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A nuclease specific to lepidopteran insects suppresses RNAi

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Running title: *RNAi efficiency related nuclease*

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ABSTRACT

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 the 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 ss and ds RNA and DNA 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 represents a target for further research on RNAi efficiency in this insect order.

During the past decade, many studies have proved that RNAi technology can be used in pest control by dsRNA spraying, feeding or via transgenic plants (1–4). However, RNAi efficiency is the most important restriction factor of this technology, especially in lepidopteran insects, since 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 sensitivity 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 RNAi pathway is a natural antiviral immunity (9). The viral dsRNA is recognized as a pathogen-associated molecular pattern and processed into siRNAs by the Dicer enzyme after which the siRNA can enter into the subsequent RNAi pathway, and cause the gene silencing effect (10–12). RNAi technology has used as a genetic tool for investigating gene function, particularly in non-model 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 an impaired cellular uptake, the presence of viral infections and a saturated RNAi machinery (14). In fact, the three major RNAi pathways (microRNA, siRNA, piRNA) are found in all 32 insect orders, but RNAi efficiency is very variable between many of these insect species (15). This means that even when the same amount of dsRNA is introduced into the insect body, the effective dsRNA that can be processed into siRNA and recruited to the 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 explaining 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*, *Leptinotarsa decemlineata* and two lepidopteran insects *Spodoptera frugiperda*, *Helicoverpa zea* were fed on dsRNA containing diets. Two days later, samples were collected and, using Northern-blot to analyse 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 could be found between two very closely related species (22, 23). These resect, but that a degree of variability in nuclease activity is observed between insects, associated with different RNAi efficiency as well.

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 non-specific 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. Knock down dsRNase activity in *L. decemlineata* 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 non-specific 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 since *eri-1* mutant *C.elegans* are more sensitive to RNAi than wild type worms (27). In *Dicer-related helicase 2* (*drh-2*) mutant nematodes, RNAi efficiency was also enhanced (28). Besides endogenous nucleases, the exogenous nucleases introduced into the body can also affect the RNAi efficiency. The PPR3 of a fish DNA virus (PPIV) belonging to the class 1 RNaseIII endoribonuclease-like proteins can suppress RNAi in the non-hosts *Nicotiana bentamiana* and *C. elegans* through cleaving double-stranded small interfering RNA (ds-siRNA) (29).

In previous research, we identified a gene which can be induced significantly by dsRNA presence, which was then 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 investigate 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 REase was obtained by 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 Supplementary 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 asteroid gathered in the other branch. All uncharacterized protein genes in the branch with REase were only present in lepidopteran insects (Fig. 1A, with red underline).

We also found that the *REase* gene from *O. furnacalis* and *uncharacterized protein* (*UP*) from *Helicoverpa armigera* were upregulated by dsEGFP (Fig. 1B). However, expression of the *protein asteroid* (*PA*) 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 *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 lie around 30%. Protein domain analysis revealed that this protein family contains a PIN domain at the 5' end (green underline). Three strictly conserved acidic residues are labeled by red asterisks. 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-MODLE. The results showed that the first 340 amino acids of REase had a similar secondary structure as 3qe9.1 of human exonuclease I (Supplementary Figure 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 indicated that the protein could have nuclease activity.

Using the Bac-to-Bac Baculovirus Expression System to express REase, a 72kDa protein was obtained (Fig. 2C). Western-blot was used to confirm that this 72KDa 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. 2F and 2G. These results indicated that about 50 percent of the dsRNA can be partially degraded after 1 day incubation and that after 2 days, all dsRNA was degraded (Fig. 2F and 2G).

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 Asian corn borer, using BSA as positive control. We observed that virus expressed REase can speed up the degradation of fluorescent labeled dsRNA in the body of the Asian corn borer (Supplementary Figure S2).

The expression level of REase can affect dsRNA degradation rate. Previous results indicated that expression of *REase* can be upregulated by introduction of dsEGFP (Fig. 1B). To investigate the relative

transcript levels of *REase* in different tissues, total RNAs were isolated from 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 upregulated in different tissues 4 hours post dsRNA treatment. However, the induced expression level in 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 the *REase* expression and dsREase was used to knockdown the *REase* expression. We discovered that *REase* expression levels in both treatments were significantly different 4 hours post treatment (Fig. 3B). Comparing with the dsEGFP-induced upregulation, treatment with dsREase led to a knockdown of the *REase* expression level (Fig. 3B). Then, the insect midgut fluid was extracted 6 hours after being induced by dsEGFP or dsREase, using midgut fluid from untreated larvae as 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. 3C and 3D at 1.5h, number 2) than the midgut fluid from dsREase-treated larvae (Fig. 3C and 3D at 1.5h, number 3), because *REase* content was enhanced by dsEGFP treatment, and reduced by dsREase treatment. These results suggest that the REase expression rates can affect nucleic acid degradation rate.

