



Core RNAi machinery and gene knockdown in the emerald ash borer (*Agrilus planipennis*)



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ABSTRACT

The RNA interference (RNAi) technology has been widely used in insect functional genomics research and provides an alternative approach for insect pest management. To understand whether the emerald ash borer (*Agrilus planipennis*), an invasive and destructive coleopteran insect pest of ash tree (*Fraxinus* spp.), possesses a strong RNAi machinery that is capable of degrading target mRNA as a response to exogenous double-stranded RNA (dsRNA) induction, we identified three RNAi pathway core component genes, *Dicer-2*, *Argonaute-2* and *R2D2*, from the *A. planipennis* genome sequence. Characterization of these core components revealed that they contain conserved domains essential for the proteins to function in the RNAi pathway. Phylogenetic analyses showed that they are closely related to homologs derived from other coleopteran species. We also delivered the dsRNA fragment of *AplaScrB-2*, a β -fructofuranosidase-encoding gene horizontally acquired by *A. planipennis* as we reported previously, into *A. planipennis* adults through microinjection. Quantitative real-time PCR analysis on the dsRNA-treated beetles demonstrated a significantly decreased gene expression level of *AplaScrB-2* appearing on day 2 and lasting until at least day 6. This study is the first record of RNAi applied in *A. planipennis*.

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1. Introduction

The emerald ash borer (*Agrilus planipennis*; Coleoptera: Buprestidae) is a devastating invasive pest of ash trees (*Fraxinus* spp.) in North America (Poland and McCullough, 2006). Since its accidental introduction in 2002 from Asia, where it is native, this insect pest has spread to 24 US states as well as two Canadian provinces as of July 2014 and has killed hundreds of millions of ash trees (Haack et al., 2002; per <http://www.emeraldashborer.info>). The task of controlling *A. planipennis* has been challenging. Most native ash trees in North America lack resistance to this invasive species (Rebek et al., 2008). It is also very difficult to detect at early stages of infestation due to its cryptic feeding habit under the bark of trees and the lack of external symptoms until trees are heavily damaged (Poland and McCullough, 2006). Furthermore, chemical insecticides are not practical for broad scale use in forested areas because systemic products must be injected individually into trees to kill insects that feed under the bark. Thus, efficient approaches are needed for *A. planipennis* management.

RNAi technology is based on the introduction of double-stranded RNA (dsRNA) into an animal to silence a target gene.

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For dsRNA to successfully achieve this goal, an organism must possess the machinery that facilitates dsRNA processing and target mRNA degradation. This RNAi machinery includes three core components: Dicer-2 (Dcr-2), Argonaute-2 (Ago-2), and R2D2, which are well documented in many insect species since the year 1998 when dsRNA-triggered gene silencing was initially discovered in *Caenorhabditis elegans* (Fire et al., 1998; Swevers et al., 2013). Dicer-2 is an RNase III like enzyme that contains two RNase III catalytic domains in the carboxy-terminal region (Kim et al., 2006). These domains are thought to be responsible for the cleavage of long dsRNAs into small interfering RNA (siRNA) duplexes which are approximately 21 nucleotides (nt) in length followed by a 3'-overhang at each end (Ji, 2008; Lee et al., 2004). Ago-2 is an endonuclease, the key component of the RNA-induced silencing complex (RISC) that cleaves target RNAs (Rand et al., 2004, 2005). Unlike Dcr-2 that cleaves dsRNA, Ago-2 is responsible for the cleavage of single-strand RNA (ssRNA) in the RNAi pathway. However, for siRNA molecules produced by Dcr-2 to be incorporated into RISC and direct the degradation of target messenger RNA, R2D2, a RNA-binding protein, is required (Liu et al., 2003). This protein forms together with Dcr-2 the Dcr-2/R2D2 heterodimer which binds and loads siRNAs into RISC. Hence, R2D2 has been shown to play a role in bridging the initiation and effector steps in the RNAi pathway (Liu et al., 2003). Moreover, R2D2 was also shown to organize small RNA networks by preventing the role of siRNAs

in the microRNA (miRNA) pathway (Okamura et al., 2011). Therefore, Dicer-2, Ago-2 and R2D2 belong to the siRNA pathway as a defense against exogenous dsRNA.

However, unlike Dicer-2 and Ago-2 that were identified in all insect species examined and appeared to be indispensable to siRNA pathway, whether R2D2 is ubiquitously expressed as an essential siRNA pathway component remains unclear. Expression studies showed that *Bombyx mori* (Lepidoptera: Bombycidae) R2D2 mRNA was barely expressed or even absent in the tissues and cells where gene silencing in response to exogenous dsRNA molecules was observed (Swevers et al., 2011). Also, R2D2 was not identified in the transcriptome analysis of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) midgut, while efficient RNAi occurs in this tissue (Swevers et al., 2013). Hence, it would be intriguing to probe whether R2D2 is indeed encoded within the *A. planipennis* genome.

RNAi technology has been widely applied in insects. Although there are several species exhibiting sensitive responses to dsRNA, the response appears to be weak in some species (Bellés, 2010), including the fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae), and the Hessian fly, *Mayetiola destructor* (Diptera: Cecidomyiidae) (Aggarwal et al., 2014; Shreve et al., 2013). In contrast, *Tribolium castaneum* has emerged as a model insect species because it has a systemic and highly penetrant RNAi pathway (Miller et al., 2012). Other reported successful coleopteran examples include the western corn rootworm (*Diabrotica virgifera virgifera*), the poplar leaf beetle (*Chrysomela populi*), the mustard leaf beetle (*Phaedon cochleariae*) and the Colorado potato beetle (*L. decemlineata*), all of which belong to the family Chrysomelidae (Bodemmann et al., 2012; Rangasamy and Siegfried, 2012; Zhu et al., 2011).

