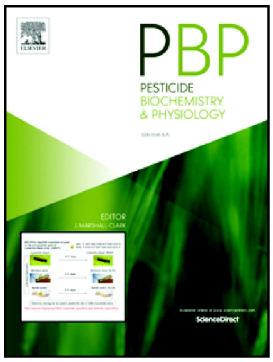

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Knockdown of *double-stranded RNases* (*dsRNases*) enhances oral RNA Interference (RNAi) in the Corn Leafhopper, *Dalbulus maidis*

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Abstract: The leafhopper *Dalbulus maidis* is a harmful pest that causes severe damage to corn crops. Conventional chemical pesticides have negative environmental impacts, emphasizing the need for alternative solutions. RNA interference (RNAi) is a more specific and environmentally friendly method for controlling pests and reducing the negative impacts of current pest management practices. Previous studies have shown that orally administered double-stranded RNA (dsRNA) is less effective than injection protocols in silencing genes. This study focuses on identifying and understanding the role of *double-stranded ribonucleases* (*dsRNases*) in limiting the efficiency of oral RNAi in *D. maidis*. Three *dsRNases* were identified and characterized, with *Dmai-dsRNase-2* being highly expressed in the midgut and salivary glands. An *ex vivo* degradation assay revealed significant nuclease activity, resulting in high instability of dsRNA when exposed to tissue homogenates. Silencing *Dmai-dsRNase-2* improved the insects' response to the dsRNA targeting the gene of interest, providing evidence of *dsRNases* involvement in oral RNAi efficiency. Therefore, administering both *dsRNases*-specific and target gene-specific-dsRNAs simultaneously is a promising approach to increase the efficiency of oral RNAi and should be considered in future control strategies.

Keywords: Hemiptera, pest management, dsRNA, co-feeding, gene silencing, *dsRNase*

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1. Introduction

The leafhopper *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera: Auchenorrhyncha) is one of the most important pests of corn (*Zea mays* L.) and has a significant economic impact (Nault, 1980; Alivizatos and Markham, 1986). In addition to yield losses resulting from feeding damage, it is also the primary vector of the mollicute *Spiroplasma kunkelii* Whitcomb, the causal agent of Corn Stunt disease (Bradfute et al., 1981; Bajet and Renfro, 1989; Oliveira et al., 1998). The increasing prevalence of Corn Stunt disease in the Americas (Bradfute et al., 1981; Hruska et al., 1996; Giménez Pecci et al., 2002) since its initial detection (Alstatt, 1945; Frazier, 1945) represents a significant challenge to corn production. Despite the use of various control strategies such as germplasm resistance and insecticide sprays to manage *D. maidis* damage (Bhirud and Pitre, 1972; Perfecto, 1990; Tsai et al., 1990; Oleszczuk et al., 2020; Carpane and Catalano, 2022), effective control of this pest remains challenging. Therefore,

there is a pressing and urgent need to establish an efficient, specific and environmentally friendly approach to control this pest.

RNA interference (RNAi) is a post-transcriptional silencing mechanism that is initiated by the presence of double-stranded RNA (dsRNA), inducing the degradation of mRNAs that are complementary to it (Zamore et al., 2000; Wilson and Doudna, 2013). Since its discovery, RNAi has become a powerful and widely used tool for gene function analysis, biomedical research, and biological pest control. RNAi technology has the potential to be applied to pest management and pathogens reduction (Baum et al., 2007; Mao et al., 2007; Bellés, 2010; Zhu et al., 2012; Zhang et al., 2017). However, RNAi efficiency varies among insects depending on the species, dsRNA delivery method and target gene selection (Huvenne and Smagghe, 2010; Yu et al., 2013; Cagliari et al., 2019; Cooper et al., 2019).

In a previous study on the application of RNAi in *D. maidis*, we confirmed that delivery of dsRNA through injection or feeding are effective ways to initiate an RNAi response (Dalaisón-Fuentes et al., 2022). Our research demonstrated that administering dsRNA specific to *Bicaudal C* (*Dmai-BicC*) induce a significant reduction in mRNA levels and oviposition, with ovaries revealing alterations in oocyte development. Together, these results provide evidence that *Dmai-BicC* plays a crucial role in *D. maidis* oogenesis. However, our data also shows that oral delivery of dsRNA^{BicC} triggers the RNAi pathway less efficiently. We hypothesize that the dsRNA may be degraded upon ingestion by some digestive nucleases before being absorbed through the gut and translocated into other body cells. Therefore, the stability of dsRNA would be critical determining the success of oral RNAi (Garbutt et al., 2013; Wang et al., 2016; Singh et al., 2017).

dsRNA-degrading nuclease activity is usually attributed to *double-stranded ribonucleases* (*dsRNases*), which belong to the DNA/RNA non-specific endonuclease (NUC) family (Arimatsu et al., 2007a, b). The activity of these *dsRNases* is mostly found in digestive fluids, indicating that they are secreted in the gut lumen for nucleic acid digestion. They are also present in other tissues such as the epidermis, fat body, brain, silk glands, and hemolymph (Arimatsu et al., 2007a, b; Allen and Walker, 2012; Liu et al., 2012; Garbutt et al., 2013; Christiaens et al., 2014; Wynant et al., 2014). *dsRNase* activity has been identified and implicated in reducing RNAi efficiency through dsRNA degradation in many insect orders (Wynant et al., 2014; Almeida Garcia et al., 2017; Luo et al., 2017; Song et al., 2017; Spit et al., 2017; Peng et al., 2018; Prentice et al., 2019; Fan et al., 2021; Sharma et al., 2021; Li et al., 2022; Zhang et al., 2022).

