Chao Li1, Yu-Jie Fan1, Shao-Ru Hu2,3, Olivier Christiaens4, Guy Smagghe2,5, and Xue-Xia Miao1,3

From the 1Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China, the 2University of the Chinese Academy of Sciences, Beijing 100049, China, and the 3Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium

More than 70% of all agricultural pests are insects in the order Lepidoptera, which, unlike other related insect orders, are not very sensitive to RNAi, limiting genetic studies of this insect group. However, the reason for this distinct lepidopteran characteristic is unknown. Previously, using transcriptome analysis of the Asian corn borer Ostrinia furnacalis, we identified a gene, termed up56, that is up-regulated in response to dsRNA. Here we report that this Lepidoptera-specific gene encodes a nuclease that contributes to RNAi insensitivity in this insect order. Its identity was experimentally validated, and sequence analysis indicated that up56 encodes a previously uncharacterized protein with homologous sequences in seven other lepidopteran species. Its computationally predicted three-dimensional structure revealed a high structural similarity to human exonuclease I. Exposure to dsRNA in O. furnacalis strongly up-regulated this gene’s expression, and the protein could digest single-stranded RNA (ssRNA), dsRNA, and dsDNA both in vitro and in vivo. Of note, we found that this up-regulation of up56 expression is faster than that of the gene encoding the key RNAi-associated nuclease Dicer. up56 knockdown in O. furnacalis significantly enhanced RNAi efficiency. Moreover, up56 overexpression in Drosophila melanogaster suppressed RNAi efficiency. Finally, up56 knockdown significantly increased the amount and diversity of small RNAs. Therefore, we renamed this protein RNAi efficiency–related nuclease (REase). In conclusion, we propose that REase may explain why lepidopterans are refractory to RNAi and that it represents a target for further research of RNAi efficiency in this insect order.

During the past decade, many studies have proven that RNAi technology can be used in pest control by dsRNA spraying or feeding or via transgenic plants (1–4). However, RNAi efficiency is the most important restriction factor of this technology, especially in lepidopteran insects, because many studies have shown that RNAi efficiency is relatively low in this insect order (5, 6). In contrast, many Orthoptera, Coleoptera, and Hemiptera seem to be more sensitive to RNAi, even though a certain degree of variability exists in these orders as well (2, 7, 8). This study attempts to disclose the reason why such a difference in RNAi efficiency is observed among different insect orders.

It is well established that the RNAi pathway is a natural antiviral immunity system (9). Viral dsRNA is recognized as a pathogen-associated molecular pattern and processed into siRNAs by the Dicer enzyme, after which the siRNA can enter the subsequent RNAi pathway and cause the gene-silencing effect (10–12). RNAi technology has been used as a genetic tool for investigating gene function, particularly in nonmodel insect species, and a potential method for pest control (6, 13). Several factors have been identified that could be related to RNAi insensitivity in insects, including impaired cellular uptake, the presence of viral infections, and a saturated RNAi machinery (14). In fact, the three major RNAi pathways (microRNA, siRNA, and piwi-interacting RNA) are found in all 32 insect species, and a potential method for pest control is the RNA-induced silencing (RISC) complex are different between insect species (16).

However, in many insects, degradation of dsRNA, notably in the digestive system, seems to play a major role in low sensitivity to RNAi. Research indicates that dsRNA can be rapidly degraded in the saliva of Lygus lineolaris (17). In the pea aphid, Acyrthosiphon pisum, both the salivary secretions and the hemolymph were able to degrade the dsRNA rapidly (14). This phenomenon was also found in the gut juice of Schistocerca gregaria (18, 19). In a comparative study between two different insect species, dsRNA was found to persist much longer in Blattella germanica hemolymph plasma than in Manduca sexta hemolymph (20). In another study, two coleopteran insects, Diabrotica virgifera virgifera and Leptinotarsa decemlineata, and two lepidopteran insects, Spodoptera frugiperda and Helicoverpa zea, were fed dsRNA-containing diets. Two days later, samples were collected and, using Northern blotting to analyze the concentration of dsRNA in insects, the results revealed that...
dsRNA was much more stable in the coleopteran species than in the two lepidopterans (21). Interestingly, a recent study also showed that dsRNA degradation in the gut plays a role in some coleopteran insects as well, and that a high degree of variability in RNAi efficiency and dsRNA stability can be found between two very closely related species (22, 23). These studies all indicate that there are nucleases that can degrade dsRNA in different tissues of insects, but that a degree of variability in nuclease activity is observed between insects.

