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Rapid and persistent RNAi response in western corn rootworm adults

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Abstract

RNA interference (RNAi) has proven effective for controlling pest insects such as western corn rootworm (WCR), *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Previous studies have shown that WCR adults display a robust RNAi response to orally-administered double-stranded RNA (dsRNA). However, it is unclear how quickly the response occurs after ingestion or how long RNAi effect lasts after WCR stop ingesting diet containing dsRNA. In the current study, WCR adult females were provided with diet treated with dsRNAs of *Laccase 2* and *Argonaute 2*, two nonessential genes, for 8 days. RNAi response in WCR females commenced as early as 10 h after exposure to dsRNA and lasted up to 40 days after exposure to dsRNA ended. Our results show that dsRNA-mediated RNAi response in WCR females is rapid and long-lasting. These findings suggest that even a short-term ingestion of transgenic plants expressing dsRNA by WCR may have a sustained impact on this insect.

1. Introduction

RNA interference (RNAi) is a process in which double-stranded RNA (dsRNA) is processed (Carrington and Ambros, 2003) into short interfering RNAs (siRNAs), which induce rapid and sustained degradation of mRNA contain-

ing sequences complementary to the siRNAs (Fire et al., 1998; Vance and Vaucheret, 2001; Yang et al., 2011). RNAi response has been postulated as a mechanism of defense against viral infection and mobilized transposable elements, as well as a process to regulate gene translation during development (Carrington and Ambros, 2003; Fire et al., 1998; Vance and Vaucheret, 2001; Liu et al., 2018).

Initially described in the nematode *Caenorhabditis elegans*, RNAi has been documented in fungi, plants, and animals (including insects) (Fire et al., 1998; Mello and Conte Jr., 2004). In many organisms, the RNAi-mediated gene silencing effect is limited to the cells where dsRNA is exogenously introduced and/or endogenously expressed. In other organisms, RNAi is more systemic in nature, with silencing signals spreading between cells and tissues. Furthermore, some organisms (e.g., *C. elegans* and some insects in the order Coleoptera) exhibit robust environmental RNAi (eRNAi), achieving sequence-specific gene silencing using dsRNA taken up from environment (e.g., via midgut) (Whangbo and Hunter, 2008). RNAi can be a powerful tool for functional genomic studies and has the potential to be used in the control of agricultural pests such as western corn rootworm (WCR, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)) where knockdown of genes with essential function results in mortality (Baum et al., 2007; Bolognesi et al., 2012; Camargo et al., 2018; Rangasamy and Siegfried, 2012; Wu et al., 2017).

WCR is an adaptive, invasive, and economically important pest of maize in the United States and Europe. An economic analysis estimates that costs of control and yield loss associated with WCR damage exceed US$1 billion annually in the U.S. alone (Sappington et al., 2006). As a univoltine pest, WCR overwinters in the soil as eggs. WCR larvae cause damage to corn by feeding on roots after they hatch in late spring to early summer. WCR adults also contribute to crop damage through feeding on corn silks.

Current approaches for controlling WCR include crop rotation, broad-spectrum soil insecticides, and transgenic crops expressing protein toxins from *Bacillus thuringiensis*. However, WCR has developed resistance to many of these practices (Gray et al., 2009; Gassmann, 2012; Gassmann et al., 2011; Siegfried and Spencer, 2012), making it necessary to develop novel and more sustainable measures to control this pest.

Alternative pest management strategies that have been proposed to control WCR include the use of transgenic plants expressing dsRNA specific to genes that are important for survival. The first proof of principle study was performed by Baum et al. (Baum et al., 2007), who tested the larvae of several insects from the order Coleoptera for their propensity for eRNAi and found that WCR larvae displayed a rapid (i.e., 12 h) and systemic RNAi response after ingesting artificial diet treated with dsRNAs of essential genes (e.g., *V-ATPases*). The silencing of these essential genes was fol-

lowed by larval stunting and mortality. Furthermore, these authors showed that transgenic plants expressing dsRNAs of these essential genes sustained reduced WCR feeding damage, suggesting that RNAi pathway can be exploited to control WCR larvae via dsRNA expressed in transgenic plants (Baum et al., 2007).