To confirm the impact of *dsRNases* on oral RNAi efficiency in *D. maidis*, our goal was to identify and characterize *dsRNases* expressed in this leafhopper. Through a transcriptome search, we identified three *Dmai-dsRNase* genes (*Dmai-dsRNase-1*, *-2* and *-3*) and evaluated their expression profiles in different tissues. We found that *Dmai-dsRNase-2* was expressed in midgut and salivary glands, *Dmai-dsRNase-1* in the fat body, and *Dmai-dsRNase-3* in both fat body and rest of body. Finally, we investigated the impact on RNAi efficiency after *Dmai-dsRNase-2* silencing. Our results demonstrate that *Dmai-dsRNase-2* reduced oral RNAi efficiency in *D. maidis*. This study provides valuable insights for improving RNAi-based pest

management strategies and must be considered for future research and developing programs in this field.

2. Materials and methods

2.1. Insect rearing

A colony of *D. maidis* was maintained on corn plants (*Zea mays* L.) in our laboratory. The colony was kept in aluminum-framed cages with a fine *voilé*-type nylon mesh and placed in a greenhouse with a temperature of 25 °C and 80% relative humidity. The photoperiod was set at 16:8 h (light: darkness). Embryogenesis is completed 11.5 ± 1.3 days after egg laying at 23 ± 3 °C (Remes Lenicov and Virla, 1993).

2.2. Identification of *dsRNase* genes in *D. maidis* transcriptome

A *D. maidis* adult transcriptome was used to perform an iterative search to identify *dsRNase* genes. A specific database was generated from different repositories (https://www.ncbi.nlm.nih.gov/protein/; https://www.uniprot.org/) containing 123 protein sequences of homologous nucleases reported in other insect species. Protein sequences predicted from the transcriptome were used as queries to perform a BLASTp search (Altschul et al., 1990), with an e-value threshold of 10^{-5}. Protein signatures were predicted by InterProScan v92.0 (Paysan-Lafosse et al., 2023) and SignalP v6.0 (Teufel et al., 2022) servers. Phylogenetic analysis was based on sequence alignments generated by Clustal Ω (Sievers and Higgins, 2014), using the software BEAST v1.10.4 (Drummond and Rambaut, 2007) in the CIPRES Science Gateway (Miller et al., 2010). Beauti v1.8.350 was used to generate the BEAST input file. One million generations were used for the run, combined with LogCombiner v1.8.350 discarding the first 6% of each chain as a burn-in. The maximum clade credibility tree was generated using TreeAnnotator v1.8.388. The result was visualized with iTol tool (Letunic and Bork, 2021).

2.3. Tissue-specific expression of *Dmai-dsRNases* and dsRNA stability assay

Total RNA samples were prepared from salivary glands, midgut, fat body and the rest of body dissected from adult females. Tissues (N=4 per biological replicate) were dissected in PBS 1X and immediately stored in TRIzol reagent (Invitrogen). Total RNA was isolated using TRIzol reagent and treated with RNase-free DNase (Qiagen, Hilden, Germany) according to manufacturer's instructions. First-strand cDNA synthesis was performed using the oligo (dT)18 primer and RevertAid Reverse Transcriptase (Thermo Scientific) based on the protocol provided by the manufacturer. RT-qPCR was carried out in technical triplicates (three wells per cDNA sample), in a 10 µL final volume reaction using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a Bio-Rad CFX-96 thermocycler. PCR cycling consisted of 30 s at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C (annealing and extension). Melting curve analysis was performed at the end using instrument default settings (65–95 °C, 0.5 °C temperature increment). A no-template control was included in all batches. All primer pairs (Table S1) were designed with Primer3Plus (Rozen and Skaletsky, 1999) and PCR Primer Stats (Stothard, 2000), and tested for dimerization, efficiency and amplification of a single product. Differences in transcript levels were calculated by a comparative Ct method (Rao et al., 2013). Expression values were normalized using *60S ribosomal protein L3* (*Dmai-RPL3*)

gene, a reference chosen after a screening of several housekeeping gene candidates, as it provided consistent results.

To investigate *ex vivo* degradation of dsRNA, salivary glands (N=100), midgut (N=100), fat body (N=10) and rest of body (N=10) were dissected from adult females and added to 75 µl of PBS 1X. Tissues were homogenized and centrifuged at 8.000 rpm for 7 min at 4 °C (Shukla et al., 2016). The fat body and rest of body were centrifuged twice. The supernatants were transferred to new tubes to estimate protein concentration by Bradford (Kruger, 2009). One µg of dsRNA^{BicC} was incubated with one µg of homogenate at room temperature and samples were collected at various time points (1, 12, and 24 h). Two µL of each sample were resolved on a 1% agarose gel. dsRNA^{BicC} exposed only to PBS 1X was used as a control.

2.4. Synthesis of dsRNA

Total RNA was isolated from adult females using TRIzol reagent (Invitrogen) according to the manufacturer's specifications. The EasyScript Reverse Transcriptase (AP-Biotech) protocol was used to synthesize cDNA which was then used as a template for reverse transcription polymerase chain reaction (RT-PCR). Specific primers for *Dmai-dsRNase-2* (Table S1) were designed to amplify a region of 330 base pairs (bp). PCR cycling was carried out as follows: 2 min at 94 °C, followed by 35 cycles of 92 °C for 15 s, 62 °C for 15 s and 72 °C for 30 s, and a final extension step for 4 min at 72 °C (Taq Pegasus, Productos Bio-Lógicos, Argentina). The integrity of the amplicon was analyzed by electrophoresis in a 1% agarose gel and sequenced to confirm its identity (Macrogen Inc., South Korea). The PCR product was used as template for a second PCR round to add a T7 promoter sequence at both ends (Table S1). dsRNA was synthesized using the TranscriptAid T7 High Yield Transcription Kit (#K0441, ThermoFisher), following the manufacturer's instructions. The template was removed by RNase-free DNase digestion (#K0441, ThermoFisher). dsRNA integrity was examined by electrophoresis in a 1% agarose gel. As reported previously, the same workflow was applied for *Dmai-BicC* gene (Dalaisón-Fuentes et al., 2022). A negative control was included using the *ß-lactamase* gene (ds*RNA^{ß}*), amplified from a pRSETb plasmid (Lavore et al., 2012).