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

Previous results indicated that in general, dsRNA is more stable in coleopteran insect 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 Lepidoptera *O. furnaclis* and *H. armigera*. The dsRNA was digested after 2h in the midgut solution of these lepidopteran insects (Supplementary Figure S4 at 2h, number 2, 3), while in the midgut extract of the coleopteran species, the dsRNA is still unaffected 6h after treatment (Supplementary Figure S4 at 6h, number 1). This result was also confirmed by an *in vivo* test. Fluorescently labeled dsEGFP was injected into *H. diomphalia* grubs and *O. furnaclis* and *H. armigera* larvae. Each insect was injected with different amounts of dsEGFP according to body weight (1 μg/gram body weight). The fluorescence can be observed in the body of the coleopteran grubs post 15 days of injection, however, the fluorescent signal disappeared just 3 days after injection (Supplementary Figure S5). In contrast, in both lepidopteran species, the fluorescent signal was much weaker and disappeared almost entirely after 48h-72h. We hypothesize that in these Lepidoptera, the specific REase or REase-like enzyme accelerate the digestion of dsRNA.

Knock down the expression of REase can enhance RNAi efficiency. To further assess the *in vivo* effects of REase on the dsRNA and RNAi efficacy, *O. furnaclis KTI* (*Kunitz trypsin inhibitor*) and *CTP8* (*Chymotrypsins 8*) were selected as marker genes. The fifth instar larvae within 12h of ecdysis were injected with dsKTI or dsCTP8. Unsurprisingly, the *REase* gene expression was found to be upregulated compared to control samples (Fig. 4A and 4B, gray column). Therefore, to suppress the expression level of the *REase* gene, a double RNAi experimental setup was used, injecting dsREase plus dsKTI or dsCTP8 (Fig. 4A and 4B, black column). The transcript level analysis indicated that the *REase* gene expression can be knocked down significantly compared to larvae that were treated only with dsKTI or dsCTP.

To discover the effect of *REase* downregulation on RNAi efficiency of dsKTI or dsCTP, the relative expression levels of *KTI* and *CTP8* after the double RNAi treatment of dsREase plus dsKTI or dsCTP8 were compared with those when only dsKTI or dsCTP8 were injected. The results showed that knocking down the expression of *REase* could enhance the RNAi efficiency of *KTI* and *CTP8* significantly ($P < 0.01$) (Fig. 4C and 4D, black column).

We were unable to find a homologous gene of *REase* in the *Drosophila melanogaster* genome (Fig. 1A). To further verify the effect of *REase* on RNAi efficiency *in vivo*, we constructed the *GMR*/+; *UAS*-

white-dsRNA/*UAS-REase* and *GMR*/+; *UAS-white-dsRNA*/+ transgenic *Drosophila* line. The expression of *white-dsRNA* and *REase* were induced in *Drosophila* compound eyes by a *GMR-GAL4* driver. Quantitative RT-PCR results indicated that *REase* could be overexpressed in these flies (Fig. 4E), at the same time, the white gene RNAi efficiency in the UAS-white dsRNA line was significantly more robust than in the *REase* overexpression line (Fig. 4F). These results indicated that *REase* overexpression can decrease the target gene RNAi efficiency *in vivo*. However, *GMR*/+; *UAS-white-dsRNA*/*UAS-REase* just can decrease the RNAi efficiency, it can't block the RNAi pathway in *Drosophila*, so we guess it is the reason why the eye color did not change from white to red.

REase affects RNAi efficiency probably by competing target dsRNA with Dicer-2. Expression levels of *REase* can be induced by introduction of dsEGFP (Fig. 1B and Fig. 5A), but cannot be induced by lipopolysaccharides (LPS) (Fig. 5A). This suggested that REase is mainly involved in the insect immune response to dsRNA, and is not involved 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 suppressed also by dsREase treatment (Fig. 5B and 5C), and all the three genes of *REase*, *Ago-2* and *Dicer-2* can be induced by dsEGFP. However, comparing the expression upregulation of these three genes in response to dsEGFP, *REase* upregulation was earlier and higher than *Ago-2* and *Dicer-2* (Fig. 5D). These results implied that REase affects RNAi efficiency probably by competing 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 dsREase, 5 μg dsEGFP, and 5 μg dsEGFP + 5μg dsREase, respectively, using un-treated insects as control. Six hours post dsRNA treatment, total RNA was extracted for small RNA sequencing by Illumina Hiseq 2000 analyzer at BGI (Shenzhen, China). The results indicated that these four samples had nearly the same amount of small RNA (Supplementary Table S2), the majority being in the 18-25 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 with Fig. 3B, dsEGFP treatment was able to upregulate the expression of *REase* compared to 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 dsEGFP was injected into the insect body, relative higher levels of REase in the dsEGFP treated larvae will digest a higher amount of dsEGFP than in 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 18-25 nt small RNA sequences which could be mapped on the EGFP gene sequence after an exogenous dsEGFP was injected into the body of the Asian corn borer were listed in Fig. 6C and 6D. Only 5019 and 90244 small RNAs were able to be mapped on EGFP sequence in the dsEGFP treatment, while for the dsEGFP + dsREase treatment, this was 12710 and 747058. 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 the dsEGFP+dsREase treatment (Fig. 6C and 6D). These results indicated that REase affects RNAi efficiency by influencing the unique and total reads of target gene siRNAs.