WCR adults may also be a target for RNAi-based pest control strategy. Lethal RNAi effects in WCR adults were first reported by Rangasamy et al. in 2012 (Rangasamy and Siegfried, 2012) who demonstrated that silencing of the *V-ATPase A* gene in WCR females commenced 24 h after they were exposed to artificial diet treated with dsRNA of *V-ATPase A* and that 95% females died within two weeks of continuous exposure to *V-ATPase A* dsRNA (Rangasamy and Siegfried, 2012). In addition to essential genes, reproductive genes may also be a target for RNAi-based control of WCR. A recent report showed that WCR females treated with dsRNAs of two reproductive genes (i.e., *dvvgr* and *dvbol*) that were delivered in diet or expressed in transgenic corn displayed reduced fecundity (Niu et al., 2017).

WCR adults apparently can pass on the dsRNA-initiated silencing signal to progeny in a process termed parental RNAi (Khajuria et al., 2015; Velez et al., 2016a). Knockdown of genes that are important for embryonic development in WCR females resulted in reduced hatching in the embryos they produced. Hence, the capacity for both eRNAi and parental RNAi makes WCR females an attractive target in RNAi-based pest control strategies that aim to affect both adults and larvae by pyramiding gene targets affecting these life stages. Despite many studies that cover various topics on RNA interference in WCR, some basic attributes of this important process in this insect are still unknown. For example, it is unclear whether sustained gene silencing in WCR adults requires continuous ingestion of dsRNA. It is also unclear whether RNAi response in WCR adults is as rapid as that in larvae.

In the current study, we profiled the expression of *Laccase 2* (*Lac2*) and *Argonaute 2* (*Ago2*) in WCR females during and after the exposure to the dsRNAs of these two genes. *Lac2* encodes a phenoloxidase required for cuticle sclerotization and pigmentation in larvae, and its silencing does not appear to affect the survival of adults (Alves et al., 2010; Velez et al., 2016b). Knockdown of *Ago2* disrupts dsRNA-mediated gene silencing in WCR (Velez et al., 2016b; Miyata et al., 2014) and does not appear to affect adult survival (Wu et al., 2017). The use of dsRNAs of two nonessential genes in the current study allowed us to characterize the RNAi response in WCR adults on a long-term basis, without being interrupted by mortality that is typically associated with the silencing of an essential gene. We found that gene suppression in WCR females occurred as early as 10 h after exposure to dsRNA and lasted up to 40 days after such exposure ceased.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

RNA from different life stages of WCR was extracted using the RNAqueous®-Micro kit (Part Number Am1931, Life Technologies, CA, USA) according to the manufacturer's instructions. cDNA was made using the cloned AMV first-strand cDNA synthesis kit (cat. no. 12328- 032, Invitrogen, CA, USA) according to the manufacturer's instructions. One microgram of total RNA isolated from a pooled sample of WCR (2 females, 2 males, 5 eggs, and 5 larvae) was used in a 20-µl reverse transcription reaction containing the manufacturer's recommended ingredients including Oligo(dT)20 primers. The reaction was performed in a thin-walled tube using a thermocycler (C1000 TouchTM Thermal Cycler, BIO-RAD). The reaction was incubated at 50 °C for 60 min, followed by incubation at 85 °C for 5 min.

cDNA for quantitative reverse transcriptase PCR (qRT-PCR) was made using high-capacity cDNA reverse transcription kits (part number 4375575, Applied Biosystems, CA, USA) according to the manufacturer's instructions. Five hundred nanograms of total RNA from individual WCR females was used in a 20-µl reaction containing all ingredients. The reaction was incubated at 25 °C for 10 min, then 37 °C for 120 min, and 85 °C for 5 min. Forty µl of TE (10mM (Wu et al., 2017; Velez et al., 2016b)Tris pH 7.5, 1mM EDTA) was then added to the cDNA reaction (1:3 dilution). cDNA synthesized by both methods was stored at −20 °C until use.