2.5. RNA interference protocol:

2.5.1. Injection followed by oral administration

dsRNA targeting *Dmai-dsRNase-2* (200 ng/µL) was administered by injection to newly molted adult females (N=40 per treatment) using the method described in Dalaisón-Fuentes et al. (2022). After 48 h, once the females were recovered from injury, they were orally exposed to an artificial diet (Dalaisón-Fuentes et al., 2022) containing dsRNA targeting *Dmai-BicC* at a final concentration of 200 ng/µL for three consecutive days with daily diet renewal. Then, the females were placed in rearing cages and mated with males.

Four different treatments were applied, the first group received an injection of dsRNA^{RNase2} followed by oral administration of dsRNA^{BicC}, and was termed dsRNA$^{RNase2/BicC}$ silenced females; the second group received an injection of dsRNA^{RNase2} followed by oral administration of dsRNA^{ß}, and was called dsRNA$^{RNase2/ß}$ silenced females; and the third group received an injection of dsRNA^{ß} followed by oral administration of dsRNA^{BicC}, called dsRNA$^{ß/BicC}$ silenced females. The fourth group served as a control, receiving an injection of dsRNA^{ß} followed by oral administration of dsRNA^{ß}, and was termed dsRNA$^{ß/ß}$ silenced females.

Female mortality was recorded at seven points over a period of 13 days. Transcript levels of *Dmai-dsRNase-2*, *Dmai-BicC* and *ß-lactamase* were quantified in salivary glands, midgut and ovaries from adult females, at one and seven days after completion of dsRNA oral administration. RNA extraction, first-strand cDNA synthesis and RT-qPCR were performed as described previously.

2.5.2. Co-feeding assay

Newly molted adult females (N=20 per treatment) were placed inside feeding chambers and fed artificial diet (Dalaisón-Fuentes et al., 2022) containing both dsRNA^{RNase2} and dsRNA^{BicC} (200 ng/µL each), referred to as dsRNA$^{RNase2/BicC}$ silenced females. Diet and dsRNA were replaced daily. After three consecutive days of dsRNA ingestion, females were placed individually in rearing cages and mated with one male each, allowing them to feed and lay eggs on corn plants. Female mortality and oviposition (i.e., number of eggs laid per female) were recorded every 48 h, when plants were renewed. Embryo lethality was monitored during the expected time of embryogenesis (Remes Lenicov and Virla, 1993). Rearing conditions were the same as for the colony. After 22 days, ovaries were dissected and fixed, as indicated in Dalaisón-Fuentes et al. (2022). Images were acquired with a stereomicroscope (Zeiss, Stemi 305). Two different treatments were applied as controls, one combined dsRNA^{ß} with dsRNA^{BicC}, called dsRNA$^{ß/BicC}$ silenced females, and the other contained only dsRNA^{ß}, termed dsRNA$^{ß/ß}$ silenced females.

A parallel experiment (N=40 per treatment) was conducted to determine *Dmai-dsRNase-2*, *Dmai-BicC* and *ß-lactamase* mRNAs expression after dsRNA delivery. Total RNA samples were prepared from salivary glands, midgut, and ovaries from adult females at one and seven days after completion of dsRNA administration. RNA extraction, first-strand cDNA synthesis and RT-qPCR were carried out as described above.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism v6.0 software (GraphPad Software, CA, USA, www.graphpad.com) and InfoStat statistical software v2020 (Di Rienzo et al., 2020). The fraction of surviving females was calculated using the Kaplan–Meier method and the *p*-values generated via the Log-rank test were used to test the null hypothesis that the survival curves were identical between the groups, with a significance level of 5%. A generalized linear mixed model was applied for the number of eggs laid to model negative binomial variables, with 'treatment', 'time' and 'interaction' as fixed effects, and 'female' as random effect. Predicted values were compared using DGC test (Di Rienzo et al., 2002), with a significance level of 5%. For RT-qPCR analysis, Shapiro-Wilk and D'Agostino & Pearson normality tests were applied to assess normality of the data set. One-way ANOVA followed by Bonferroni multiple comparison *post-hoc* test was performed to analyze group differences. Data that did not meet the assumptions of normality were subjected to a Kruskal–Wallis test followed by Dunn's multiple comparison test for pairwise comparison.

3. Results

3.1. Identification and characterization of *D. maidis dsRNase* genes

Orthologues of *double-stranded ribonucleases* (*dsRNases*) were identified in a transcriptome of adult *D. maidis* (Table 1). Three transcript sequences were retrieved, namely *Dmai-dsRNase-1*, *Dmai-dsRNase-2* and *Dmai-dsRNase-3*. All predicted proteins were similar in length and contained a signal peptide and single DNA/RNA non-specific endonuclease (PF01223) domain. Multiple sequence alignments of the predicted *dsRNase* proteins revealed that only the active key site's first and fifth residues varied among them. *Dmai-dsRNase-2* and *Dmai-dsRNase-3* have an alanine (A) at position one in the active site, while *Dmai-dsRNase-1* has a serine (S). At position five, *Dmai-dsRNase-1* and *Dmai-dsRNase-2* both have a glutamine (Q) in the active site, but *Dmai-dsRNase-3* has a methionine (M) (Fig. 1). Phylogenetic analysis indicated that the three *Dmai-dsRNases* cluster in the hemipteran clade (Fig. 2). Overall, these results indicate that *dsRNases* are conserved in *D. maidis*.