DISCUSSION

RNAi technology is a promising approach using 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 efficiency among insects. Previous research showed that dsRNA may stay stable much longer in many Coleoptera compared to most Lepidoptera (16, 20, 21). Our research results suggest that REase or REase-like enzyme in the Lepidoptera

insect midgut is 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 insect to some extent. From this research, we can get the following conclusions:

REase is a new type nuclease belonging to the PIN family. In this research, we found a new nuclease only present in lepidopteran insects. Sequence analysis predicted that this protein, which we called REase, contains a PIN-domain at the 5' end using the Pfam and UniProt databases. Further sequence analysis, using the I-TASSER server to predict its protein tree-dimensional structure, revealed that the highest score threading template is 3qe9Y, a human exonuclease 1 (EXO 1). The REase protein was expressed by 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 Supplementary Figure S3). In general, PIN domain proteins have the function of nucleases enzyme. 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 *NMD4p* and *smg-2* orthologous gene *Upf1p* have PIN domains and participle 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 ºC for 3 days to thoroughly degrade 200ng dsRNA. This may be due to the purity of the enzyme by recombinant expression. It may also due to the enzymatic reaction system, such as the mono or divalent ions concentration, and may even due to inappropriate enzyme substance concentration or reaction temperature. All of these reasons should be consider and test for further study.

REase is a Lepidoptera-specific nuclease. REase and its homologous genes were found only in 7 lepidopteran insects according to the 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.

The RNAi efficiency is very different between species. In plants and many nematodes, RNAi-mediated gene knockdown is easily achieved due to 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. published a large study on 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 the RNAi in Lepidopteran insect.

REase probably has a competitive relationship with Dicer-2. In vivo experiments indicated that decreasing the expression level of *REase* will enhance the RNAi efficiency in the Asian corn borer (Fig. 4C and 4D); and overexpression of *REase* leads to a suppression of the RNAi efficiency (Fig. 4E and 4F). These results indicated 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 upregulated by exogenous dsEGFP (Fig. 5A-C). However, *REase* can be induced earlier and stronger than *Ago-2*, *Dicer-2* by dsEGFP (Fig. 5D). Furthermore, using small RNA sequencing technology, we fur-

ther analyzed the type and number of small RNA. When we knocked down the expression of *REase* by dsREase, both the unique and total reads of EGFP small RNAs were found to be increased (Fig. 6C and 6D). 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 a variable RNAi efficiency. In a recent study, WCR (D*iabrotica virgifera virgifera* LeConte), CPB (*Leptinotarsa decemlineat*a) and FAW (*Spodoptera frugiperda*) were fed on 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 WCR and CPB, but not in FAW. These results demonstrated 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 FAW (21). In another study, P^{32} labeled dsRNA was incubated in lepidopteran and coleopteran cells, after which total RNA was isolated after 72 hours 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. The *Ostrina 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 on a modified artificial diet (120 g maize granules, 32 g maize flour, 120 g soybean flour, 4 g vitamin C, 12 g agar, 72 g yeast powder, 4 g sorbic acid, 60 g glucose, 1.6 mL formaldehyde, and 1000 mL water). Moths were fed a 10% (vol/vol) honey solution.

Helicoverpa armigera and *Holotrichia diomphalia* were grown under the same conditions as the *Ostrina furnacalis*.

dsRNA and fluorescently labelled 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 green fluorescent protein (dsEGFP) was generated using pPigbacA3EGFP as the template. All the primer sequences are listed in Supplementary Table S3. Template DNA and single-strand RNA were removed from the transcription reaction by DNase and RNase treatments, respectively. dsRNA was purified using MEGAclear columns (Ambion, Austin, USA) and eluted in nuclease free water. dsRNA concentrations were measured using a Biophotometer (Eppendorf, Hamburg, Germany). The synthesis procedure of labeled dsRNA was the same as described earlier except that 0.25mL 10mM fluorescent UTP (Uridine triphosphate) labelled 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 6 insect orders were selected, and three other sequences were retrieved from *Helicoverpa armigera* and *Agrotis ypsilon* transcriptome databases. All the sequence information is listed in Supplementary Table S1.