In the silkworm, desert locust, and Colorado potato beetle, Bm-dsRNAse, Sg-dsRNAse, and Ld-dsRNAse were found in the gut, respectively (8, 19, 24, 25). All of these dsRNases belong to the category of DNA/RNA nonspecific nucleases. In this nuclease family, dsRNA is the most preferred substrate, even though ssRNA, ssDNA, and dsDNA can be degraded as well (26). These dsRNA degradation nucleases are likely to affect RNAi efficiency. Knockdown of dsRNAse activity in the L. deceiplineata midgut could enhance RNAi efficiency, but a similar effect was not found in S. gregaria (8). In bacteria, nematodes, and mammals, members of the DNA/RNA nonspecific nuclease group were also found (24).

In fact, nucleases have been found to affect RNAi efficiency in many species. In Caenorhabditis elegans, the eri-1 (enhanced RNAi-1) gene, which encodes a nuclease, seems to inhibit RNAi efficiency because eri-1 mutant C. elegans are more sensitive to RNAi than WT worms (27). In Dicer-related helicase 2 (drh-2)–mutant nematodes, RNAi efficiency was also enhanced (28). Besides endogenous nucleases, exogenous nucleases introduced into the body can also affect RNAi efficiency. PPR3 of a fish DNA virus (PPIV) belonging to the class 1 RNaseIII endonucleases can suppress RNAi in the nonhosts Nicotiana benthamiana and C. elegans through cleaving double-stranded siRNA (29).

In previous research, we identified a gene that can be induced significantly by dsRNA presence and that we named up56 (30). Here we selected Ostrinia furnacalis as the major research subject and present data indicating that up56 is a new type of nuclease, called RNAi efficiency–related nuclease (REase) and that this nuclease is one of the factors that can explain why Lepidoptera are so refractory to RNAi. We investigated the expression profile of this gene, examined the enzyme activity of the translated protein, and investigated whether knocking down this nuclease could enhance RNAi efficiency in O. furnacalis.

**Results**

**REase is a lepidopteran-specific gene**

The full-length 1866-nucleotide cDNA sequence of REase was obtained by rapid amplification of cDNA ends (RACE) technology (GenBank accession no. F682492). Sequence similarity searches in GenBank (via NCBI) indicated that the highly identical genes were annotated as uncharacterized proteins and protein asteroid homologs. To investigate the correct identification, a phylogenetic tree was constructed using 41 genes from 40 species belonging to six insect orders by the neighbor-joining method (Fig. 1A and Table S1). The results indicated that REase gathered together in one branch with six other uncharacterized proteins. All other 34 proteins, which were named as protein asteroids, gathered in the other branch. All uncharacterized protein genes in the branch with REase were only present in lepidopteran insects (Fig. 1A, red underline).

We also found that the REase gene from O. furnacalis and the uncharacterized protein from Helicoverpa armigera were upregulated by dsEGFP (Fig. 1B). However, expression of the protein asteroid genes from O. furnacalis, H. armigera, and Tribolium castaneum was not affected by dsEGFP exposure (Fig. 1C). Although these two types of genes have similar sequences, their function must be different.

To further study the function of the REase gene, multiple sequence alignment analysis was done for the seven uncharacterized protein genes in these Lepidoptera (Fig. 1D). Their amino acid sequence similarity was found to be around 30%. Protein domain analysis revealed that this protein family contains a PiT N terminus (PIN) domain at the 5′ end (Fig. 1D, green underline). Three strictly conserved acidic residues are also labeled (Fig. 1D, red crosses). Apart from these three residues in the PIN domain, this gene shows a very high sequence diversity between different species (31). PIN domain proteins are found in eukaryotes and prokaryotes, where they function as nucleases that are involved in nonsense-mediated mRNA decay (32), so we deduced that REase is likely to be a new member of the PIN domain family and has nuclease activity.

**REase protein is capable of degrading various types of nucleic acid**

To further study the function of REase, the three-dimensional structure of REase was predicted by SWISS-MODEL. The results showed that the first 340 amino acids of REase had a similar secondary structure as 3qe9.1 of human exonuclease I (Fig. S1). The first 340 amino acids were then used to predict the three-dimensional structure using the I-TASSER service. The highest score-threading template was 3qe9Y, human exonuclease I. The predicted protein model was compared with 3qe9Y, and we found that these were highly consistent (Fig. 2A). Furthermore, the 2-, 30-, 76-, 83-, 90-, 111-, 138-, 156-, and 169-amino acid sites are capable of binding to nucleic acid sequences (Fig. 2B). This result indicates that the protein could have nuclease activity.