3.2. Analysis of *Dmai-dsRNases* transcript levels and dsRNA degradation in different tissues

Quantitative analysis of *dsRNases* expression revealed that *Dmai-dsRNase-2* had the highest expression in salivary glands followed by the midgut. The transcript expression in the salivary glands was nearly 600-fold higher than in the midgut. *Dmai-dsRNase-1* expression was detectable only in the fat body, and *Dmai-dsRNase-3* was expressed in the fat body and rest of body, but undetectable in the other tissues (Fig. 3).

An *ex vivo* dsRNA stability assay was conducted to assess nuclease activity in the above mentioned tissues. After 1 h of incubation a fraction of dsRNA^{BicC} was already degraded in all samples (Fig. S1); however, degradation was more evident in the sample exposed to salivary gland contents (Fig. S1). Degradation in the midgut was faster than the fat body and rest of body, although not to the same extent as in salivary glands, which showed complete degradation of dsRNA^{BicC} after 12 h of incubation.

The increased expression of *Dmai-dsRNase-2* in the digestive tract of *D. maidis*, along with the strong ability for the rapid degradation of dsRNA by the salivary glands, suggests that *Dmai-dsRNase-2* may exhibit robust nuclease activity responsible for dsRNA degradation.

3.3. Measurement of RNAi efficiency after dsRNA^{RNase2} injection

To investigate the potential enhancement of RNAi efficiency by suppressing *Dmai-dsRNase-2*, adult virgin females were injected with a specific dsRNA targeting *Dmai-dsRNase*-2 and then were transferred to an artificial diet containing dsRNA^{BicC} –the gene of interest-. Female mortality and mRNA levels of *Dmai-dsRNase-2* and *Dmai-BicC* were recorded. In salivary glands, the *Dmai-dsRNase-2* expression level on the first day after oral diet decreased by 23.6 and 121 times in dsRNA$^{RNase2/ß}$ and dsRNA$^{RNase2/BicC}$ silenced females, respectively, compared to the control group (Fig. 4A). In addition, the *Dmai-dsRNase-2* level in the midgut was reduced by 33.1 times in dsRNA$^{RNase2/ß}$ silenced females and by 15 times in dsRNA$^{RNase2/BicC}$ silenced females compared to the control group (Fig. 4B). A significant reduction of *Dmai-BicC* transcripts was detected seven days after dsRNA^{BicC} oral administration, with a fold change decrease of nearly 16 times in dsRNA$^{ß/BicC}$ silenced females compared to the control ones (Fig. 4C). At the same time point, dsRNA$^{RNase2/BicC}$ silenced females evidenced no expression of *Dmai-BicC* transcripts (Figure 4C).

Female mortality was evaluated at seven time points over a period of 13 days to rule out a possible lethal effect of *Dmai-dsRNase-2* silencing. The results showed no significant differences in the proportion of living females throughout the experiment (p=0.5304) (Fig. 5).

These results demonstrate that *Dmai-dsRNase-2* and *Dmai-BicC* were successfully silenced at the mRNA level, and the survival of dsRNA$^{RNase2/BicC}$ silenced females was unaffected. Taken together, our findings suggest that *Dmai-dsRNase-2* could modulate the RNAi response.

3.4. RNAi efficiency after simultaneous silencing of *Dmai-dsRNase-2* and *Dmai-BicC* genes by co-feeding

An RNAi co-feeding approach was applied to further assess the effects of nuclease activity in the digestive tract on the sensitivity and efficiency of the RNAi response. In this, the artificial diet was mixed with dsRNA^{BicC} and dsRNA^{RNase2}, simultaneously. After mating, female survival, egg deposition, embryo lethality and ovary morphology were evaluated. No significant differences were found in the percentage of living females throughout the experiment (p=0.809) (Fig. 6).

Compared to dsRNA$^{\beta/\beta}$ and dsRNA$^{\beta/BicC}$ silenced, females fed dsRNA$^{RNase2/BicC}$ deposited significantly fewer eggs (Fig.7A; Table S2A). Furthermore, the number of viable eggs laid by dsRNA$^{RNase2/BicC}$ silenced females was significantly lower than that of the dsRNA$^{\beta/\beta}$ and dsRNA$^{\beta/BicC}$ groups (Fig. 7B). In other words, only 64.42% of the eggs corresponding to dsRNA$^{Rnase2/BicC}$ silenced females completed the embryogenesis and reached the first-instar larvae on time, against 74.66% and 94.79% in females silenced with dsRNA$^{\beta/BicC}$ and dsRNA$^{\beta/\beta}$, respectively (Table S2A). The eggs which did not hatch were smaller, thinner and had abnormal, large bubbles in the yolk, without distinguishable embryonic structures, indicating that their development was arrested (Figure S2D). On the 12th day, a point of inflection was observed, as the eggs laid by dsRNA$^{\beta/BicC}$ silenced females started to complete the embryogenesis, showing no significant differences compared to the control ones. After 22 days, 82.35% of the dsRNA$^{\beta/BicC}$ silenced females laid embryonated eggs and were fully recovered, whereas the dsRNA$^{RNase2/BicC}$ silenced females continued to lay mainly non-embryonated eggs (12.5%), or were sterile (18.75%) (Table S2B). The morphology of the ovary showed, compared to the control (dsRNA$^{\beta/\beta}$), a vitellarium composed of a large number of immature oocytes that failed to progress toward choriogenesis (Fig. S2B), suggesting that oocyte maturation was affected.