2.2. dsRNA synthesis and feeding

Primers with the T7 promoter sequence added at the 5′ ends (Table 1) were used to amplify fragments of *Ago2* and *Lac2* genes that were later used for dsRNA synthesis in a manner similar to those described previously (Wu et al., 2017; Velez et al., 2016b). PCR products for WCR genes were amplified from 1 µl of cDNA prepared as described above. PCR products for *GFP* gene (negative control) were amplified from 50 ng of pGLO plasmid (Cat. No. 1660405, BIO-RAD). Bands of the expected size (~500 bp for *Ago2* and *GFP*; ~180 bp for *Lac2*) were extracted and purified using a Gel Extraction kit (Qiagen, Valencia, California) according to the manufacturer's protocol. Direct sequencing of the purified PCR products was performed at the Interdisciplinary Center for Biotechnology and Research at the University of Florida using the primers used for PCR amplification. dsRNAs were synthesized from 1 µg of purified PCR products (500 bp) using MEGAscript RNAi kit (cat. No. AM1626, Life Technologies, CA, USA) according to the manufacturer's instructions. Sizes of purified dsRNAs were confirmed by gel electro-

phoresis on a 1% agarose gel containing 1× TBE buffer. Concentrations of purified dsRNAs were determined by spectrophotometry (NanoDrop 1000, Thermo Scientific, USA), and purified dsRNAs were stored in elution buffer at −20 °C until further use.

Newly emerged, non-diapausing WCR adults (mixed sexes) that were purchased from Crop Characteristics Inc. (Farmington, MN) were provided with fresh sweet corn ears and allowed to mate for at least 4 days before bioassays. The rearing of WCR and experiments were all performed at 25 ± 1 °C, RH > 80% and L:D 16:8. WCR females (5–6 days old and in groups of 10) were transferred into 118 ml soufflé cups, with each cup counted as one biological replicate, and starved for 24 h. They were then provided with dsRNA over an 8-day period (2 days of sucrose+dsRNA and 6 days of artificial diet+dsRNA). For the first 2 days of the 8-day period, WCR females were provided with 8.3 µl of solution containing 300 ng dsRNA/µl in 20% sucrose (Sigma) per female per day, in a manner similar to those used in previously studies (Wu et al., 2017; Velez et al., 2016b). For the next 6 days of the 8-day period, WCR females were provided with one artificial diet plug (~4mm in diameter and 2mm in height) for every two WCR every other day. Each plug was coated with 20 µl of dsRNA (100 ng/µl). The artificial diet was prepared as described previously (Khajuria et al., 2015). For WCR females that completed the 8-day dsRNA treatment period, each received a total of 8 µg of dsRNA. After the dsRNA exposure period, WCR females were provided with an untreated artificial diet plug for every two females, every other day, for up to 40 days.

One WCR (one biological replicate) from each soufflé cup was collected at different time points. A total of 4 biological replicates were processed for RNA extraction and cDNA synthesis at each time point (see above).

2.3. qRT-PCR analysis

qRT-PCR analysis was performed in a similar manner as described previously (Wu et al., 2017). The sequences for the forward and reverse primers used for the qRT-PCR are listed in Table 1. The primers were validated using standard curves, based on serial dilutions of cDNA to determine the primer annealing efficiencies. A no-template control was included in each experiment to check for possible contamination. qRT-PCR (in technical duplicates) was performed using conditions that were previously described (Wu et al., 2017). Relative quantification of the transcripts was calculated using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and was normalized to β-actin (Rangasamy and Siegfried, 2012; Velez et al., 2016a; Velez et al., 2016b; Rodrigues et al., 2014). The specificity of qRT-PCR was confirmed by melting-curve analyses after each reaction.