Using the Bac-to-Bac baculovirus expression system to express REase, a 72-kDa protein was obtained (Fig. 2C). Western blotting was used to confirm that this 72-kDa protein was in fact REase (Fig. 2D). The purified REase was then incubated at 37 °C with dsRNA, ssRNA, dsDNA, ssDNA, and plasmid DNA. Three days later, the stability of these nucleic acids was investigated by agarose gel electrophoresis, and we observed that all the types of nucleic acids could be degraded by REase (Fig. 2E). The enzyme degradation rates for dsRNA are shown in Fig. 2, F and G. These results indicate that about 50% of the dsRNA

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4The abbreviations used are: ssRNA, single-stranded RNA; ssDNA, single-stranded DNA; cDNA, complementary DNA; qRT-PCR, real-time quantitative PCR; KT1, Kunitz trypsin inhibitor; chymotrypsin; RNA-Seq, RNA sequencing; RdRP, RNA-dependent RNA polymerase; FAW, fall armyworm; dsEGFP, double-stranded enhanced GFP; qPCR, quantitative PCR; REase, RNAi efficiency–related nuclease.
can be partially degraded after 1 day of incubation and that, after 2 days, all dsRNA was degraded (Fig. 2, F and G).

According to the above results, we confirmed that REase is a new nuclease belonging to the PIN domain family. More important is that REase was only found in Lepidoptera insects according to current database information.

The only problem is that enzyme activity was found to be relatively low. This may be due to the purity or structure effects on the enzyme activity by the in vitro expression system. To check whether virus-expressed REase can degrade dsEGFP in vivo, the purified REase and fluorescently labeled dsEGFP were injected into fifth-instar larvae of the Asian corn borer, using BSA as positive control. We observed that virus-expressed REase can speed up the degradation of fluorescently labeled dsRNA in the body of the Asian corn borer (Fig. S2).

Figure 1. REase is a Lepidoptera-specific gene belonging to the PIN family. A, phylogenetic tree constructed using 41-amino acid sequences from 40 insect species (Table S1) by the neighbor-joining method (1000 bootstrap repeats). All proteins came from six insect orders, which are shown in different colors. REase is grouped in one branch with six other uncharacterized proteins, which are underlined in red. B, REase from the Asian corn borer (O. furnacalis) and the homologous gene in the cotton bollworm (H. armigera) cannot be induced by dsEGFP. D, multiple sequence alignment of the seven genes coding for the uncharacterized protein from Lepidoptera insects. The underlined part shows the PIN domain at the 5’ end of REase. Three red crosses show the strictly conserved acidic residues. Data are mean ± S.D., n = 3; **, p < 0.01.
The expression level of REase can affect the dsRNA degradation rate

Previous results indicated that expression of REase can be up-regulated by introduction of dsEGFP (Fig. 1B). To investigate the relative transcript levels of REase in different tissues, total RNAs were isolated from the blood, brain, head, fat body, and midgut of dsRNA-treated and untreated fifth-instar O. furnacalis larvae. Gene transcript levels were then analyzed by qRT-PCR. REase was found to be mainly expressed in the midgut before dsRNA treatment (Fig. 3A). Expression was found to be up-regulated in different tissues 4 h after dsRNA treatment; however, the induced expression level in the midgut remained higher than in other tissues at this time point (Fig. 3A). These results suggest that REase is mainly functional in the midgut.

To clearly understand the relationship between its expression pattern and its function in the midgut, dsEGFP was used to induce REase expression, and dsREase was used to knock down REase expression. We discovered that REase expression levels with both treatments were significantly different 4 h after treatment (Fig. 3B). Compared with dsEGFP-induced up-regulation, treatment with dsREase led to a knockdown of the REase expression level (Fig. 3B). Then the insect midgut fluid was extracted 6 h after being induced by dsEGFP or dsREase, using midgut fluid from untreated larvae as a control. Three different midgut extracts were incubated with dsRNA or DNA. The results showed that the extracts from dsEGFP-treated larvae were able to degrade dsRNA or DNA more quickly (Fig. 3, C and D, 1.5 h, lane 2) than the midgut fluid from dsREase-treated larvae (Fig. 3, C and D, 1.5 h, lane 3) because REase content was enhanced by dsEGFP treatment and reduced by dsREase treatment. These results suggest that REase expression rates can affect the nucleic acid degradation rate.

We further repeated this experiment in the cotton bollworm (H. armigera) and found similar results. dsEGFP treatment also accelerated dsRNA degradation by midgut extracts in H. armigera (Fig. S3, 1 h, lane 2).