In order to confirm that *Dmai-dsRNase-2* and *Dmai-BicC* were silenced, their transcript levels were measured at different times after being orally administered. The result showed a significant decrease in the abundance of *Dmai-RNase-2* transcripts in the midgut of dsRNA$^{RNase2/BicC}$ silenced females on day seven compared to the control group (Fig. 8A). While there was also a reduction in expression in the salivary glands, this difference was not statistically significant (Fig. 8B). The transcript level of *Dmai-BicC* was significantly reduced on day seven in the silenced females (dsRNA$^{\beta/BicC}$ and dsRNA$^{RNase2/BicC}$) compared to the control group (Fig. 8C).

The incorporation of dsRNA^{Rnase2} in the artificial diet enhances the insect's response to the dsRNA targeting *Dmai-BicC*, thus indicating that silencing of *Dmai-dsRNase-2* improves the oral RNAi efficiency in *D. maidis*.

4. Discussion

Dalbulus maidis is a significant pest that can cause yield losses of up to 90% in corn production through direct feeding and/or pathogen transmission (Nault and Bradfute, 1979; Summers and Stapleton, 2002; Tsai and Capinera, 2008; Pérez-López et al., 2018). Currently, there are no effective control methods for this pest, but RNAi shows excellent potential as a strategy for controlling its spread (Dalaisón-Fuentes et al., 2022). Unfortunately, oral administration of dsRNA is less efficient compared to injection. Therefore, the objective of the present study was to investigate the factors influencing the response of *D. maidis* to oral RNAi.

Several potential modulators of the oral RNAi response in insects have been proposed, including dsRNA instability, incomplete dsRNA internalization, impaired systemic spread of the RNAi signal, deficient dsRNA exposure, and refractory target genes (Huvenne and Smagghe, 2010; Yu et al., 2013; Cagliari et al., 2019; Cooper et al., 2019). Among those factors, dsRNA instability has been attributed to the enzymatic activity of *dsRNases* and physiological pH (Singh et al., 2017; Peng et al., 2018; Cooper et al., 2019; Kunte et al., 2020). Previous research has demonstrated that dsRNA must persist certain time in body fluids, such as hemolymph or gut contents before being taken up by cells and triggering a RNAi response (Ren et al., 2014). In this time frame, *dsRNases* are strong candidates to suppress oral RNAi response by rapidly degrading dsRNA before it can be internalized (Wynant et al., 2014; Almeida Garcia et al., 2017; Luo et al., 2017; Song et al., 2017; Spit et al., 2017; Peng et al., 2018; Prentice et al., 2019; Fan et al., 2021; Sharma et al., 2021; Li et al., 2022; Zhang et al., 2022). Therefore, we hypothesized that the lower efficiency of oral administration, compared to injection, can be attributed to the presence of *dsRNases* in the tissues of *D. maidis*.

In this work we identified three *dsRNase* genes in the transcriptome of *D. maidis*: *Dmai-dsRNase-1*, *-2* and *-3*. Similar to *Acyrthosiphon pisum* (Christiaens et al., 2014) and *Bemisia tabaci* (Luo et al., 2017), *D. maidis* had multiple *dsRNases*, whereas some heteropterans, as *Euschistus heros* (Cagliari et al., 2019) and *Nezara viridula* (Sharma et al., 2021), express only one. Outside of Hemiptera, the number of *dsRNases* ranges from two to ten (Song et al., 2019; Giesbrecht et al., 2020; Peng et al., 2020a, b; Yoon et al., 2021). Characterization of the *Dmai-dsRNases* showed that the predicted proteins present the non-endonuclease domain and a signal peptide for extracellular secretion, suggesting a role in extracellular dsRNA degradation (Arimatsu et al., 2007a, b). Multiple *dsRNase* genes are known to have tissue-specific expression, functional divergence, and adaptation to different environments (Song et al., 2017, 2019; Giesbrecht et al., 2020). Interestingly, our analysis of the active sites revealed conservation throughout the sequences, with variability only at residues one and five. The variability in residue one, also a substrate binding site, suggests that *Dmai-dsRNases* may differ in their substrate specificity, as observed in *Locusta migratoria* (*Lm-dsRNases*) (Song et al., 2019). *Dmai-dsRNase-2* and *Dmai-dsRNase-3* shared common residues with *Lm-dsRNase-1*, which has substrate-specificity to dsRNA and dsDNA, while *Dmai-dsRNase-1* exhibited common residues with *Lm-dsRNase-2*, which is involved in the digestion of dsRNA and siRNA. These similarities suggest that *Dmai-dsRNases* may have enzymatic activities like those of *L. migratoria*. Functional experiments to confirm substrate specificity have been limited to *Bombyx mori* (Arimatsu et al., 2007a, b) and *L. migratoria* (Song et al., 2019), hence performing it in *D. maidis* would be interesting.