RNAi is a powerful tool allowing genome-wide loss-of-function screening and has been used with great success in annotating genes with unknown functions, especially in organisms exhibiting robust systematic RNAi response such as the nematode *C. elegans* and the red flour beetle *T. castaneum* (Coleoptera: Tenebrionidae) (Knorr et al., 2013; Lee et al., 2004). A phenotypic effect resulting from RNAi-induced gene silencing is informative for characterizing the function of an endogenous gene in an organism. In addition, employing RNAi provides an alternative method for insect control and the related research has made significant progress in recent years (Katoch et al., 2013). For example, using plant-mediated RNAi to silence expression of a cotton bollworm (*Helicoverpa zea*, Lepidoptera: Noctuidae) P450 monooxygenase gene has been shown to impair larval tolerance of gossypol (Mao et al., 2007).

To reveal whether *A. planipennis* demonstrates strong RNAi response, we conducted a genome-wide screening of the three RNAi core component genes, Dcr-2, Ago-2 and R2D2. Characterization of these *A. planipennis* genes (*Apla-Dcr-2*, *Apla-Ago-2* and *Apla-R2D2*) indicates that they are structurally and evolutionally conserved. We have previously identified and characterized *AplaScrB-2*, a β -fructofuranosidase gene that was acquired by *A. planipennis* through the horizontal gene transfer (HGT) mechanism and is highly expressed in the beetle midgut (Zhao et al., 2014). Here, we demonstrated that the expression of *AplaScrB-2* was significantly reduced through micro-injection of dsRNA into the *A. planipennis* adults. This study, according to our knowledge, is the first report of RNAi application in the invasive beetle, *A. planipennis*.

2. Materials and methods

2.1. Identification of *Apla-Dcr-2*, *Apla-Ago-2* and *Apla-R2D2*

To retrieve the sequence of Dcr-2, Ago-2 and R2D2 in *A. planipennis*, sequence similarity searches were conducted by running

tBLASTn (E-value threshold: 1×10^{-10}) in a local computer using the query protein sequences *T. castaneum* Dcr-2 (Tcas-Dcr-2; NCBI accession number: NP_001107840.1), Ago-2a (Tcas-Ago-2a; NCBI accession number: NP_001107842.1) and R2D2 (Tcas-R2D2; NCBI accession number: NP_001128425.1) to search against the *A. planipennis* genome database (author's unpublished data). These searches hit two *A. planipennis* genomic DNA scaffolds, #2024918 (E-value = 5×10^{-143}) and #2025360 (E-value = 1×10^{-74}) exhibiting sequence similarity to Tcas-Dcr-2, three scaffolds, #2040102 (E-value = 3×10^{-97}), #2047353 (E-value = 1×10^{-43}) and #2000557 (E-value = 7×10^{-20}) exhibiting sequence similarity to Tcas-Ago-2a, and one scaffold #2043176 (E-value = 4×10^{-32}) exhibiting sequence similarity to Tcas-R2D2. These genomic DNA scaffold sequences were retrieved for manual annotation with bioinformatics tool supports including the DNA translation tool (<http://web.expasy.org/translate/>), the GENESH gene prediction server (<http://linux1.softberry.com/berry.phtml?topic=fgenes&group=programs&subgroup=gfind>) (Solovyev et al., 2006), and gene homology search using the BLAST program (<http://www.ncbi.nlm.nih.gov/>). The putative *A. planipennis* protein sequences showing similarity to Dcr-2, Ago-2 and R2D2 were obtained.

Given that the tBLASTn searches identified two Dcr-2-like and three Ago-2-like genes in the *A. planipennis* genome, in order to determine whether they are *Apla-Dcr-2* or *Apla-Ago-2* genes, phylogenetic analyses were performed (methodology of phylogenetic analyses described below in Section 2.2). For the identification of *Apla-Dcr-2*, phylogeny of the two putative Dcr-2-like protein sequences derived from genomic DNA scaffolds #2024918 and #2025360, as well as the protein sequences of *D. melanogaster* Dcr-1 (Dmel-Dcr-1; NCBI accession number: AAF56056.1), Dmel-Dcr-2 (NCBI accession number: NP_523778.2), *T. castaneum* Dcr-1 (Tcas-Dcr-1, NCBI accession number: XP_968993.2), Tcas-Dcr-2, and *C. elegans* Dcr-1 (Cele-Dcr-1; NCBI accession number: CCD71200.2) was tested. It should be noted that although Dcr-1 and Dcr-2 share a certain degree of sequence similarity, they are functionally distinct: Dcr-1 is involved in miRNA pathway while Dcr-2 is required for processing of siRNA precursor (Lee et al., 2004). This phylogenetic analysis attempted to identify the *Apla-Dcr-2* protein(s) and exclude *Apla-Dcr-1* for further characterization.

Similarly, the phylogenetic relationship was determined for the three Ago-2-like proteins derived from *A. planipennis* genome i.e. DNA scaffolds #2040102, #2047353 and #2000557 with Dmel-Ago-1 (NCBI accession number: AAF58314.1), Dmel-Ago-2 (NCBI accession number: AGB94575.1), Dmel-Ago-3 (NCBI accession number: NP_001163498.1), Dmel-Piwi (P-element induced wimpy testis; NCBI accession number: AAF53043.1), Tcas-Ago-1 (NCBI accession number: XP_971295.2), Tcas-Ago-2a (NCBI accession number: NP_001107842.1), Tcas-Ago-2b (NCBI accession number: NP_001107828.1), Tcas-Ago-3 (NCBI accession number: XP_968053.2) and Tcas-Piwi (NCBI accession number: EFA07425.1). Among these Argonaute proteins, only Ago-2 is essential in the RNAi pathway. In contrast, Ago-3 and Piwi are important for transcriptional silencing (Hutvagner and Simard, 2008). Therefore, the *A. planipennis* Ago-2 protein was identified based on the phylogenetic clustering and selected for further characterization.

It should be noted that our assembly of the *A. planipennis* genome (not yet deposited at NCBI) is independent from the one deposited at NCBI (*Apla_1.0*; GenBank #: GCA_000699045.1). To validate the gene sequences retrieved from our database, the derived sequences were further searched within the *Apla_1.0* sequence assembly using BLASTn and high sequence similarities (sequence identity > 99%, E-value = 0) (Table 2).

2.2. Phylogenetic analyses

The amino acid sequences of proteins were edited in FASTA format and loaded to MEGA 6 software. Sequences were aligned using MUSCLE (Edgar, 2004) and phylogenetic trees were subsequently constructed in MEGA 6 using the neighbor-joining method with pairwise deletion (Saitou and Nei, 1987; Tamura et al., 2013). Bootstrap value, the percentage of replicate trees in which the associated taxa clustered together, was tested for 5000 replicates.