Previous results indicated that, in general, dsRNA is more stable in coleopteran insects than in lepidopteran insects (21). Our results also confirmed that dsRNA is more stable in the midgut solution of the larvae of the coleopteran Holotrichia diomphalia than in the lepidopteran O. furnacalis and H. armigera. The dsRNA was digested after 2 h in the midgut solution of these lepidopteran insects (Fig. S4, 2 h, lanes 2 and 3), whereas in the midgut extract of the coleopteran species, the dsRNA was still unaffected 6 h after treatment (Fig. S4, 6 h, lane 1). This result was also confirmed by an in vivo test. Fluorescently labeled dsEGFP was injected into H. diomphalia grubs and...
Each insect was injected with different amounts of dsEGFP according to body weight (1 μg/g of body weight). The fluorescence could be observed in the bodies of the coleopteran grubs after 15 days of injection; however, the fluorescent signal disappeared just 3 days after injection (Fig. S5). In contrast, in both lepidopteran species, the fluorescent signal was much weaker and disappeared almost entirely after 48–72 h. We hypothesize that, in these lepidopterans, the specific REase or REase-like enzyme accelerates the digestion of dsRNA.

Knockdown of the expression of REase can enhance RNAi efficiency

To further assess the in vivo effects of REase on dsRNA and RNAi efficacy, *O. furnaclis KTI* (Kunitz trypsin inhibitor) and *CTP8* (chymotrypsin 8) were selected as marker genes. Fifth-instar larvae within 12 h of ecdysis were injected with dsKTI or dsCTP8. Not surprisingly, the REase gene expression was found to be up-regulated compared with control samples (Fig. 4, A and B, gray columns). Therefore, to suppress the expression level of the REase gene, a double RNAi experimental setup was used, injecting dsREase plus dsKTI or dsCTP8 (Fig. 4, A and B, black columns). Transcript-level analysis indicated that REase gene expression can be knocked down significantly compared with larvae that were treated only with dsKTI or dsCTP.

To discover the effect of REase down-regulation on the RNAi efficiency of dsKTI or dsCTP, the relative expression levels of dsKTI or dsCTP after double RNAi treatment with dsREase plus dsKTI or dsCTP were compared with those when only dsKTI or dsCTP were injected. The results showed that knocking down the expression of REase could significantly enhance the...
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**Figure 4. The expression levels of REase will affect the RNAi efficiency of other genes in vivo.** A and B, double-injected 10 μg of dsKTI together with 10 μg of dsREase will enhance the RNAi efficiency for KTI. C and D, double-injected 10 μg of dsCTP8 together with 10 μg of dsREase will enhance the RNAi efficiency for CTP8. Compared with dsEGFP and CK, KTI was not suppressed significantly just by dsKTI (p > 0.05), but CTP8 was suppressed significantly by dsCTP8 (p < 0.01). E and F, overexpression of REase in D. melanogaster will reduce RNAi efficiency for the white gene. Data are mean ± S.D., n = 3; **, p < 0.01.

**REase affects RNAi efficiency, probably by competition of target dsRNA with Dicer-2**

The expression levels of REase can be induced by introduction of dsEGFP (Figs. 1B and 5A) but not by lipopolysaccharides (Fig. 5A). This suggests that REase is mainly involved in the insect immune response to dsRNA but not in the bacterial immune response. To further illustrate the mechanism of REase influence on RNAi efficiency, RNAi core genes were investigated. We discovered that, compared with dsEGFP treatment, the expression levels of Ago-2 and Dicer-2 were also suppressed by dsREase treatment (Fig. 5, B and C), and all three genes, REase, Ago-2, and Dicer-2, can be induced by dsEGFP. However, when comparing the expression up-regulation of these three genes in response to dsEGFP, REase up-regulation was earlier and higher than Ago-2 and Dicer-2 (Fig. 5D). These results imply that REase affects RNAi efficiency, probably by competition of target dsRNA with Dicer-2.

**REase affects RNAi efficiency by influencing the unique and total reads of target gene siRNAs**

To further understand the function of REase in vivo, fifth-instar larvae of the Asian corn borer were injected with 5 μg of dsREase, 5 μg of dsEGFP, and 5 μg of dsEGFP + 5 μg of dsREase, using untreated insects as control. Six hours after dsRNA treatment, total RNA was extracted for small RNA-Seq by an Illumina Hiseq 2000 analyzer at BGI (Shenzhen, China). The results indicated that these four samples had nearly the same amount of small RNA (Table S2), the majority being in the 18- to 25-nt range (Fig. 6A). These lengths are consistent with the processing of dsRNA into siRNA in the RNAi pathway.