Our findings on the tissue-specific expression profile of *dsRNases* suggest that *Dmai-dsRNAse-2,* expressed in the digestive system, could be a critical factor limiting the efficiency of oral RNAi. The substantial difference in expression level in the salivary glands compared to other tissues (i.e., midgut, fat body, and rest of body) suggests that salivary glands are the primary source of this *dsRNase*, as reported previously in *N. viridula* (Sharma et al., 2021). In agreement, an *ex vivo* degradation assay demonstrated high instability of dsRNA when exposed to homogenates prepared from different tissues of adult females. The rapid degradation of dsRNA by *D. maidis* nucleases also suggests an important role for these enzymes in crucial physiological mechanisms, such as defense against pathogens (Lomate and Bonning, 2018). These results are consistent with previous studies on other insect species in which tissue specificity of *dsRNase* genes and their impact on oral RNAi efficiency have been described (Prentice et al., 2019; Song et al., 2019; Peng et al., 2020a, b; Sharma et al., 2021; Li et al., 2022).

Two independent experiments were carried out to determine whether silencing of *Dmai-dsRNase-2* could protect dsRNAs from degradation and thus enhance the insect's response to oral RNAi. Initially, we evaluated the potential of *Dmai-dsRNAse-2* through injection. This delivery method was selected to guarantee effective gene silencing (Dalaisón-Fuentes et al., 2022). We chose *Dmai-BicC* as target gene to compare the results obtained here with our previous work (Dalaisón-Fuentes et al., 2022). Moreover, we administered the same concentration of dsRNA, but at a larger volume, to ensure ingestion. Our results confirmed the successful silencing of both genes at mRNA level without affecting female survival, indicating that the silencing of *Dmai-dsRNase-2* could enhance the response of *D. maidis* to oral RNAi. All of this evidence led us to propose a co-feeding bioassay.

The results obtained by co-feeding share similarities with the studies conducted on *Bactrocera tryoni* (Tayler et al., 2019) and *B. tabaci* (Luo et al., 2017), in which the simultaneous silencing of *dsRNases* and target genes improved the efficiency of oral RNAi. Both treatments -dsRNA$^{\delta/BicC}$ and dsRNA$^{RNase2/BicC}$- significantly reduced the number of oviposited and embryonated eggs compared to the control group, accompanied by a decrease in transcript levels. Interestingly, a moderate decrease in *Dmai-dsRNAse-2* expression in the salivary glands increased the efficiency of orally delivered dsRNA$^{RNase2/BicC}$ in silencing *Dmai-BicC*. In this regard, we hypothesize that the significant suppression of *Dmai-dsRNAse-2* in the midgut acts as a compensatory mechanism, contributing to the overall silencing effect of *Dmai-BicC*. In addition, this could be attributed to the short half-life of *Dmai-dsRNAse-2* proteins in salivary glands, leading to a considerable decline in nuclease activity even in the absence of significant changes in transcript abundance, as proposed in *B. tabaci* (Luo et al., 2017).

In our first report, the ovaries of females silenced through oral administration of dsRNA^{BicC} exhibited a range of morphological defects (Dalaisón-Fuentes et al., 2022). However, in the present study, we observed consistent effects on ovarian morphology in all silenced females, which we attribute to the increased availability of diet. Specifically, the vitellarium showed immature oocytes that failed to progress to the chorionic stage, like in injected females (Dalaisón-Fuentes et al., 2022). Therefore, the administration of a larger volume of diet

containing specific dsRNA targeting the gene of interest also enhance the efficiency of oral RNAi.

Silencing of *Dmai-dsRNase-2* not only enhances the effect of dsRNA^{BicC}, resulting in reduced oviposition and embryonated eggs but also prolongs this effect. Our findings revealed that, 22 days after silencing, 31.25% of dsRNA$^{Rnase2/BicC}$ females still maintained the gene silencing effect, compared to only 17% of females treated with dsRNA$^{\beta/BicC}$. These results not only provide further confirmation on the contribution of *Dmai-dsRNase-2* in the RNAi response, but also demonstrate an improvement in the efficiency of oral RNAi response in *D. maidis*.

Oral delivery of dsRNA has emerged as the most practical approach and has become the preferred method for implementing RNAi technology in pest control applications. Our study demonstrates that the co-delivery of dsRNA^{BicC} and dsRNA^{Rnase2} enhances the efficiency of oral RNAi in *D. maidis*. However, our results do not exclude the possibility of a synergistic effect between this enzyme and other *dsRNases* present in *D. maidis*. In a subsequent phase, we propose to further investigate the simultaneous silencing of multiple dsRNA-degrading nucleases to enhance RNAi efficiency. Moreover, alternative methods such as nanoparticle-formulated dsRNA or engineered microorganisms/viruses expressing specific dsRNA can be explored to prevent degradation by extracellular nucleases (Kunte et al., 2020; Pugsley et al., 2021; Silver et al., 2021; Yan et al., 2021).

In conclusion, our research validates the effectiveness of *Dmai-dsRNase-2* silencing as a strategy to enhance the insect's response to oral RNAi, and reinforces our previous findings on the considerable potential of RNAi-based systems to become a successful approach to *D. maidis* management.