2.3. Double-stranded RNA synthesis

AplaScrB-2 (treatment, NCBI accession number: KJ634683) and the green fluorescent protein (*GFP*, control, NCBI accession number: M62653.1) genes were amplified by PCR using primers containing T7 RNA polymerase promoter (Table 1). The PCR products, 475-bp for *AplaScrB-2* and 535-bp for *GFP*, were gel purified using the QIAquick Gel Extraction Kit (QIAGEN Inc, Valencia, CA, USA). With the purified PCR products serving as templates, the dsRNA was synthesized and purified using the MEGAscript® RNAi Kit (Ambion, Austin, TX, USA) according to the protocol provided. The synthesized dsRNA was then quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and its purity and integrity were examined by agarose gel electrophoresis.

2.4. Microinjection of double-stranded RNA

Ash trees, *Fraxinus pennsylvanica* Marsh, infested with *A. planipennis* that were overwintering in pupal cells as mature larvae or pre-pupae were located during winter 2013 at a field site in Beaverton, MI, USA. Trees were felled, cut into 90 cm logs, and then transported to the USDA Forest Service laboratory at Michigan State University where they were stored in a cold room (4 °C). When adults were needed, logs were placed into rearing tubes held in the laboratory at room temperature for 4–6 weeks until beetle emergence. Freshly emerged *A. planipennis* beetles were immediately separated by sex and held in 295 ml plastic cups with an evergreen ash, *Fraxinus uhdei*, leaf in a vial of water for feeding. When beetles were 2–4 days old they were selected for dsRNA microinjection. By making use of apparent death response shown by *A. planipennis* adults to outward touch, we immobilized the beetles on a wooden plate under a micro-injector using adhesive tape and performed dsRNA microinjections using the Nanoject II™ Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). The concentration of dsRNA solution used for injection was 1 µg/µl dissolved in the elution buffer which was supplied in the MEGAscript® RNAi Kit. RNA was injected into the soft suture

region between head and prosternum of *A. planipennis* adults. Per individual, 200 ng of dsRNA was injected. Insects were kept in plastic cages covered with nylon net at room temperature after injection and supplied with fresh evergreen ash tree leaves. Collection of dsRNA-injected *A. planipennis* adults started on the next day after microinjection (day 1 post-injection) and continued for six consecutive days (day 1 to day 6 post-injection). On each day, 4 live *AplaScrB-2* dsRNA-treated beetles (treatment) including 2 males and 2 females were pooled as one biological replicate for RNA isolation. In the same way, 4 *GFP* dsRNA-treated beetles (control) were pooled as one biological replicate on each day for RNA isolation. Four biological replicates in both treatment and control were performed for statistical analysis.

2.5. Total RNA Isolation and first-strand cDNA synthesis

Double stranded RNA-injected beetles were homogenized in liquid nitrogen and the total RNA was extracted using 1 ml of Trizol reagent (Invitrogen, Grand Island, NY) following the protocol provided. Turbo DNase treatment (Ambion, Austin, TX) was then employed to obtain DNA-free RNA samples. RNA concentration and purity were determined using the Nanodrop 2000 spectrophotometer. All the samples were verified to have A_{260}/A_{280} ratio of 1.8–2.0. Approximately 0.5 µg of total RNA was used to synthesize the first strand cDNA using the SuperScript™ First-strand synthesis system following the manufacturer's protocol (Invitrogen, Grand Island, NY). Quantification of the first-strand cDNA samples was performed with the Nanodrop 2000 spectrophotometer.

2.6. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to examine the gene expression profiles of *A. planipennis* adults treated with *GFP* or

Table 2
Putative Dicer, Argonaute and R2D2 proteins identified in *A. planipennis*.

Putative protein	NCBI accession#	Protein Size (Amino acids)	Genomic DNA [*] Scaffold#	Apla_1.0 [^] Scaffold#
Apla-Dcr-1	KP036489	1860	#2025360	#207
Apla-Dcr-2	KP036490	1625	#2024918	#302
Apla-Ago-1	KP036491	925	#2047353	#398
Apla-Ago-2	KP036492	867	#2040102	#39
Apla-Ago-3	KP036493	860	#2000557	#3514
Apla-R2D2	KP036494	314	#2043176	#205

^{*} Authors' *A. planipennis* genome assembly.

[^] *A. planipennis* genome assembly Apla_1.0; GenBank; #: GCA_000699045.1

Table 1
Primers used for PCR analysis in this study.

Primer	Sequence (5' to 3')
GFP-T7-F [*]	TAATACGACTCACTATAGGAGAGAGTGGAGGGTGAAGGTGA
GFP-T7-R [^]	TAATACGACTCACTATAGGAGAGAAAGGGCAGATTGTGTGGAC
AplaScrB-2-T7-F	TAATACGACTCACTATAGGAGAGATCATTGAATTGGATCATGGA
AplaScrB-2-T7-R	TAATACGACTCACTATAGGAGAGAAAGCTTCAGGATTATTTCG
AplaScrB-1-qRT-F	ATGAATTCGGTTTGTGTTG
AplaScrB-1-RT-R	AGGTTTGTCTGTTTCGTTG
AplaScrB-2-qRT-F	TTCGTCTTATGATCTATTTCG
AplaScrB-2-RT-R	GAAAGTAGCAATATATGGAAG
ApTEF1α-F	CATTGAAACCTACGTTGTCGC
ApTEF1α-R	ACTGGAGTGCTTAAACCTGG

The underlined nucleotides are T7 RNA polymerase promoter sequence.

^{*} Forward primer.

[^] Reverse primer.