To clearly understand and further analyze these data, the gene expression levels of REase were analyzed by qRT-PCR (Fig. 6B). Similar as in Fig. 3B, dsEGFP treatment was able to up-regulate the expression of REase compared with control check (CK) and dsREase treatment. However, when the insects were treated with dsEGFP + dsREase, the expression level of REase was lower than when they were treated with dsEGFP. So we hypothesized that, although the same dose of 5 μg of dsEGFP was injected into the insect body, relative higher levels of REase in dsEGFP-treated larvae will digest a higher amount of dsEGFP than dsEGFP + dsREase treatment, which will be reflected in the amount of small RNAs.

Our hypothesis was confirmed by the unique and total reads of EGFP small RNAs. The 18- to 25 nt-small RNA sequences that could be mapped on the EGFP gene sequence after exogenous dsEGFP was injected into the body of the Asian corn borer are listed in Fig. 6, C and D. Only 5019 and 90,244 small RNAs were able to be mapped on the EGFP sequence in dsEGFP treatment, whereas, for dsEGFP + dsREase treatment, this was 12,710 and 747,058. This may be due to the increased content of REase in dsEGFP treatment digesting more dsEGFP. In accordance with our hypothesis, the total number and different types of small RNA in the dsEGFP treatment were significantly lower than in dsEGFP + dsREase treatment (Fig. 6, C and D). These results indicated that REase affects RNAi efficiency by influencing the unique and total reads of target gene siRNAs.
RNAi technology is a promising approach used in agricultural pest control \((6, 33)\). However, RNAi efficiency is relatively low in lepidopteran insects compared with many other species \((5)\). dsRNA degradation and cellular uptake and transport seem to be the main reasons for various RNAi efficiencies among insects. Previous research showed that dsRNA may stay stable much longer in many Coleoptera compared with most Lepidoptera \((16, 20, 21)\). Our research results suggest that REase or an REase-like enzyme in the Lepidoptera insect midgut is

**Figure 5. The interaction of REase with Dicer-2 and Ago-2 at the gene expression level.** A, REase expression can be induced by dsEGFP but not by lipopolysaccharide (LPS). B and C, the expression levels of Dicer-2 and Ago-2 can be up-regulated by dsEGFP but will be suppressed by dsREase. D, the expression levels of REase, Dicer-2, and Ago-2 can be induced by dsEGFP, but in terms of response time and intensity, REase was earlier and higher than Ago-2 and Dicer-2. Data are mean ± S.D., \(n = 3\); *, \(p < 0.05\); **, \(p < 0.01\).

**Figure 6. The REase expression level affects the unique and total reads of target siRNAs.** A, the length distribution of small RNAs in four samples. B, gene expression level of REase in larvae that were injected with 5 \(\mu\)g of dsREase, 5 \(\mu\)g of dsEGFP, or 5 \(\mu\)g of dsEGFP + 5 \(\mu\)g of dsREase per insect, using untreated insects as controls. Four hours after dsRNA treatment, REase expression levels were tested by qRT-PCR. Data are mean ± S.D., \(n = 3\); *, \(p < 0.05\). C and D, the unique and total reads of 18- to 25-nt small RNA that can be mapped against the EGFP gene sequence.

**Discussion**

RNAi technology is a promising approach used in agricultural pest control \((6, 33)\). However, RNAi efficiency is relatively low in lepidopteran insects compared with many other species \((5)\). dsRNA degradation and cellular uptake and transport seem
RNAi efficiency–related nuclease

probably the major reason for this aspect (Figs. 3 and 4). The Lepidoptera-specific nuclease REase could digest dsRNA before processing by Dicer and thus affect RNAi efficiency; this gene can explain the low RNAi efficiency in lepidopteran insects to some extent. From this research, we can draw the following conclusions.

REase is a new type of nuclease belonging to the PIN family

In this research, we found a new nuclease only present in lepidopteran insects. Sequence analysis using Pfam and UniProt databases predicted that this protein, which we called REase, contains a PIN domain at the 5’ end. Further sequence analysis, using the I-TASSER server to predict its protein three-dimensional structure, revealed that the highest score-threading template is 3qeryY, a human exonuclease 1 (EXO 1). The REase protein was expressed by the Bac-to-Bac baculovirus expression system, and enzyme activity was measured in vitro and in vivo. The results showed that the REase protein has nuclease activity and that it can degrade various types of nucleic acids, including dsDNA, ssDNA, dsRNA, ssRNA and plasmid (Fig. 2E and Fig. S3). In general, PIN domain proteins have the function of nucleases enzymes. Some studies have confirmed that PIN domains are found to be involved in nonsense-mediated mRNA decay in eukaryotes (31, 32, 34).