5. References

Alivizatos, A. S., Markham, P. G., 1986. Acquisition and transmission of corn stunt spiroplasma by its leafhopper vector *Dalbulus maidis*. Annals of Applied Biology, 108(3), 535–544. https://doi.org/https://doi.org/10.1111/j.1744-7348.1986.tb01992.x

Allen, M. L., Walker, W. B., 2012. Saliva of *Lygus lineolaris* digests double stranded ribonucleic acids. Journal of Insect Physiology, 58(3), 391–396. https://doi.org/https://doi.org/10.1016/j.jinsphys.2011.12.014

Almeida Garcia, R., Lima Pepino Macedo, L., Cabral do Nascimento, D., Gillet, F. X., Moreira-Pinto, C. E., Faheem, M., Moreschi Basso, A. M., Mattar Silva, M. C., Grossi-de-Sa, M. F., 2017. Nucleases as a barrier to gene silencing in the cotton boll weevil, *Anthonomus grandis*. PLOS ONE, 12(12), e0189600-. https://doi.org/10.1371/journal.pone.0189600

Alstatt, G. E., 1945. A new corn disease in the Rio Grande Valley. Plant Disease. Rep, 29, 533–534.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J., 1990. Basic local alignment search tool. Journal of Molecular Biology, 215(3), 403–410. https://doi.org/https://doi.org/10.1016/S0022-2836(05)80360-2

Arimatsu, Y., Furuno, T., Sugimura, Y., Togoh, M., Ishihara, R., Tokizane, M., Kotani, E., Hayashi, Y., Furusawa, T., 2007a. Purification and Properties of Double-stranded RNA-degrading Nuclease, dsRNase, from the Digestive Juice of the Silkworm, *Bombyx mori*. Journal of Insect Biotechnology and Sericology, 76(1), 1_57-1_62. https://doi.org/10.11416/jibs.76.1_57

Arimatsu, Y., Kotani, E., Sugimura, Y., Furusawa, T., 2007b. Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*. Insect Biochemistry and Molecular Biology, 37(2), 176–183. https://doi.org/https://doi.org/10.1016/j.ibmb.2006.11.004

Bajet, N. B., Renfro, B. L., 1989. Occurrence of corn stunt spiroplasma at different elevations in Mexico. Plant Disease, 73(11), 926–930.

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., Roberts, J., 2007. Control of coleopteran insect pests through RNA interference. Nature Biotechnology, 25(11), 1322–1326. https://doi.org/10.1038/nbt1359

Bellés, X., 2010. Beyond *Drosophila*: RNAi *in vivo* and functional genomics in insects. Annual Review of Entomology, 55, 111–128.

Bhirud, K. M., Pitre, H. N., 1972. Bioactivity of systemic insecticides in corn: Relationship to leafhopper vector control and corn stunt disease incidence. Journal of Economic Entomology, 65(4), 1134–1140.

Bradfute, O. E., Tsai, J. H., Gordon, D. T., 1981. Corn stunt spiroplasma and viruses associated with a maize disease epidemic in Southern Florida. Plant Disease, 65(10), 837–841.

Cagliari, D., Dias, N. P., Galdeano, D. M., Dos Santos, E. Á., Smagghe, G., Zotti, M. J., 2019. Management of pest insects and plant diseases by non-transformative RNAi. Frontiers in Plant Science, 10, 1319.

Carpane, P., Catalano, M. I., 2022. Probing behavior of the corn leafhopper *Dalbulus maidis* on susceptible and resistant maize hybrids. PLOS ONE, 17(5), e0259481-. https://doi.org/10.1371/journal.pone.0259481

Christiaens, O., Swevers, L., Smagghe, G., 2014. DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. Peptides, 53, 307–314.

Cooper, A. M. W., Silver, K., Zhang, J., Park, Y., Zhu, K. Y., 2019. Molecular mechanisms influencing efficiency of RNA interference in insects. In Pest Management Science (Vol. 75, Issue 1, pp. 18–28). John Wiley and Sons Ltd. https://doi.org/10.1002/ps.5126

Dalaisón-Fuentes, L. I., Pascual, A., Gazza, E., Welchen, E., Rivera-Pomar, R., Catalano, M. I., 2022. Development of efficient RNAi methods in the corn leafhopper *Dalbulus maidis*, a promising application for pest control. Pest Management Science, 78(7), 3108–3116. https://doi.org/https://doi.org/10.1002/ps.6937

Di Rienzo, J. A., Guzmán, A. W., Casanoves, F., 2002. A multiple-comparisons method based on the distribution of the root node distance of a binary tree. Journal of Agricultural, Biological, and Environmental Statistics, 7(2), 129–142.

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., Tablada, M., Robledo, C.W., 2020. InfoStat versión 2020. Centro de Transferencia InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL http://www.infostat.com.ar

Drummond, A. J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology, 7(1), 214. https://doi.org/10.1186/1471-2148-7-214

Fan, Y. H., Song, H. F., Abbas, M., Wang, Y. L., Li, T., Ma, E. B., Cooper, A. M. W., Silver, K., Zhu, K. Y., Zhang, J. Z., 2021. A dsRNA-degrading nuclease (*dsRNase2*) limits RNAi efficiency in the Asian corn borer (*Ostrinia furnacalis*). Insect Science, 28(6), 1677–1689. https://doi.org/https://doi.org/10.1111/1744-7917.12882

Frazier, N., 1945. A streak disease of corn in California. Plant Dis. Rep, 29, 212–213.

Garbutt, J. S., Bellés, X., Richards, E. H., Reynolds, S. E., 2013. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from *Manduca sexta* and *Blattella germanica*. Journal of Insect Physiology, 59(2), 171–178. https://doi.org/https://doi.org/10.1016/j.jinsphys.2012.05.013

Giesbrecht, D., Heschuk, D., Wiens, I., Boguski, D., LaChance, P., Whyard, S., 2020. RNA interference is enhanced by knockdown of double-stranded RNases in the yellow fever mosquito *Aedes aegypti*. Insects, 11(6), 327.

Giménez Pecci, M. de la P., Laguna, I. G., Avila, A. O., Marino de Remes Lenicov, A. M., Virla, E. G., Borgogno, C., Nome, C. F., Paradell, S. L., 2002. Difusión del Corn Stunt Spiroplasma del maíz (*Spiroplasma kunkelii*) y del vector (*Dalbulus maidis*) en la República Argentina. Revista de La Facultad de Agronomía, 105.