AplaScrB-2 dsRNA. To test the expression of *AplaScrB-2* by qRT-PCR, *AplaScrB-2*-qRT-F and *AplaScrB-2*-RT-R (Table 1, Zhao et al., 2014) were used as the forward and reverse primers, respectively. Quantification of *AplaScrB-1* was also performed by qRT-PCR to test off-target effects of *AplaScrB-2* dsRNA using *AplaScrB-1*-qRT-F and *AplaScrB-1*-RT-R as the forward and reverse primers, respectively (Table 1, Zhao et al., 2014). The total volume of each qRT-PCR reaction was 10 μ L, consisting of 1 μ L forward primer (5 μ M), 1 μ L reverse primer (5 μ M), 2 μ L cDNA template (20 ng/ μ L), 5 μ L SYBR green master mix (Bio-Rad, Hercules, CA, USA) and 1 μ L of nuclease free water. The PCR reactions were performed at the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 min, and final step at 72 °C for 10 min. Raw data was obtained as cycle threshold (Ct) values determined by single threshold mode from quantitative real-time PCR. The primer efficiency was tested by running a standard curve for each set of primers. Relative expression values were calculated by comparative Ct method (Livak and Schmittgen, 2001). The expression of the genes across different samples analyzed was normalized using the *A. planipennis* translation elongation factor 1- α gene (primers are ApTEF1 α -F and ApTEF1 α -R listed in Table 1) as internal control (Rajarapu et al., 2012).

2.7. Statistical analysis

The statistical differences among gene expression levels were analyzed by one-way analysis of variance (ANOVA) with a significance level (α) of 0.05 using Minitab software (version 16, Minitab Inc, State College, PA, USA).

3. Results

3.1. Identification of *Apla-Dcr-2*, *Apla-Ago-2* and *Apla-R2D2* genes

We identified *A. planipennis* genomic DNA scaffolds showing significant sequence similarity (E -value $< 10^{-10}$) to *T. castaneum* core RNAi machinery genes by using *T. castaneum* Dcr-2, Ago-2a and R2D2 protein sequences as tBLASTn search queries. Through manual annotation, the putative protein sequences were predicted and retrieved, which are two Dcr-2-like proteins from Scaffolds #2024918 and #2025360, three Ago-2-like proteins from Scaffolds #2040102, #2047353 and #2000557, and one R2D2-like protein from Scaffold #2043176 (Table 2). To examine whether or not all these proteins are *A. planipennis* RNAi machinery core components, they were used to construct phylogenetic trees along with the Dcr and Ago homologs derived from *T. castaneum*, *D. melanogaster*, and/or *C. elegans* (Fig. S1). Phylogenetic analyses showed that the *A. planipennis* Dicer protein retrieved from Scaffold #2025360 is clustered with the Dcr-1 proteins of *T. castaneum*, *D. melanogaster* and *C. elegans* with a strong bootstrap value support (100%), indicating that it is the *A. planipennis* Dcr-1 homolog (*Apla-Dcr-1*). In contrast, the dicer protein derived from *A. planipennis* genomic DNA Scaffold #2024918 is perfectly (bootstrap value = 100%) clustered with Tcas-Dcr-2 and therefore is named *Apla-Dcr-2* (Fig. S1A). In the phylogeny of Ago proteins (Fig. S1B), the *A. planipennis* Ago protein located in Scaffold # 2047353 forms a unique subclade with Tcas-Ago-1 and Dmel-Ago-1, and is named *Apla-Ago-1*. Similarly, *Apla-Ago-2* was identified as the one derived from Scaffold #2040102 and *Apla-Ago-3* is from Scaffold #2000557. The identification of *Apla-Dcr-2* and *Apla-Ago-2* through phylogenetic analyses agrees with the tBLASTn search results which showed that the best hit of Tcas-Dcr-2 in the *A. planipennis* genome database is Scaffold #2024918 (E -value = 5×10^{-143}) and that the best hit of Tcas-Ago-2a is Scaffold #2040102 (E -value = 3×10^{-97}).

3.2. Characterization and phylogenetic analysis of *Apla-Dcr-2*

The putative *Apla-Dcr-2* protein sequence was submitted for the detection of domain architecture using the ScanProsite tool (<http://prosite.expasy.org/scanprosite/>, De Castro et al., 2006). Like most other Dcr-2 identified in coleopteran insects (Tomoyasu et al., 2008), *Apla-Dcr-2* contains two amino-terminal helicase domains, a Dicer double-stranded RNA-binding fold (Dicer_DSRBF) domain, a PAZ domain and two carboxy-terminal RNase III domains (Fig. 1A). This domain signature appeared to be conserved in coleopteran Dcr-2 proteins except that, according to the PrositeScan analysis, *L. decemlineata* Dicer-2 (Ldec-Dicer-2) lacks the Dicer_DSRBF domain but owns a carboxy-terminal dsRNA binding domain (DSRBD) that other coleopteran species examined thus far do not possess. However, compared to *T. castaneum* Dcr-1 (Tcas-Dcr-1), the miRNA pathway Dicer protein that has similar domain architecture, the coleopteran Dcr-2 proteins have high Prosite profile hit scores (16.6–24 versus 8.4) in the first N-terminal helicase domain but low scores (14.4–17.2 versus 25.4) in the PAZ domain.

The neighbor-joining phylogenetic analysis showed that all the selected insect Dcr-2 proteins form a unique clade, separating from *D. melanogaster* and *T. castaneum* Dcr-1 proteins (Fig. 1B). As expected, the Dcr-2 proteins clustered according to the insect Orders they were derived from and specifically, the four coleopteran Dcr-2 including *Apla-Dcr-2* were clustered together with a bootstrap value of 100%.

3.3. Characterization and phylogenetic analysis of *Apla-Ago-2*

Function domain detection using the ScanProsite tool identified two domains, PAZ and PIWI, in *Apla-Ago-2*, as well as other coleopteran homologs (Fig. 2A). Sequence alignment of these two domains indicated that they are highly conserved in coleopteran Ago-2 proteins (Fig. S2). The amino acid residues involved in binding with siRNA (Ma et al., 2004) are especially conserved in the PAZ domain; specifically, *Apla-Ago-2* shared nearly identical amino acid residues with Tcas-Ago-2 proteins (Fig. S2A). In the PIWI domain, several amino acids are believed to interact with three non-bridging oxygen atoms at 5' phosphate of siRNA and are important in binding with siRNA (Elkayam et al., 2012). Sequence alignment demonstrated that these signature amino acid residues are present and identical in all coleopteran Ago-2 proteins including *Apla-Ago-2* (Fig. S2B).