The 41 selected sequences were aligned with the MUSCLE alignment software, Phylogenetic analysis was performed using MEGA version 5.2, 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-htb, then the recombinant plasmid was transformed into DH10Bac, using PCR

to analyse recombinant Bacmid DNA and transfect it into sf9 cells. P1 viruses were added to sf9 suspending cells and incubated in a 27°C shaking incubator. Then P2 viruses were added to sf9 suspending cells, western-blot was used to detect signal, Ni-NTA superdex 200 was used to purify REase and the His-tag was cleaved by TEV. Protein was stored in protein buffer (50 mM Tris, 150 mM NaCl, 0.5 mM TCEP, 5% (vol/ vol) glycerol, pH 8.0).

REase antibody production and Western-blot. The sequence segment of 1-555bp of *REase* was constructed in pET32a plasmid and the recombinant vector was then transformed into E. coli cell of BL21(DE3). The monoclonal cells were incubated at 37°C and the target protein was inducted by 0.5 mM IPTG. The protein was purified using a NTA 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-blot.

REase activity assays on nucleic acid in vitro. 200 ng of DNA, ssDNA, dsRNA, or RNA was respectively incubated with 5 μg REase in a 20 μL volume of 67 mM Glycine-KOH, 6.7 mM $MgCl_2$, 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 fluorescent 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, dsREase, respectively. After 6 hours, 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 as those performed on *O. furnacalis*.

In vivo degradation of REase on dsRNA.To investigate whether in vitro produced REase could degrade dsRNA in vivo, the fifth instar larvae were injected with 5 μg REase and 100 ng fluorescent-labelled dsEGFP at the same time. As a control, BSA was injected. Fluorescence intensity was recorded by a fluorescent 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 fluorescent-labelled dsEGFP per gram of body weight. The fluorescence intensity was recorded by a fluorescent microscope at different times.

Transgenic Drosophila lines construction and test. The full length 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 Core Facility of Drosophila Resource and Technology, SIBCB, CAS. *UAS-white-dsRNA Drosophila* Line (THU1985) was received from TsingHua Fly Center. 5906, 5907 and *GMR-GAL4* were purchased from Bloomington Drosophila Stock Center.

5906 were crossed with 5907, F1 *+/Sco*; *+/TM6C, Sb* female were crossed with *UAS-white-dsRNA* male, F2 *+/Sco*; *UAS-white-dsRNA/TM6C, sb* male 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* male and *GMR/+*; *UAS-white-dsRNA/+* male were collected for further RNA extraction and reverse transcription. REase or white gene expression level was tested by quantitative real-time PCR. The primer sequences were listed in Supplementary Table S3.

Drosophila stocks and crosses were maintained on standard cornmeal agar medium. Crosses were performed at 25℃.

Sample collection and RNA isolation. The samples of the *O. furnacalis*, *H. armigera* and *T. castaneum* were collected, immediately frozen in liquid nitrogen and stored at -80°C until the 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, Ipswich, USA) for 30 min at 37°C to remove residual DNA.

Real-time quantitative PCR (qRT-PCR).Total RNA was extracted using TRIzol® reagent (Invit-

rogen) 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 the SYBR® Premix Ex Taq™ II (Takara). To ensure the 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 the Supplementary Table S1. Melting-curve analyses were performed for all of the primers. To normalize 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 & Schmittgen (2001) and Bustin et al (2009) (40, 41). The data were analyzed using a one-way analysis of variance (ANOVA) to look for treatment effects compared with the untreated control.

Small RNA sequencing and data analysis.Fifth-instar *O. furnacalis* larvae, 12 hours post-molting, were injected with 5 μg dsEGFP, 5 μg dsREase, 5 μg dsEGFP + 5 μg dsREase, respectively, using untreated insect as control. Six hours post dsRNA treatments, samples were collected to extract total RNA for small RNA sequencing. Six larvae were treated per treatment. Samples were sequenced by Illumina Hiseq 2000 analyzer at BGI (Shenzhen, China). The sequencing information is listed in Supplementary Table S2. All small RNA sequencing files are available from the NCBI SRA database (accession number: SRP117224).

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

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Author contributions: # These authors contributed equally to this work. X.M. designed research; R.G., H.L, Y.F. and S.H performed research; R.G. analyzed data; X.M., G.S., R.G. and O.C. wrote and revised the paper.