Table 1. Sequences of PCR primers used in the current study.

Gene	Primer sequence
dsRNA synthesis[a]	
Argonaute 2	DvvAgo2F
	5′-TAATACGACTCACTATAGGGAGTATCCTCAGATGCCGACACTA-3′
	DvvAgo2R
	5′-TAATACGACTCACTATAGGGAGGGTTGTTCTGCTTCACCAATC-3′
Laccase 2	DvvLac2F
	5′-TAATACGACTCACTATAGGGATGTGCAAGAGCTTGTAGGG-3′
	DvvLac2R
	5′-TAATACGACTCACTATAGGGATGCGATTGGCTGTTAGAAG-3′
GFP	GFPF
	5′-TAATACGACTCACTATAGGGAGAGGTGATGCTACATACGGAAAG-3′
	GFPR
	5′-TAATACGACTCACTATAGGGAGACAGGTAATGGTTGTCTGGTAAA-3′
Quantitative reverse transcriptase PCR	
Argonaute 2	rtDvvAgo2F
	5′-CCGACGTACTATGCCCATTTAG-3′
	rtDvvAgo2R
	5′-CTTCTGGATACTGTCCTGGATTT-3′
Laccase 2	rtDvvLac2F
	5′-GTTATCCGTCAACCTCCTTCTC-3′
	rtDvvLac2R
	5′-CTTCGTGCATCCAGTCACTTA-3′
Actin	rtDvvActinF
	5′-TCCAGGCTGTACTCTCCTTG-3′
	rtDvvActinR
	5′-CAAGTCCAAACGAAGGATTG-3′

a. T7 polymerase promoter sequence (TAATACGACTCACTATAGGGAG) was added to 5′ end of the oligonucleotide sequence to facilitate subsequent dsRNA transcription as described in manufacturer's protocol (MEGAScript, Ambion).

2.4. Statistical analysis

The means and standard errors of means (SEM) were analyzed by analysis of variance (ANOVA) (JMP 8; SAS Institute, Cary, NC), and means were separated using Student's *t*-test ($p < 0.05$) in manners similar to previous studies (Wu et al., 2017).

3. Results and discussion

Western corn rootworm females treated with *Lac2* dsRNA exhibited robust gene silencing (a 76% knockdown relative to *GFP* dsRNA controls) as early as 10 h after exposure to dsRNA (Fig. 1A). By comparison, a significant knockdown (69%) of *Ago2* transcripts did not occur until 24 h after the dsRNA treatment (Fig. 1B). The percent of gene knockdown for *Lac2* and *Ago2* ap-