The phylogenetic tree constructed using the Neighbor-joining method analysis demonstrated that all the insect Ago-2 proteins analyzed are closely related to one another but distantly related to Tcas-Ago-1 and Tcas-Ago-3 (Fig. 2B). Five Ago-2 proteins derived from four coleopteran species are clustered in a subclade with a high bootstrap value support (97%), and are separated from Ago-2 of non-coleopteran insects.

3.4. Characterization and phylogenetic analysis of *Apla-R2D2*

The domain analysis on the putative *Apla-R2D2* protein at ScanProsite showed the presence of two dsRNA binding domains (DSRBD), a feature conserved in insect R2D2 including Tcas-R2D2 and Dpon-R2D2 and dissimilar to Tcas-PASHA, a miRNA pathway protein that contains only one DSRBD domain (Fig. 3A). The DSRBD domains in coleopteran R2D2 proteins have similar Prosite profile hit scores ranging from 13.8 to 15.8. Additionally, the positions of both DSRBD domains are relatively close to the N-termini of R2D2, in contrast to Tcas-PASHA whose DSRBD domain is closer to the C-terminus (Fig. 3A).

Phylogenetic analysis clustered *Apla-R2D2* with Tcas-R2D2 and Dpon-R2D2 (bootstrap value = 91%), homologs of another two

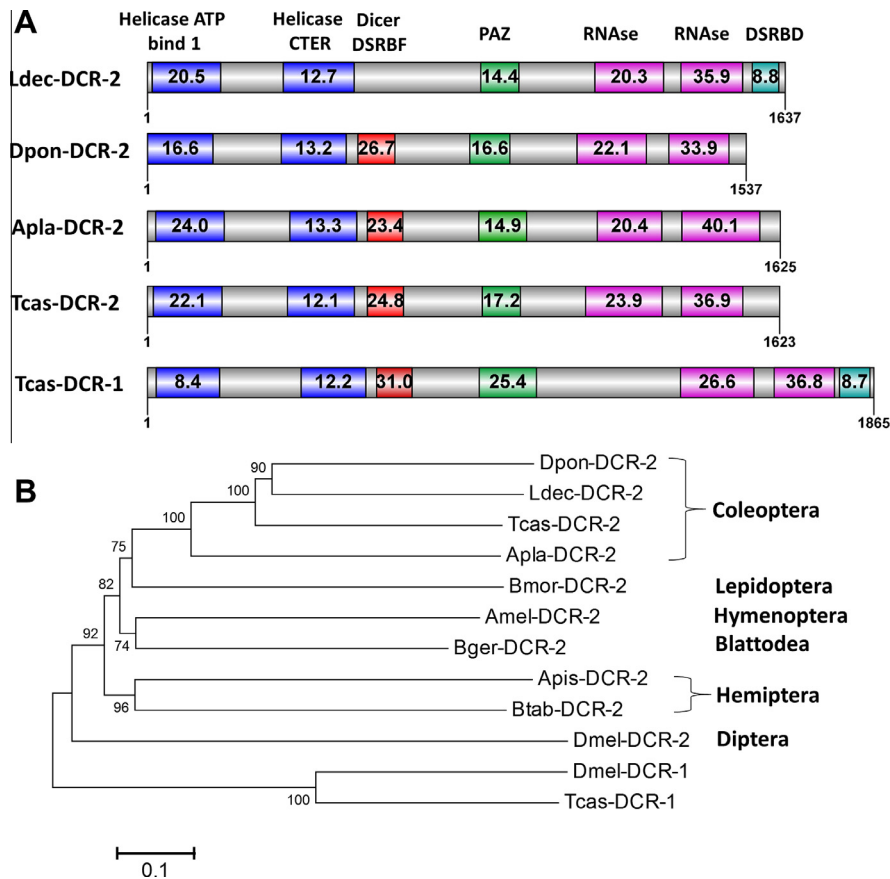


Fig. 1. Domain architecture and phylogenetic analysis of the *A. planipennis* Dicer-2 protein. (A) Domain architecture of the putative *A. planipennis* (Apla) Dicer-2 (DCR-2) protein compared to *Leptinotarsa decemlineata* (Ldec) DCR-2 (Swevers et al., 2013), *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae) (Dpon) DCR-2 (NCBI accession number: ERL92210.1), *Tribolium castaneum* (Tcas) DCR-2 (NCBI accession number: NP_001107840.1) and Tcas-DCR-1 (NCBI accession number: XP_968993.2). Conserved domains and their Prosite profile hit scores are indicated. (B) Neighbor-joining inferred phylogeny of insect Dicer proteins, including four coleopteran DCR-2 proteins, as well as *Bombyx mori* (Lepidoptera: Bombycidae) (Bmor) DCR-2 (NCBI accession number: NP_001180543.1), *Apis mellifera* (Hymenoptera: Apidae) (Amel) DCR-2 (NCBI accession number: XP_006571379.1), *Blattella germanica* (Blattodea: Blattellidae) (Bger) DCR-2 (NCBI accession number: CCF23094.1), *Acyrtosiphon pisum* (Hemiptera: Aphididae) (Apis) DCR-2 (NCBI accession number: XP_003240110.1), *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Btab) DCR-2 (NCBI accession number: AIC07485.1), Dmel-DCR-2, Dmel-DCR-1 (NCBI accession number: AAF56056.1) and Tcas-DCR-1. Bootstrap values (5000 replicates) are shown next to the branches.

coleopteran species (Fig. 3B). These coleopteran R2D2 proteins are closely related to other insect R2D2 homologs, but distantly related to Tcas-PASHA in the phylogenetic tree constructed (Fig. 3B).