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FIGURES

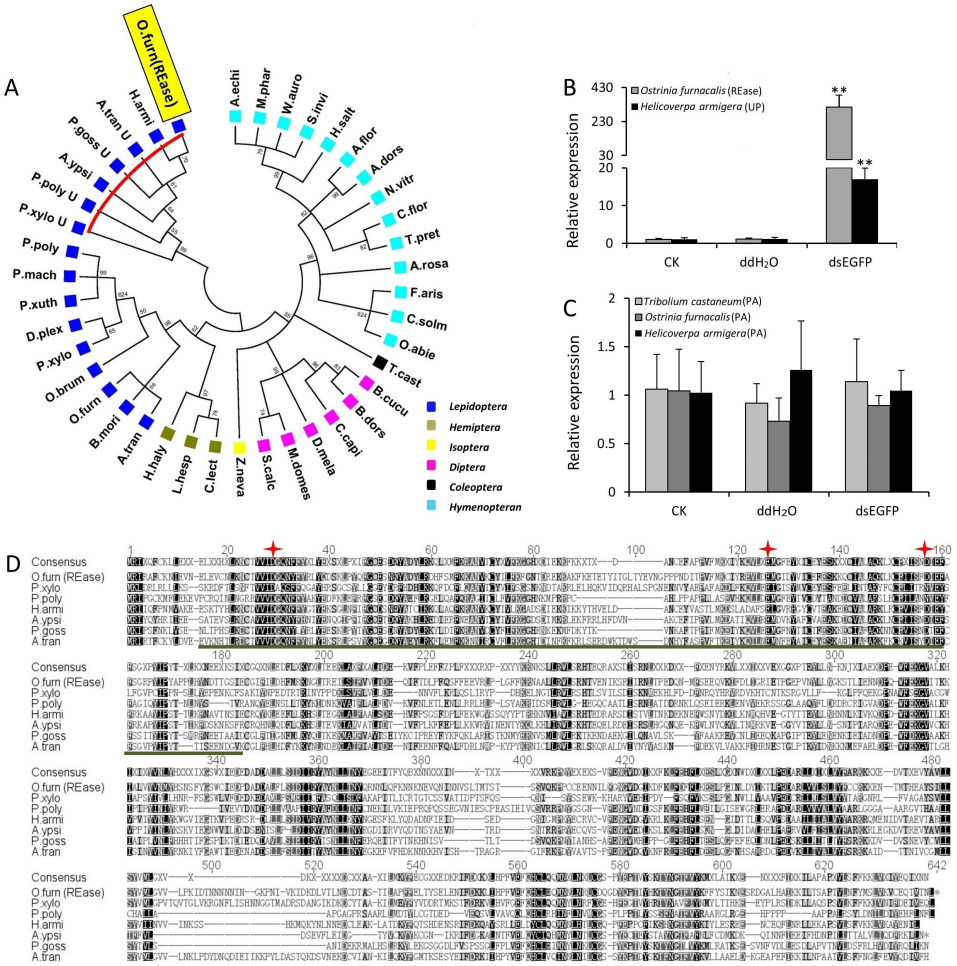

Figure 1: *REase* is a Lepidoptera-specific gene belonging to the PIN family. *A*, Phylogenetic tree constructed using 41 amino acid sequences from 40 insect species (S1 Table) by the neighbor-joining method (1000 bootstrap repeats). All the proteins came from 6 insect orders which are shown in different colors. REase is grouped in one branch with six other uncharacterized proteins which are red underlined. *B*, *REase* from the Asian corn borer (*O. furnacalis*) and the homologous gene in the cotton bollworm (*H. armigera*) can be upregulated by dsEGFP. UP: uncharacterized protein. *C*, Genes named as *protein asteroid* in *O. furnacalis*, *H. armigera* and *T. castaneum* 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 asterisks show the strictly conserved acidic residues. (Mean ± SD, n=3; **P < 0.01)