In C. elegans, the PIN domain protein smg-5 is involved in regulating RNAi, and the yeast NMID4p and smg-2 orthologous gene Upf1p have PIN domains and participate in RNAi as well (35, 36). PIN domains appear to be common components in proteins involved in both nonsense-mediated mRNA decay and the RNAi pathway (37). The discovery of this new PIN domain–containing protein and the results obtained in this study can provide a reference for us to further study RNAi efficiency in lepidopteran insects. However, the in vitro assay indicated that the enzyme activity of REase was very low, and it required incubation at 37°C for 3 days to thoroughly degrade 200 ng of dsRNA. This may be due to the purity of the enzyme by recombinant expression. It may also be due to the enzymatic reaction system, such as the mono- or divalent ion concentration, and may even be due to inappropriate enzyme substance concentration or reaction temperature. All of these reasons should be considered and tested for further study.

REase is a Lepidoptera-specific nuclease

REase and its homologous genes are found only in seven lepidopteran insects according to current public databases (Fig. 1A). We proved that knockdown of REase can improve RNAi efficiency, and therefore the presence of this gene may be an important reason why the RNAi efficiency in lepidopteran insects is different from that in many other insect species.

RNAi efficiency is very different between species. In plants and many nematodes, RNAi-mediated gene knockdown is easily achieved because of the presence of RNA-dependent RNA polymerase (RdRP)–mediated synthesis of secondary siRNAs (38, 39). A similar RdRP has never been found in insects, but based on the high sensitivity to RNAi in some insect species, e.g., several coleopteran insects, one could wonder whether an alternative system of amplification of a silencing signal could be present in these insects, allowing them to compensate for the lack of RdRP. In 2016, Dowling et al. (15) published a large study of transcriptomic data of 100 insect species in which they presented an overview of the distribution and diversity of 11 core RNAi pathway genes. In Lepidoptera, there was no evidence that the lower RNAi sensitivity could be easily explained by the RNAi machinery repertoire (15). This further highlights the possible importance of this newly discovered nuclease, specific for Lepidoptera and able to rapidly degrade dsRNA in the insect body. The presence of this nuclease could represent an evolutionary response to a heavy viral load (20) and can help us to further understand RNAi in Lepidopteran insects.

REase probably has a competitive relationship with Dicer

In vivo experiments indicated that decreasing the expression level of REase will enhance the RNAi efficiency in the Asian corn borer (Fig. 4, C and D), and overexpression of REase leads to suppression of RNAi efficiency (Fig. 4, E and F). These results indicate that the REase level is closely related to RNAi efficiency. In addition to this, real-time quantitative PCR results show that REase, Ago-2, and Dicer-2 expression can be up-regulated by exogenous dsEGFP (Fig. 5, A–C). However, REase can be induced earlier and stronger than Ago-2 and Dicer-2 by dsEGFP (Fig. 5D). Furthermore, using small RNA-Seq technology, we further analyzed the type and number of small RNA. When we knocked down expression of REase by dsREase, both the unique and total reads of EGFP small RNAs were found to be increased (Fig. 6, C and D). These results imply that a large part of the introduced dsRNA could be digested by REase before it could be processed by Dicer, therefore reducing the siRNA amount able to be active in the RNAi pathway.

The stability of dsRNA in insects is clearly related to RNAi efficiency (8), but the rate of dsRNA processing into siRNAs is the direct reason for variable RNAi efficiency. In a recent study, Western corn rootworms (D. virgifera virgifera LeConte), Colorado potato beetles (L. decemlineata), and FAWs (S. frugiperda) were fed corn roots, tomato leaves, and corn leaves, respectively. After 11–12 days, samples were collected for sRNA sequencing. Mapping these sRNA reads to the corresponding plant genome revealed a significant accumulation of host plant–derived siRNAs in Western corn rootworms and Colorado potato beetles but not FAWs. These results demonstrate that the plant-endogenous long dsRNAs can be processed into 21-nt siRNAs and accumulate in high quantities in these coleopteran herbivores, but no accumulation of siRNAs was observed in the lepidopteran FAWs (21). In another study, P132-labeled dsRNA was incubated in lepidopteran and coleopteran cells, after which total RNA was isolated after 72 h before being analyzed on denaturing gels. Detection of the radioactivity revealed a band of ~23 nt in the RNA sample isolated from coleopteran cells. In contrast, small RNAs were not found in lepidopteran cells (16). The reasons leading to this difference in lepidopteran and coleopteran were not clear at the time. With the discovery of this Lepidoptera-specific REase, able to digest dsRNA before dsRNA was processed into siRNA by Dicer-2, we might have provided at least part of an explanation for these previous observations.
Experimental procedures