3.5. Gene knockdown induced by dsRNA microinjection

Recently, two HGT-associated β -fructofuranosidase genes, *AplaScrB-1* and *AplaScrB-2*, were identified in *A. planipennis* (Zhao et al., 2014). Unlike *AplaScrB-1* whose expression is neither stage-specific nor tissue-specific, *AplaScrB-2* is highly expressed in the adult midgut. To test the RNAi-mediated gene silencing in *A. planipennis*, we injected 200 ng of 485-bp *AplaScrB-2* dsRNA into each freshly emerged beetles. A 535-bp dsRNA fragment of *GFP* was injected into beetles as a control, which should not silence *AplaScrB-2*. Quantitative real-time PCR was performed to determine the gene expression level of *AplaScrB-2* in beetles injected with *AplaScrB-2* dsRNA compared with those injected with *GFP* dsRNA. On day 1 post-injection, no significant ($p > 0.05$) gene expression difference was observed between the treatment (*AplaScrB-2* dsRNA injection) and the control (*GFP* dsRNA injection). However, starting from day 2 to day 6, the expression of *AplaScrB-2* was significantly ($p < 0.05$) repressed in *AplaScrB-2* dsRNA-treated beetles compared to the control, and the decreased level ranged from 73% reduction (day 3) to 90% reduction (day 6) (Fig. 4).

In order to check whether *AplaScrB-2* dsRNA delivered into *A. planipennis* has off-target effects, we performed qRT-PCR analysis on the same beetle samples but using a pair of PCR primers specific to *AplaScrB-1*, a member in the same gene family as *AplaScrB-2* (Zhao et al., 2014). Results showed that the gene expression profiles of *AplaScrB-1* are not significantly ($p > 0.05$) influenced by *AplaScrB-2* dsRNA compared to by *GFP* dsRNA in the six days examined, further supporting our findings that *AplaScrB-2* dsRNA may trigger the *A. planipennis* RNAi machinery, specifically degrading *AplaScrB-2* mRNA molecules *in vivo* (Fig. 5).

4. Discussion

RNAi-mediated gene silencing is an important mechanism of post-transcription gene regulation that has been identified in organisms ranging from fungi to mammals (Carthew and Sontheimer, 2009). This mechanism and associated RNAi pathway genes have been identified and characterized in many insect species, e.g., *D. melanogaster*, *T. castaneum* and *B. mori* (Kim et al., 2006; Kolliopoulou and Swevers, 2013; Tomoyasu et al., 2008). Among the coleopteran species, *T. castaneum* is one of the most successful examples that exhibits a highly sensitive RNAi response to introduction of exogenous dsRNA (Miller et al., 2012). In this study, we identified three RNAi pathway core components, Dcr-2, Ago-2 and R2D2, in another coleopteran species, *A. planipennis*,

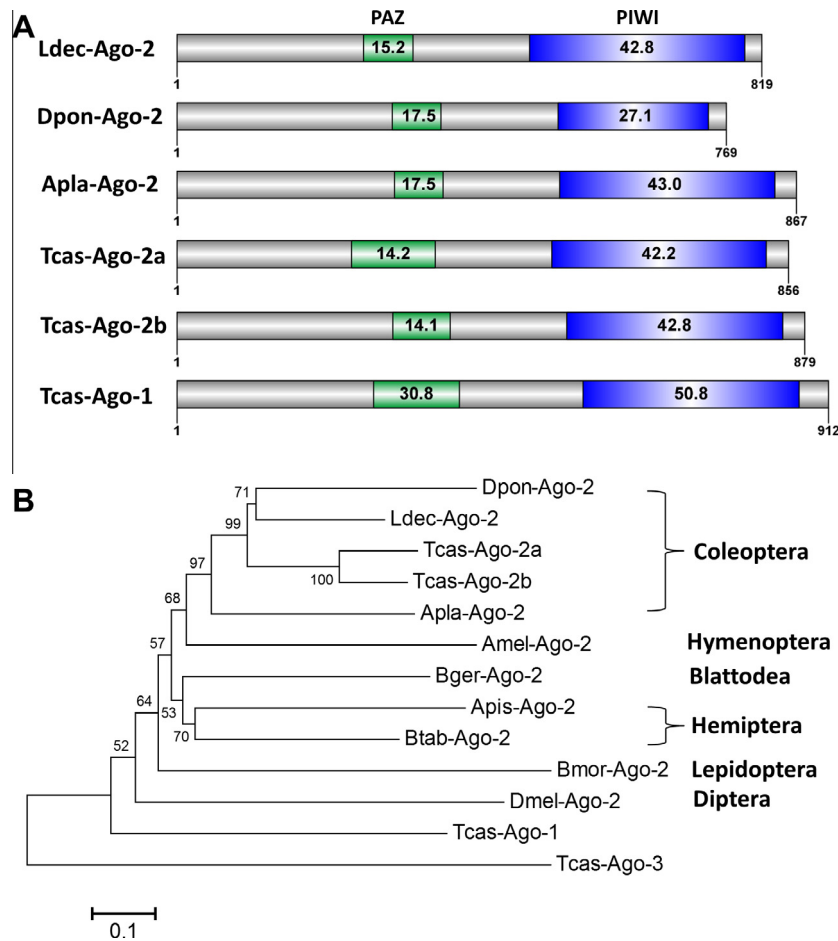


Fig. 2. Domain architecture and phylogenetic analysis of the *A. planipennis* Argonaute-2 (Ago-2) protein. (A) Domain architecture of the putative Apla-Ago-2 protein compared to Ldec-Ago-2 (Swevers et al., 2013), Dpon-Ago-2 (NCBI accession number: ENN72015.1), Tcas-Ago-2a (NCBI accession number: NP_001107842.1), Tcas-Ago-2b (NCBI accession number: NP_001107828.1) and Tcas-Ago-1 (NCBI accession number: XP_971295.2). The PAZ and PIWI domains and their Prosite profile hit scores are indicated. (B) Neighbor-joining inferred phylogeny of insect Argonaute proteins, including five coleopteran Ago-2 proteins, as well as Amel-Ago-2 (NCBI accession number: XP_395048.4), Bger-Ago-2 (NCBI accession number: CCV01213.1), Apis-Ago-2 (NCBI accession number: XP_001944852.2), Btab-Ago-2 (NCBI accession number: AHY18683.1), Bmor-Ago-2 (NCBI accession number: NP_001036995.2), Dmel-Ago-2 (NCBI accession number: NP_648775.1), Tcas-Ago-1 (NCBI accession number: XP_971295.2) and Tcas-Ago-3 (NCBI accession number: XP_968053.2). Bootstrap values (5000 replicates) are shown next to the branches.

based on genome-wide homology searches. Characterization and phylogenetic analyses of these putative *A. planipennis* protein sequences, along with their homologs taken from other coleopteran species, revealed the conservative properties of these proteins during evolution. The strong evidence for conservation of the RNAi pathway genes in coleopterans may suggest a robust RNAi phenotype in beetles.