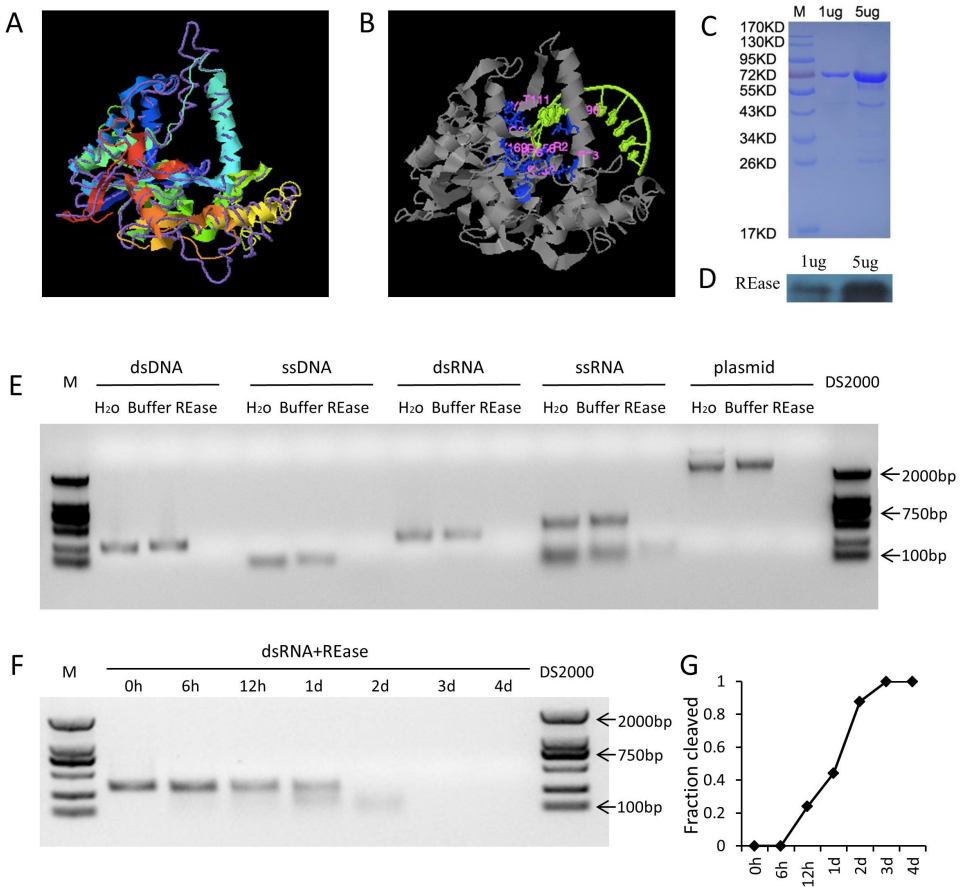

Figure 2: REase can degrade various types of nucleic acid *in vitro*. *A*, Three-dimensional structure comparison between 3qe9Y and REase predicted by I-TASSER service. The purple skeleton is the predicted model corresponding to the first 340 amino acid of REase protein. The color cartoon is 3qe9Y of human exonuclease I. *B*, The ligand binding sites of REase predicted by I-TASSER. The 2, 30, 76, 83, 90, 111, 138, 156 and 169 amino acid sites are capable of binding to nucleic acid sequences (marked in pink), the green ring is their structure of nucleic acid. *C*, *D*. REase was expressed using the Baculovirus expression system, and tested by SDS-PAGE and Western-blot. *E*, Purified REase can digest different kinds of nucleic acid. 5 μg REase incubated with 200 ng different kinds of nucleic acid at 37°C for 3 days. M is DNA marker of DS200. dsDNA is double stranded DNA, ssDNA is single stranded DNA, dsRNA is double stranded RNA, ssRNA is single stranded RNA, plasmid is a circular plasmid. *F*, *G*. The degradation rate of REase enzyme mediated dsRNA digestion.

