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Instar-dependent systemic RNA interference response in *Leptinotarsa decemlineata* larvae

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ABSTRACT

RNA interference (RNAi) is a promising approach to control *Leptinotarsa decemlineata*. In this study, RNAi efficiency by double-stranded RNA (dsRNA) targeting *S-adenosyl-L-homocysteine hydrolase* (*LdSAHase*) was compared among *L. decemlineata* first- to fourth-instar larvae. Ingesting ds*LdSAHase* successfully decreased the target gene expression, caused lethality, inhibited growth and impaired pupation in an instar- and concentration-dependent manner. To study the role of *Dicer2* and *Argonaute2* genes in RNAi efficiency, we identified *LdDcr2a*, *LdDcr2b*, *LdAgo2a* and *LdAgo2b*. Their expression levels were higher in young larvae than those in old ones. Exposure to ds*egfp* for 6 h significantly elevated *LdDcr2a*, *LdDcr2b*, *LdAgo2a* and *LdAgo2b* mRNA levels in the first-, second-, third- and fourth-instar larvae. When the exposure periods were extended, however, the expression levels were gradually reduced. Continuous exposure for 72 h significantly repressed the expression of *LdAgo2a* and *LdAgo2b* in the first, second and third larval instars, and the four genes in final instars. Moreover, we found that ds*LdSAHase*-caused *LdSAHase* suppressions and larval mortalities were influenced by previous ds*egfp* exposure: 12 h of previous exposure increased *LdSAHase* silencing and mortality of the final instar larvae, whereas 72 h of exposure reduced *LdSAHase* silencing and mortality. Thus, it seems the activities of core RNAi-machinery proteins affect RNAi efficiency in *L. decemlineata*.

1. Introduction

RNA interference (RNAi) is a mechanism for sequence-specific suppression of gene expression. During the process of RNAi, Dicer (Dcr) cleaves double-stranded RNAs (dsRNAs) into small interfering RNAs (siRNAs) of approximately 21–23 nucleotides. These siRNAs are then incorporated into an RNA-induced silencing complex (RISC). Argonaute proteins (Agos), the catalytic components of RISC, use siRNA as a template to recognize and degrade the complementary mRNA [1]. Therefore, *Dicer2* (*Dcr2*) [2–4] and *Argonaute2* (*Ago2*) [2,5,6] are among the core RNAi genes. In insects, Dcr2 and Ago2 have been studied in *Drosophila melanogaster* and *Tribolium castaneum* [7,8]. Moreover, silencing *SgDcr2* and *SgAgo2* decreased RNAi efficiency in *Schistocerca gregaria* [9].

Given its capacity to suppress genes in a sequence-specific manner, RNAi offers great opportunities for insect science, especially to analyze gene function [10–12], manage pest populations [13–15], and reduce insect-borne infectious diseases [10]. It has been known that RNAi efficiency varies dramatically among insect life stages [10]. However, very few results have been documented. To the best of our knowledge, the only example has been reported in *Apis mellifera* [16]. When a 504 bp of vitellogenin-dsRNA is injected at the preblastoderm stage, 15% of *A. mellifera* workers have a strongly reduced level of vitellogenin mRNA. In contrast, 96% individuals show the RNAi phenotype when dsRNA is introduced by intra-abdominal injection in newly emerged bees [16]. In agricultural pests, bacterially expressed dsRNAs have been used to feed neonates [17,18], the second instars [19–21], the third instars [17,22], the fourth instars [23], the fifth instars [24], and the adults [25–29] of Lepidopteran and Coleopteran insect species. However, these studies are limited in specific genes and at particular life stages. No comparison to RNAi efficiency has been made among different life stages in agricultural insects.

Leptinotarsa decemlineata (Say) is a notorious insect defoliator of potato in most major potato-growing areas of the world. RNAi by dietary introduction of bacterially expressed dsRNAs is able to effectively knock down several target genes in *L. decemlineata* [19,21,25,28–33]. Moreover, almost all genes involved in the RNAi pathway are expressed in the gut [34]. It seems that RNAi is a promising approach in the management of *L. decemlineata*.

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Our previous results showed that ingestion of bacterially-expressed dsRNA targeting *S-adenosyl-L-homocysteine hydrolase* (*SAHase*) in *L. decemlineata* significantly decreases *LdSAHase* transcript, causes larval death, and impairs pupation and adult emergence [19]. In this study, RNAi efficiency mediated by ds*LdSAHase* was compared among the first, second, third and fourth larval instars, and the possible mechanism underlining the sensitive differences was analyzed. The results of this study will help realize the potential applications of RNAi in functional genetics and *L. decemlineata* management.

2. Methods and materials

2.1. Insects rearing

L. decemlineata larvae and adults were routinely reared in an insectary according to a previously described method [19], using potato foliage at vegetative growth or tuber initiation stages.

2.2. Molecular cloning and phylogenetic analysis

Expressed sequence tags of putative *LdDcr2a*, *LdDcr2b*, *LdAgo2a* and *LdAgo2b* were obtained from *L. decemlineata* transcriptome data [34–37]. To verify the correctness of the sequence, total RNA was extracted from the fourth larval instars and first-strand cDNA was synthesized using the reverse transcriptase (M-MLV RT) (Takara Bio., Dalian, China) and an oligo (dT)18 primer, and was used as a template for polymerase chain reaction (PCR) using primers in Table 1. The resulting sequences were submitted to GenBank (*LdDcr2a*, KP230552; *LdDcr2b*, KP230553; *LdAgo2a*, KP274882; *LdAgo2b*, KP274883).