However, unlike *T. castaneum* that possesses two Ago-2 genes, *Tcas-Ago-2a* and *Tcas-Ago-2b*, we were only able to identify a single Ago-2 gene in the *A. planipennis* genome. Among the three putative *A. planipennis* Ago proteins which were found through a sequence homology search using *Tcas-Ago-2a* as the query sequence, only the best hit, the one derived from the genomic DNA scaffold #2040102, is phylogenetically clustered with other insect Ago-2 homologs. Screening the genome database of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae: Scolytinae) (NCBI accession numbers: GCA_000355655.1 for male genome assembly and GCA_000346045.2 for female genome assembly; Keeling et al., 2013), we also identified a single Ago-2 gene in both sexes of genome assembly, i.e., the male sequence Seq01054111 (NCBI accession number: APGK01054101) and the female sequence Seq01029088 (NCBI accession number: APGL01029028). Similarly, from the Colorado potato beetle (*L. decemlineata*) gut transcriptome, only one Ago-2 sequence was retrieved (Swevers et al., 2013). Hence, we hypothesize that

coleopteran species may have only one Ago-2 gene as an ancestral and common feature with an exception in *T. castaneum* that may have experienced a linear-specific gene duplication event to produce an additional paralog in the genome. This hypothesis is supported by the phylogenetic analysis which showed that *Tcas-Ago-2a* and *Tcas-Ago-2b* form a unique subclade in the constructed tree (Fig. 2B). Considering that Ago-2 is an essential component in the RNAi pathway and is required for siRNA duplex unwinding and specific mRNA cleavage (Okamura et al., 2004), retaining two copies of Ago-2 in the genome may account for a very efficient RNAi response in *T. castaneum*; delivering as little as 0.6 ng of dsRNA into a larva could significantly repress the expression of the target gene (Miller et al., 2012).

In this study, we successfully demonstrated the first RNAi example within the family Buprestidae, the largest and most diverse family of beetles. By injecting 200 ng of dsRNA per *A. planipennis* adult, the target gene expression level was reduced by 73%–90% and the RNAi response started to appear on day 2 and lasted at least until day 6. Although further experiments are needed to determine the minimum quantity of dsRNA required to significantly induce RNAi response, *A. planipennis* appeared to be sensitive in triggering the RNAi machinery in response to dsRNA introduction, when compared to some other insect species such as the Hessian fly, *M. destructor* (Aggarwal et al., 2014). Although both *AplaScrb-2* and *AplaScrb-1* share high sequence similarity

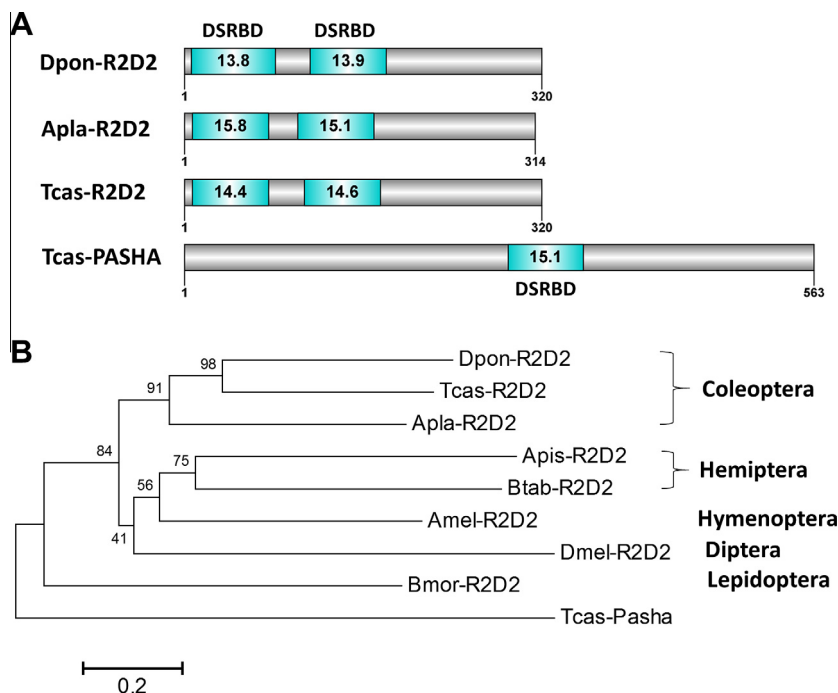


Fig. 3. Domain architecture and phylogenetic analysis of the *A. planipennis* R2D2 protein. (A) Domain architecture of the putative AplA-R2D2 protein compared to Dpon-R2D2 (NCBI accession number: ENN77206.1), Tcas-R2D2 (NCBI accession number: NP_001128425.1) and Tcas-PASHA (NCBI accession number: XP_971282.1). The dsRNA binding domains (DSRBD) and their Prosite profile hit scores are indicated. (B) Neighbor-joining inferred phylogeny of insect dsRNA binding proteins, including three coleopteran R2D2 proteins, as well as Apis-R2D2 (NCBI accession number: NP_001155644.1), Btab-R2D2 (NCBI accession number: AIC07486.1), Amel-R2D2 (NCBI accession number: XP_001121349.2), Dmel-R2D2 (NCBI accession number: NP_609152.1), Bmor-R2D2 (NCBI accession number: NP_001182007.1), and Tcas-PASHA. Bootstrap values (5000 replicates) are shown next to the branches.