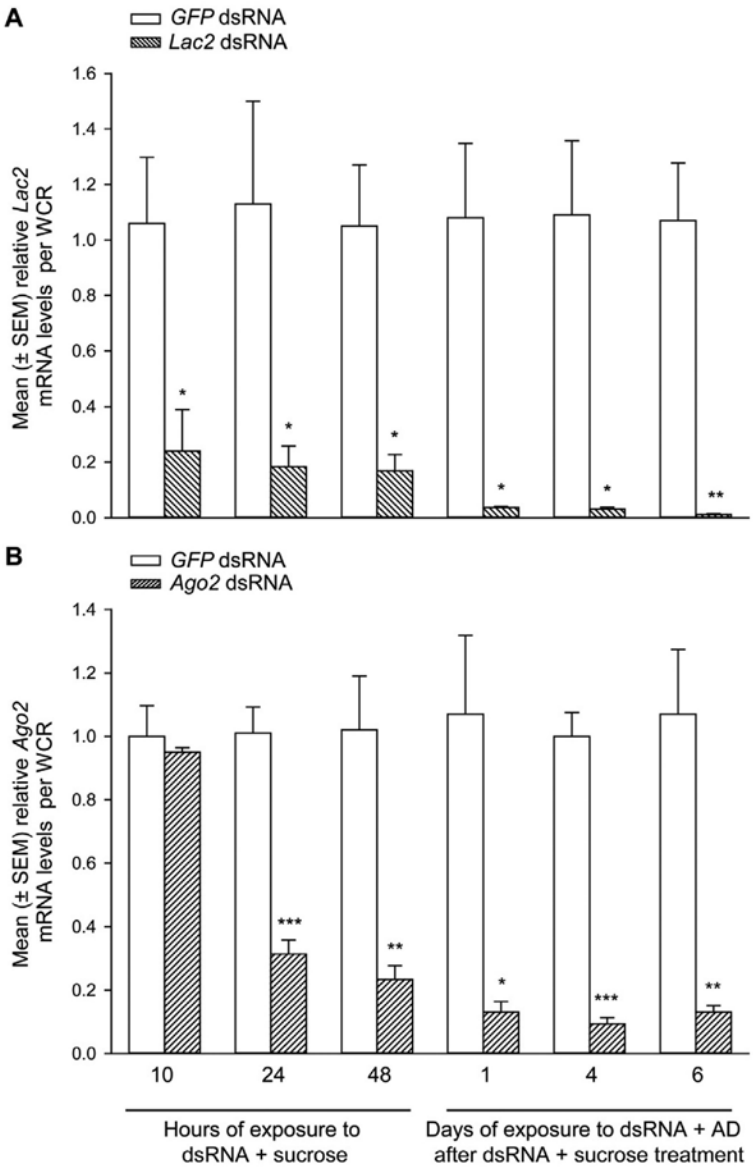

Fig. 1. The effects of *Lac2* and *Ago2* dsRNA deliveries on the mRNA levels of *Lac2* and *Ago2*, respectively, during the dsRNA treatment period.

peared to increase continuously throughout the 8-day dsRNA treatment period, reaching 99% and 87%, respectively, on day 8 (Fig. 1).

We further investigated the duration of gene silencing post dsRNA treatment. Three days after dsRNA treatment ended, WCR previously treated with *Lac2* and *Ago2* dsRNAs displayed a knockdown of 93% and 88% of respective mRNAs (Fig. 2). Similar degrees of gene suppression persisted for another 7 days. Twenty days after dsRNA treatment ended, the knockdown