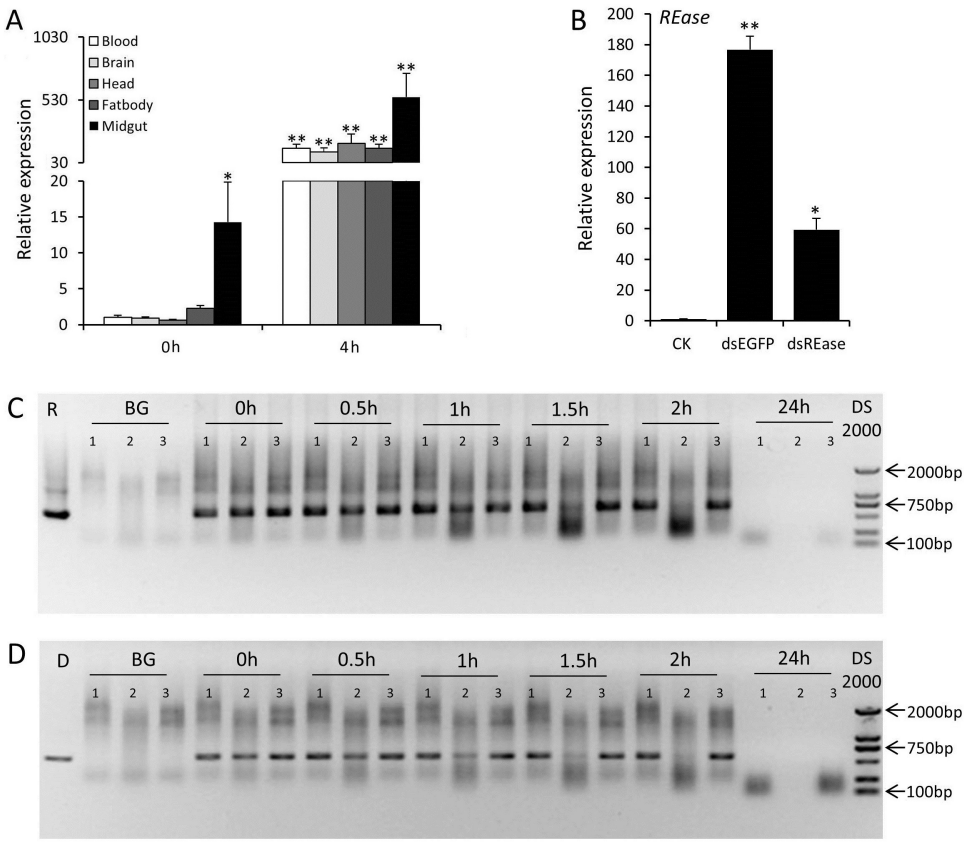

Figure 3: The expression level of REase affects enzyme activity and degradation rate of nucleic acids by midgut extraction solution. *A*, Tissue expression levels of *REase* before and after induction by dsEGFP. *B*, Four hours after dsEGFP or dsREase treatment, *REase* can be induced or suppressed in the midgut of Asian corn borer. *C*, *D*. Four hours after dsEGFP or dsREase treatment, Asian corn borer midgut extraction solution (10 μL) incubated with 200 ng dsRNA (C) or 200 ng DNA (D). R means dsRNA; D means DNA; BG means back ground of midgut extraction solution without any nucleic acid; 1 is untreated midgut extraction solution incubated with nucleic acid; 2 and 3 are nucleic acid incubated with midgut extraction solution treated with dsEGFP or dsREase. (Mean ± SD, n=3; **P < 0.01)

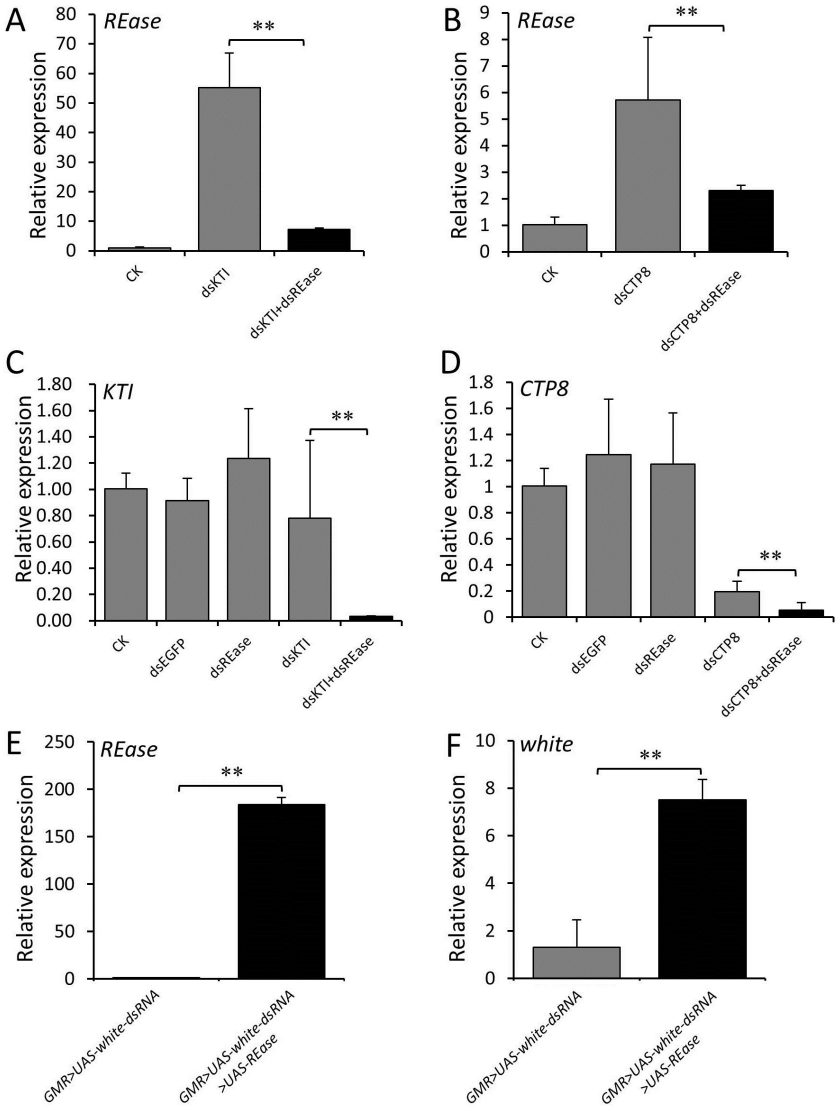

Figure 4: The expression levels of *REase* will affect RNAi efficiency of other genes *in vivo*. *A*, *B*. Double injected 10 μg dsKTI together with 10 μg dsREase will enhance the RNAi efficiency for *KTI*. *C*, *D*. Double injected 10 μg dsCTP8 together with 10 μg dsREase will enhance the RNAi efficiency for *CTP8*. Comparing with dsEGFP & CK, *KTI* was not suppressed significantly just by dsKTI ($P > 0.05$), but *CTP8* was suppressed significantly by dsCTP8 ($P < 0.01$). *E*, *F*. Overexpression of REase in *Drosophila melanogaster* will reduce the RNAi efficiency for white gene.(Mean ± SD, n=3; **$P < 0.01$)