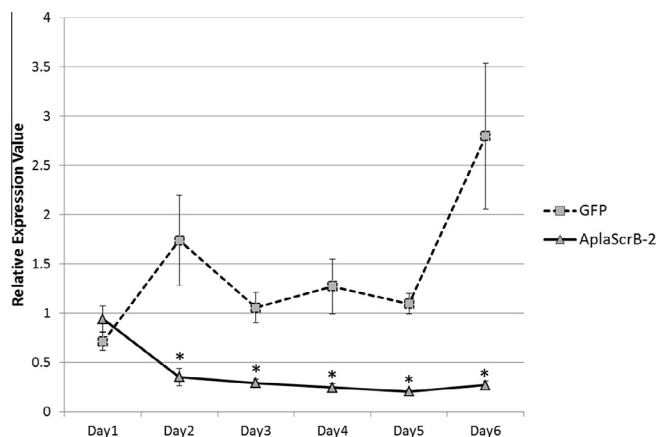


Fig. 4. Validation of RNAi-induced gene knockdown at different time points. Quantitative real-time PCR analysis was performed to assess the *AplAcrB-2* gene expression levels of *A. planipennis* adults injected with *AplAcrB-2* dsRNA (treatment, unbroken line) in comparison with those injected with GFP dsRNA (control, dashed line). Transcript levels of insect whole body collected from day 1 to day 6 after injection were measured. Data shown are the mean $\pm$ S.E.M. ($n = 4$) and an asterisk indicates significant difference ($P < 0.05$) from control.

(63% nucleotide identity), the 475-bp *AplAcrB-2* dsRNA fragment injected was highly target specific and did not influence the expression of *AplAcrB-1*. *AplAcrB-2* is almost exclusively expressed in the midgut of *A. planipennis* adults (Zhao et al., 2014); however, its expression was repressed by dsRNA molecules injected through the suture region between head and prothorax, implying a mechanism for systemic dsRNA uptake and spreading in the insect. Therefore, future investigations should aim at identifying and characterizing *A. planipennis* homologs of the systemic RNAi response genes including a multi transmembrane protein gene *sid-1* (systemic RNA interference deficient-1) and several

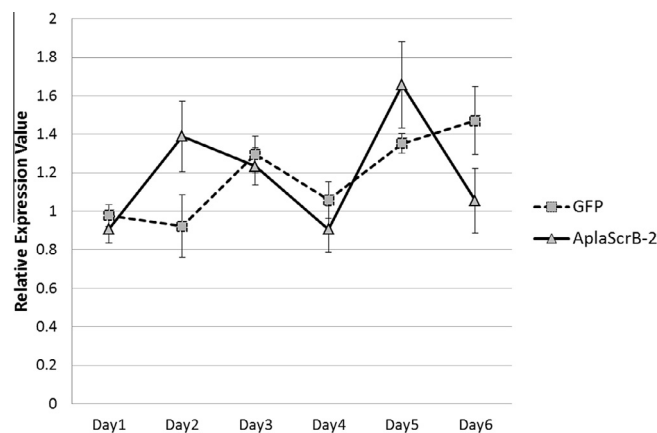


Fig. 5. Assessment of off-target effects of *AplAcrB-2* dsRNA at different time points. Quantification of *AplAcrB-1* expression level to assess off-target effects of *AplAcrB-2* dsRNA in *A. planipennis* adults at different time points (from day 1 to day 6 post-injection). Whole body *AplAcrB-1* transcript levels in adults from day 1 to day 6 after injection with *AplAcrB-2* dsRNA (treatment, unbroken line) and GFP dsRNA (control, dashed line) are shown.

RNAi spreading defective genes (Tijsterman et al., 2004; Winston et al., 2002).

Peak expression of *AplAcrB-2* appears in the adult stage and in the midgut tissue, and thus it is hypothesized that *A. planipennis* gained this gene from bacteria to efficiently digest dietary sucrose (Zhao et al., 2014). Hence, we anticipated that the RNAi-mediated *AplAcrB-2* gene knockdown would lead to digestive disorders and high insect mortality rate. However, based on a 10-day window observation, the mortality rate of beetles treated with *AplAcrB-2* dsRNA was not statistically different ($p > 0.05$, t -test) from that of beetles treated with GFP dsRNA (Table 3). The absence of RNAi phenotype may be owing to two possible alternative factors. One is the

Table 3Ten-day mortality rate of *A. planipennis* beetles post RNAi microinjections.

Group	Mortality rate	Average mortality (%)	Standard deviation (%)
Treatment-1	40% (N = 20)	48.8	12.4
Treatment-2	57.5% (N = 40)		
Control-1	35% (N = 20)	41.7	9.5
Control-2	48.4% (N = 31)		

Note: *p*-value = 0.293 (*t*-test); *N* = number of beetles injected.

incomplete repression of RNAi on *AplaScrB-2* expression, which gave rise to certain amounts of “escaped” mRNA molecules that produced β -fructofuranosidases sufficient for dietary sucrose digestion. The other factor is the upregulation of *AplaScrB-2* paralogs and/or other sucrose-breaking enzyme genes, which compensates the knockdown of *AplaScrB-2*. A genome-wide survey identified three *ScrB* paralogs in *A. planipennis*, in addition to *AplaScrB-1* and *AplaScrB-2* that we have previously reported. Although these newly found sequences contain either incomplete coding regions due to sequence gaps or contain a frame-shift mutation (data not shown) and their expression profiles are unknown, the possibility exists that they, or one or two of them, encode digestive β -fructofuranosidases in the adult midgut. Moreover, the presence of over 10 α -glucosidase-encoding genes (data not shown), which belong to another family (Glycoside hydrolase family 31) of sucrose-breaking enzymes (Terra and Ferreira, 1994), in the *A. planipennis* genome may also contribute to the compensation for the RNAi-induced *AplaScrB-2* knockdown.

In conclusion, we identified and characterized three RNAi pathway core component genes, *Apla-Dcr-2*, *Apla-Ago-2* and *Apla-R2D2*, in *A. planipennis*, an invasive coleopteran insect species. The structural and evolutionary conservation of these genes indicate that *A. planipennis* has a sensitive RNAi machinery, which can be triggered by exogenous dsRNA molecules to knock down the expression of a target gene. This is supported by our experimental evidence that injecting the *AplaScrB-2* dsRNA fragment significantly reduced expression of the target gene in *A. planipennis*. This study provides the foundation for RNAi-based functional genomics research in *A. planipennis*. Further, for the application of RNAi in *A. planipennis* control, future studies are needed including the examination of specific candidate genes that debilitate its development; and perhaps other modes of dsRNA delivery including feeding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2014.12.002>.

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