Insect culture

*O. furnacalis* eggs were originally obtained from fields in Shanghai, China, and reared in the laboratory at 25 °C and 75% relative humidity on a 14/10-h light/dark cycle. The larvae were fed a modified artificial diet (120 g of maize granules, 32 g of maize flour, 120 g of soybean flour, 4 g of vitamin C, 12 g of agar, 72 g of yeast powder, 4 g of sorbic acid, 60 g of glucose, 1.6 ml of formaldehyde, and 1000 ml of water). Moths were fed a 10% (v/v) honey solution. *H. armigera* and *H. diomphalia* were grown under the same conditions as *O. furnacalis*

dsRNA and fluorescently labeled dsRNA preparation

dsRNAs were synthesized using the MEGAscript® RNAi kit (Ambion, Huntingdon, UK) according to the manufacturer’s instruction. T7 promoter sequences were tailed to each 5’ end of the DNA templates by PCR amplifications. Double-stranded enhanced GFP (dsEGFP) was generated using pPigbacA3EGFP as the template. All primer sequences are listed in Table S3. Template DNA and single-stranded RNA were removed from the transcription reaction by DNase and RNase treatments, respectively. dsRNA was purified using MEGAclear columns (Ambion) and eluted in nuclease-free water. dsRNA concentrations were measured using a Biophotometer (Eppendorf, Hamburg, Germany). The synthesis procedure for labeled dsRNA was the same as described earlier, except that 0.25 ml of 10 mM fluorescent UTP labeled with Cy3 was added.

Phylogenetic analysis

Phylogenetic analysis was performed using the tBlast-N algorithm to search all public NCBI databases, using the *O. furnacalis* REase protein sequence as a query. Thirty-seven protein sequences from six insect orders were selected, and three other sequences were retrieved from *H. armigera* and *Agrotis ipsilon* transcriptome databases. All sequence information is listed in Table S1.

The 41 selected sequences were aligned with the MUSCLE alignment software. Phylogenetic analysis was performed using MEGA version 5.2, and a neighbor-joining tree was constructed using the Poisson model and tested by the bootstrap method with 1000 replications. All gaps were treated as missing data.

Protein expression and purification

The Bac-to-Bac baculovirus expression system was used to express the REase protein. The full-length of *REase* was cloned into pFastbac-hbt, and then the recombinant plasmid was transformed into DH10Bac, using PCR to analyze recombinant Bacmid DNA and transfect it into sf9 cells. P1 viruses were added to sf9-suspending cells and incubated at a 27 °C shaking incubator. Then P2 viruses were added to sf9-suspending cells, and Western blotting was used to detect the signal. Nickel-nitritolactacetic acid Superdex 200 was used to purify REase, and the His tag was cleaved by tobacco etch virus. Protein was stored in protein buffer (50 mM Tris, 150 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine, and 5% (v/v) glycerol (pH 8.0)).

REase antibody production and Western blotting

The sequence segment of 1–555 bp of *REase* was constructed in the pET32a plasmid, and the recombinant vector was then transformed into *Escherichia coli* cell of BL21(DE3). The monoclonal cells were incubated at 37 °C, and the target protein was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. The protein was purified using a nitritolactacetic acid column. The purified protein was used to immunize New Zealand rabbits to produce polyclonal antibody (at Shanghai YouKe Biotechnology Co. Ltd.). Using the Bac-to-Bac baculovirus expression system to express the full-length gene of REase, the protein was checked by SDS-PAGE and Western blotting.

REase activity assays on nucleic acid in vitro

200 ng of DNA, ssDNA, dsRNA, or RNA was incubated with 5 μg of REase in a 20-μl volume of 67 mM glycine-KOH, 6.7 mM MgCl₂, and 1 mM DTT at pH 7.5. Reactions were incubated at 37 °C. Samples were collected at different time points. Reactions were analyzed by agarose gel. Gels were scanned using a fluorescence laser scanner (Genosens 1860 Gel Doc System, CLiNX), and the bands were quantified with software (GenoSensCapture).