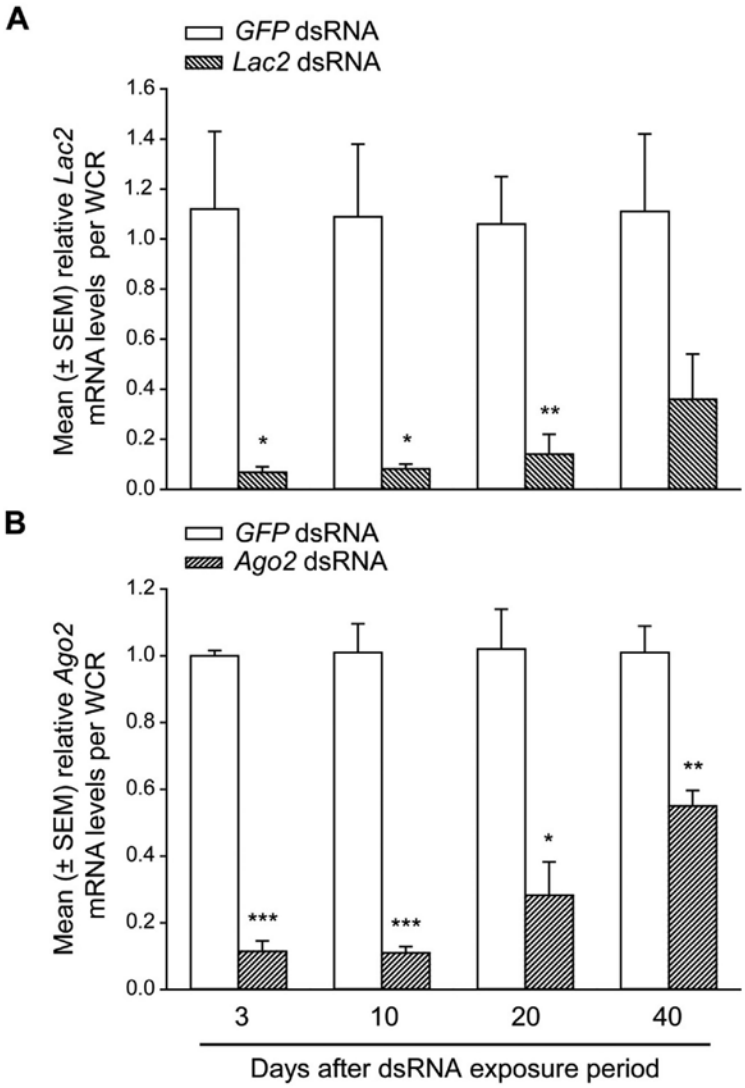

Fig. 2. Long-term suppression of *Lac2* and *Ago2* mRNA levels in WCR females after the dsRNA treatment period.

rates for *Lac2* and *Ago2* were still at 86% and 72%, respectively (Fig. 2). Forty days after dsRNA treatment terminated, the *Lac2* knockdown rate was at 74% (Fig. 2), although the difference only approached significance level (p = 0.082, 2-tailed t-test). Remarkably, the *Ago2* mRNA levels in WCR previously treated with *Ago2* dsRNA were still significantly lower than those of controls by 45% at this time. Taken together, these results demonstrate that gene silencing mediated by these dsRNAs is rapid and long-term in WCR females.

The data suggest that RNAi response in WCR females can be as rapid as that in larvae (Baum et al., 2007). Interestingly, the earliest time that gene silencing was detected appears to be gene-specific. The discrepancy in the earliest detection time is possibly due to a difference in the expression levels of *Ago2* and *Lac2* mRNAs. For example, if the expression levels of *Ago2* mRNA are higher than those of *Lac2* and, *Ago2* and *Lac2* dsRNAs induced similar rates of mRNA degradation, it may take *Ago2* mRNA a longer time to show a decline than *Lac2*. Alternatively, *Ago2* and *Lac2* dsRNAs may produce different rates of mRNA degradation, which may also result in a discrepancy in the earliest time gene silencing could be detected.

The result, which show gene knockdown being sustained 20–40 days after previous exposure to the dsRNAs of two different genes, suggest that extended gene silencing following brief dsRNA treatment is likely an intrinsic feature of RNAi response in WCR adults. Future studies are needed to test whether persistent RNAi can also be achieved with other genes in WCR adults. Similar studies are also needed in WCR larvae, where developmental changes and significant increases in body mass may impact its long-term persistence.

A previous study showed that RNAi response in larvae occurred across a range of dsRNA concentrations (Bolognesi et al., 2012). It would be interesting to test whether persistent gene knockdown can be achieved in WCR adults and larvae fed on lower concentrations of dsRNA. Similarly, additional studies are needed to characterize the RNAi response under field conditions, where WCR likely consume less amounts of dsRNA than those used in the current study.

The current findings raise the interesting question of how WCR is able to sustain prolonged gene silencing without a continuous input of dsRNA and the production of secondary siRNAs or transitive RNAi (Li et al., 2016). It is possible that the same molecular machinery participating in the systemic spread of RNAi signal is also involved in maintaining the prolonged gene silencing in WCR. To date, the molecular components involved in the systemic spread of RNAi in WCR (or other insects) remain poorly defined (Fishilevich et al., 2016).