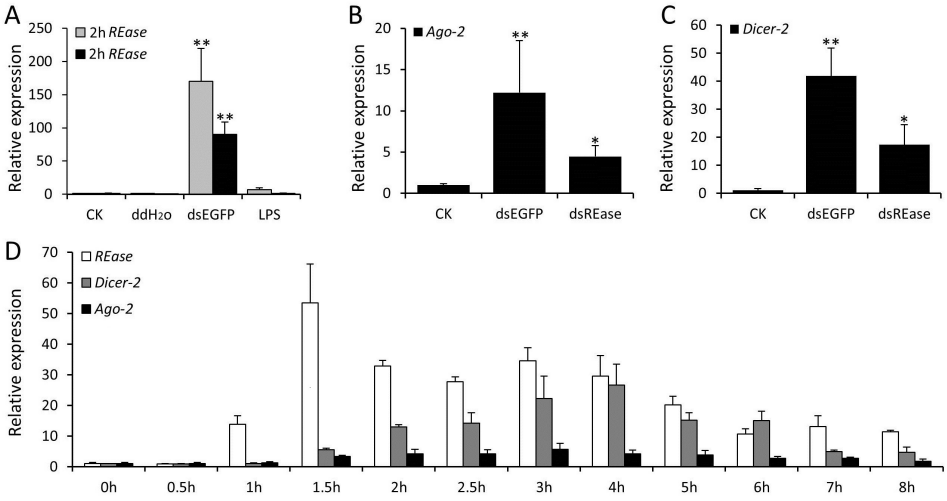

Figure 5: The interaction of *REase* with *Dicer-2* and *Ago-2* at gene expression level. *A*, *REase* expression can be induced by dsEGFP, but not by LPS. *B*, *C*. The expression levels of *Dicer-2* and *Ago-2* can be upregulated by dsEGFP, but their expression levels will be suppressed by dsREase. *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*. (Mean ± SD, n=3; **P < 0.01)

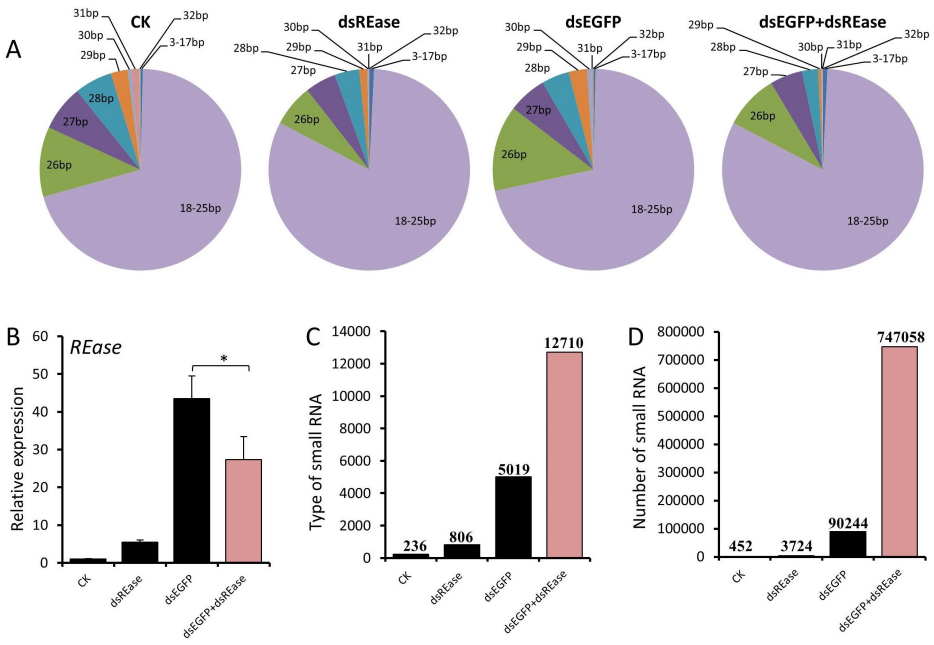

Figure 6: The *REase* expression level affects the unique and total reads of target siRNAs. *A*, The length distribution of small RNAs in four samples. *B*, Gene expression level of *REase* in larvae which were injected with 5 μg dsREase, 5 μg dsEGFP, 5 μg dsEGFP + 5 μg dsREase per insect, using untreated insects as control. Four hour post dsRNA treatment, *REase* expression levels were tested by qRT-PCR. (Mean ± SD, n=3; **P < 0.01). *C*, *D*. The unique and total reads of 18-25 nt small RNA which can be mapped against the EGFP gene sequence.

A nuclease specific to lepidopteran insects suppresses RNAi

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