In vitro degradation of REase on dsRNA

Fifth-instar *O. furnacalis* larvae were treated with CK, dsEGFP, or dsREase. After 6 h, midgut juice samples were collected. 200 ng of dsRNA was incubated with midgut juice samples at 37 °C. The samples and the dsRNA integrity were analyzed by means of 1% agarose gel electrophoresis. As a positive control, 200 ng of dsRNA was incubated in nuclease-free water. The experiments performed on *H. armigera* and *H. diomphalia* were identical to those performed on *O. furnacalis*.

In vivo degradation of REase on dsRNA

To investigate whether in vitro-produced REase could degrade dsRNA in vivo, fifth-instar larvae were injected with 5 μg of REase and 100 ng of fluorescently labeled dsEGFP at the same time. As a control, BSA was injected. Fluorescence intensity was recorded by a fluorescence microscope at different times.

To investigate the correlation between REase expression and in vivo dsRNA degradation, *H. diomphalia* Bates, *H. armigera*, and *O. furnacalis* were injected with 1 μg of fluorescently labeled dsEGFP per gram of body weight. The fluorescence intensity was recorded by a fluorescence microscope at different times.

Transgenic Drosophila line construction and test

The full-length coding DNA sequence (CDS) of *REase* was constructed into the pUAS-T plasmid. A P-element insertion on chromosome 3, pUAST-REase, was performed by the platform of *Drosophila* in the Core Facility of Drosophila Resource and Technology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The UAS-white-dsRNA *Drosophila* line (THU1985) was received from the TsingHua Fly Center. 5906, 5907, and GMR-GAL4 were purchased from the Bloomington Drosophila Stock Center.
5906 were crossed with 5907. F1 +/Sco; +/TM6C, Sb females were crossed with UAS-white-dsRNA males. F2 +/Sco; UAS-white-dsRNA/TM6C, sb males were crossed with female GMR/Cyo; GE25979/TM6B. F3 GMR/Sco; UAS-white-dsRNA/TM6B were crossed with pUAST-REase. The adult heads of F4 GMR/+; UAS-white-dsRNA/UAS-REase males and GMR/+; UAS-white-dsRNA/+ males were collected for further RNA extraction and reverse transcription. The REase or white gene expression level was tested by quantitative real-time PCR. The primer sequences are listed in Table S3. Drosophila stocks and crosses were maintained on standard cornmeal agar medium. Crosses were performed at 25 °C.

Sample collection and RNA isolation

The samples of O. furnacalis, H. armigera, and T. castaneum were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Total RNA was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions. Samples were treated with RNase-free DNaseI (New England Biolabs) for 30 min at 37 °C to remove residual DNA.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was made from 1 μg of RNA primed by oligo(dT)18 using M-MLV reverse transcriptase (Takara, Kyoto, Japan). A qPCR assay for multiple genes was performed with SYBR® Premix Ex Taq™ II (Takara). To ensure qRT-PCR quality, two or three primer pairs were designed for all of the amplification segments, but only one pair was used in the final test. The sequences information is list in Table S1. Melting curve analyses were performed for all of the primers. To normalize cycle threshold (Ct) values obtained for each gene, 18S rRNA expression levels were used. RT-qPCR was carried out using a Mastercycler® ep realplex (Eppendorf). All qPCR assays were repeated three times. To assess the extent of RNAi, RNA was extracted from pools of three dsRNA-treated and surviving larvae using TRIzol® reagent (Invitrogen), and each treatment was repeated three times. The samples were then treated with DNase I (Invitrogen) to remove any genomic DNA contamination, and reverse transcriptase (Fermentas) was added to make first-strand cDNA using random primers. RT-qPCR reactions and data were analyzed according to the methods of Livak and Schmittgen [40] and Bustin et al. [41]. The data were analyzed using a one-way analysis of variance to look for treatment effects compared with the untreated control.

Small RNA-Seq and data analysis

Fifth-instar O. furnacalis larvae, 12 h post-molting, were injected with 5 μg of dsEGFP, 5 μg of dsREase, and 5 μg of dsEGFP + 5 μg dsREase, using untreated insect as controls. Six hours after dsRNA treatments, samples were collected to extract total RNA for small RNA-Seq. Six larvae were treated per treatment.

Samples were sequenced by an Illumina Hiseq 2000 analyzer at BGI. The sequencing information is listed in Table S2. All small RNA-Seq files are available from the NCBI SRA database (accession number SRP117224).

In this experiment, small RNAs 18–25 nt long were used for analysis. Small RNAs from samples were mapped back to the dsEGFP sequence used in this experiment, and the unique and total reads of EGFP small RNAs were calculated.

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