In *C. elegans*, several SID (systemic interference deficient) proteins are involved in dsRNA uptake and the systemic spread of RNAi signal. Putative SID or SID-like proteins have been identified in insects, including WCR (Miyata et al., 2014; Xu and Han, 2008; Tomoyasu et al., 2008). However, it appears that these proteins are involved in the RNAi process in only some of the insects that possess them (Fishilevich et al., 2016; Cappelle et al., 2016).

Two SID-like genes were identified in WCR and they appear to play a minor role in the RNAi pathway (Miyata et al., 2014). It is possible that other proteins (e.g., clathrin heavy chain) are involved in dsRNA (and pos-

sibly secondary silencing signal) uptake and the systemic spread of RNAi signal in WCR and some other insects (Fishilevich et al., 2016; Cappelle et al., 2016; Saleh et al., 2009; Saleh et al., 2006; Ulvila et al., 2006; Xiao et al., 2015). The fact that silencing of *Ago2* did not appear to affect the extent and duration of its own knockdown, when compared to *Lac2*, suggests that Ago2 likely plays no or only a limited role in the systemic spread of silencing signal in WCR.

The current findings indicate that continuous or repeated dsRNA exposures may not be needed in RNAi-based functional genomic studies in WCR. This will likely reduce the costs associated with synthesizing and applying large amounts of dsRNA previously thought necessary for continuous gene silencing during such studies. Obviously, any possible long-term silencing of a gene of interest has to be verified individually when conducting such studies.

4. Conclusions

The data support the notion that long-term persistency is likely an intrinsic feature of RNAi response in WCR. For RNAi-based control approaches, current results suggest that continuous feeding of transgenic plants expressing dsRNAs of essential or nonessential target genes may not be necessary for achieving a lasting effect on WCR adults. Currently, rootworm traits are deployed as refuge-in-a-bag (RIB). The sustained RNAi response in WCR supports the development of dsRNA as an RIB trait. In field conditions where transgenic and non-transgenic food sources coexist, RNAi-based WCR control measures may have the added benefit of long-term effectiveness.

(A) Exposure to *Lac2* dsRNA resulted in significant knockdown in the mRNA levels of *Lac2* in WCR females throughout the dsRNA treatment period.

(B) *Ago2* dsRNA treatment resulted in significant knockdown in the *Ago2* mRNA levels at all but the earliest (10h) time points of the exposure period.

Two-tailed Student's *t*-tests were used to compare the mRNA levels of WCR treated with *Lac2* (or *Ago2*) dsRNA *vs. GFP* dsRNA. *denotes $p < 0.05$; **denotes $p < 0.005$; ***denotes $p < 0.0005$. The *Lac2* (or *Ago2*) mRNA levels in *GFP* dsRNA control females were scaled to 1. The numbers of biological replicates were 3 for the following treatment groups: *GFP* dsRNA controls at 10 h and 48 h of exposure to dsRNA + sucrose, and *Lac2* dsRNA at 10 h of exposure to dsRNA + sucrose. The numbers of biological replicates for the rest of the treatment groups were 4. AD: artificial diet.

(A) The *Lac2* mRNA levels in WCR females that were treated with *Lac2* dsRNA previously were consistently lower than those of controls for up 20 days.

(B) The *Ago2* mRNA levels in females that were treated with *Ago2* dsRNA previously were consistently lower than those of controls for up to 40 days.

Two-tailed Student's *t*-tests were used to compare the mRNA levels of WCR treated with *Lac2* (or *Ago2*) dsRNA *vs. GFP* dsRNA. *denotes $p < 0.05$; **denotes $p < 0.005$; ***denotes $p < 0.0005$. The *Lac2* (or *Ago2*) mRNA levels in *GFP* dsRNA-treated controls were scaled to 1. The number of biological replicates was 3 for the *Ago2* group at 20-day time point. The numbers of biological replicates for the rest of the treatment groups were 4.

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