The Dcr and Ago sequences were retrieved from NCBI, and were respectively aligned with the predicted *Ld*Dcr or *Ld*Ago using ClustalX (2.1) [38]. Neighbor-joining (NJ) trees were constructed using MEGA6 [39] under the Poisson correction method. The reliability of NJ tree topology was evaluated by bootstrapping a sample of 1000 replicates.

Table 1
Primers used for RT-PCR and qPCR.

Fragment name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Sequence position
RT-PCR			
LdAgo2a	TTTTCCCAAACGATACCC	TCCGAATGACATCCTGAA	310–2558
LdAgo2b	ATGATTTGCCACCTATGC	TCACTTTGGAAGCGGATG	59–2027
LdDcr2a-1	GCAGTGGTATCAACGCAGAG	AAAGTGCATCGGGGAATG	3–1709
LdDcr2a-2	ACCCTTACAACGACACCA	TCAAACCGAACAGCGAGT	1568–4066
LdDcr2a-3	ACATCGTCAACTTGGAGC	AGCTTGCCAAATGTAGGA	3764–5837
LdDcr2a-ORF	GATGGCTTAGCAGATGTAGT	AGCTTGCCAAATGTAGGA	934–5837
LdDcr2b-1	ACGGTTGCTCTTGTTGAT	AACTCCGAGACTGGTTTT	306–2414
LdDcr2b-2	TGATAGGAAAGAACCAGGAG	ACCGACGTATTGACTCCC	1672–3995
LdDcr2b-3	GAATCATCCCACTGTCCG	GCCGTTGAGCATAACTCT	4090–4868
LdDcr2b-ORF	ACGGTTGCTCTTGTTGAT	GTGCCGTTGAGCATAACT	306–4870
qPCR			
LdSAHase	TGCATTTGGGAGGCGTGAGATAGA	CACCCAGAGCAGCCAAAGTTTCAA	465–611
LdAgo2a	TCGAAGCGATTACTGTGGAG	CTGAAACGCCATCTCTGAAA	1953–2029
LdAgo2b	ACGCAGCAAACAAGAGTGTC	ACTGCATAGGTGGCAAATCA	4–60
LdDcr2a	AAGGCCGCTGTATCTCACTT	CTTCATGCTGTCCTCCAGAA	1204–1319
LdDcr2b	GGAGGAGAAGCTTGAGTTGG	TATGCAGAATCCCGATACCA	2636–2697

2.3. Bioassays using dsRNA

The same method and primer pairs as described [19] were used to express ds*LdSAHase* and ds*egfp* derived from a 521 bp fragment of *LdSAHase*, and a 414 bp fragment of enhanced green fluorescent protein gene, using *Escherichia coli* HT115 (DE3) competent cells lacking RNase III. Individual colonies of HT115 (DE3) were inoculated, and induced to express dsRNA. The expressed dsRNA was extracted, confirmed by electrophoresis on 1% agarose gel, and quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE). Bacteria cells were centrifuged at 5000 *g* for 10 min, and resuspended in 0.05 M phosphate buffered saline (PBS, pH 7.4) at different ratios to obtain a series of bacterial suspensions containing dsRNAs at the final concentrations of 6.0, 12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 μg/ml.

Three independent bioassays were carried out with the same protocol as described recently [31]:

1. Newly-eclosed first-, second-, third- and fourth-instar larvae were used to compare the RNAi efficiency among different developing stages. Larvae were allowed to ingest bacterial suspensions containing ds*LdSAHase* at 5 different concentrations (equivalent to 6.0, 12.5, 25.0, 50.0 and 100.0 μg/ml of dsRNA for the first and second larval instars, and 25.0, 50.0, 100.0, 200.0 and 400.0 μg/ml of dsRNA for the third and fourth larval instars). Larvae feeding on PBS and ds*egfp* were used as blank and negative control. Each treatment was repeated 9 times, a total of 90 larvae were treated. Three replicates were used to extract total RNA after being continuously fed for 3 days. The remaining 6 replicates were used for bioassay. The mortalities were recorded 6 days after the initiation of feeding and the weights of the survivors used for bioassay were measured. After 6 days of experiment, the survivors were kept in the laboratory with normal foliage. The initiation of pupation was indicated by the soil-digging behavior. The pupation individuals were recorded during a 4-week period.
2. Newly-eclosed first-, second-, third- and fourth-instar larvae were also used to test the responses of core RNAi genes to ds*egfp* at the concentration of 170 μg/ml. Larvae were allowed to feed ds*egfp*-, or PBS-immersed foliage. Each treatment was repeated 15 times, a total of 150 larvae at each instar stage were treated. After being exposed for 6, 12, 24, 48 and 72 h, 3 replicates were used to extract total RNA.
3. After being previously exposed to ds*egfp* at the concentration of 170 μg/ml at the third-instar stage, the resulting fourth-instar larvae were then exposed to ds*LdSAHase* to examine the differences of larval mortality. There were three treatments. (1) The larvae were exposed to ds*egfp* at the last 12 h of the third-instar stage. (2) The larvae were allowed to feed ds*egfp* throughout all the third-instar stage. (3) Controls were allowed to feed PBS-immersed foliage at the third-instar stage. After ecdysis, pre-treated larvae were then confined in dishes containing foliage immersed with bacterial suspension containing ds*LdSAHase* at the concentration of 170 μg/ml, equivalent to LC_{50} value for the fourth larval instars. Each treatment was repeated 6 times, a total of 60 larvae at each instar stage were treated. After being exposed for 3 days, 3 replicates were used to extract the total RNA. The remaining 3 replicates were used to record the mortalities 6 days after the initiation of feeding.

For each of the three independent bioassays, three biological replicates were carried out.