Hruska, A. J., Gladstone, S. M., Obando, R., 1996. Epidemic Roller Coaster: Maize Stunt Disease in Nicaragua. American Entomologist, 42(4), 248–252. https://doi.org/10.1093/ae/42.4.248

Huvenne, H., Smagghe, G., 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. Journal of Insect Physiology, 56(3), 227–235.

Kruger, N. J., 2009. The Bradford Method For Protein Quantitation. In J. M. Walker (Ed.), The Protein Protocols Handbook (pp. 17–24). Humana Press. https://doi.org/10.1007/978-1-59745-198-7_4

Kunte, N., McGraw, E., Bell, S., Held, D., Avila, L. A., 2020. Prospects, challenges and current status of RNAi through insect feeding. Pest Management Science, 76(1), 26–41. https://doi.org/https://doi.org/10.1002/ps.5588

Lavore, A., Pagola, L., Esponda-Behrens, N., Rivera-Pomar, R., 2012. The gap gene *giant* of *Rhodnius prolixus* is maternally expressed and required for proper head and abdomen formation. Developmental Biology, 361(1), 147–155.

Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Research, 49(W1), W293–W296. https://doi.org/10.1093/nar/gkab301

Li, J., Du, J., Li, S., Wang, X., 2022. Identification and Characterization of a Double-Stranded RNA Degrading Nuclease Influencing RNAi Efficiency in the Rice Leaf Folder *Cnaphalocrocis medinalis*. International Journal of Molecular Sciences, 23(7), 3961.

Liu, J., Swevers, L., Iatrou, K., Huvenne, H., Smagghe, G., 2012. *Bombyx mori* DNA/RNA non-specific nuclease: Expression of isoforms in insect culture cells, subcellular localization and functional assays. Journal of Insect Physiology, 58(8), 1166–1176. https://doi.org/https://doi.org/10.1016/j.jinsphys.2012.05.016

Lomate, P. R., Bonning, B. C., 2018. Proteases and nucleases involved in the biphasic digestion process of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Archives of Insect Biochemistry and Physiology, 98(3), e21459.

Luo, Y., Chen, Q., Luan, J., Chung, S. H., Van Eck, J., Turgeon, R., Douglas, A. E., 2017. Towards an understanding of the molecular basis of effective RNAi against a global insect pest, the whitefly *Bemisia tabaci*. Insect Biochemistry and Molecular Biology, 88, 21–29.

Mao, Y. B., Cai, W. J., Wang, J. W., Hong, G. J., Tao, X. Y., Wang, L. J., Huang, Y. P., Chen, X. Y., 2007. Silencing a cotton bollworm *P450 monooxygenase* gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nature Biotechnology, 25(11), 1307–1313. https://doi.org/10.1038/nbt1352

Miller, M. A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop (GCE), 1–8. https://doi.org/10.1109/GCE.2010.5676129

Nault, L., Bradfute, O., 1979. Corn stunt: involvement of a complex of leafhopper-borne pathogens. In Maramorosch K. and Harris K. F. (Eds.), Leafhopper Vectors and Plant Disease Agents (pp. 561–585). Academic Press.

Nault, L. R., 1980. Maize bushy stunt and corn stunt: a comparison of disease symptoms, pathogen host ranges, and vectors. Phytopathology, 70(7), 659–662.

Oleszczuk, J. D., Catalano, M. I., Dalaisón, L., Di Rienzo, J. A., Giménez Pecci, M. de la P., Carpane, P., 2020. Characterization of components of resistance to Corn Stunt disease. PLOS ONE, 15(10), e0234454-. https://doi.org/10.1371/journal.pone.0234454

Oliveira, E., Waquil, J. M., Fernandes, F. T., Paiva, E., Resende, R. O., Kitajima, E. W., 1998. “Enfezamento pálido” e "enfezamento vermelho" na cultura do milho no Brasil Central. Fitopatologia Brasileira, 23(1), 45–47.

Paysan-Lafosse, T., Blum, M., Chuguransky, S., Grego, T., Pinto, B. L., Salazar, G. A., Bileschi, M. L., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D. H., Letunić, I., Marchler-Bauer, A., Mi, H., Natale, D. A., Orengo, C. A., Pandurangan, A. P., Rivoire, C., … Bateman, A., 2023. InterPro in 2022. Nucleic Acids Research, 51(D1), D418–D427. https://doi.org/10.1093/nar/gkac993

Peng, Y., Wang, K., Chen, J., Wang, J., Zhang, H., Ze, L., Zhu, G., Zhao, C., Xiao, H., Han, Z., 2020a. Identification of a double-stranded RNA-degrading nuclease influencing both ingestion and injection RNA interference efficiency in the red flour beetle *Tribolium castaneum*. Insect Biochemistry and Molecular Biology, 125, 103440. https://doi.org/https://doi.org/10.1016/j.ibmb.2020.103440

Peng, Y., Wang, K., Fu, W., Sheng, C., Han, Z., 2018. Biochemical Comparison of dsRNA Degrading Nucleases in Four Different Insects. Frontiers in Physiology, 9. https://www.frontiersin.org/articles/10.3389/fphys.2018.00624

Peng, Y., Wang, K., Zhu, G., Han, Q., Chen, J., Elzaki, M. E. A., Sheng, C., Zhao, C., Palli, S. R., Han, Z., 2020b. Identification and characterization of multiple dsRNases from a lepidopteran insect, the tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). Pesticide Biochemistry and Physiology, 162, 86–95. https://doi.org/https://doi.org/10.1016/j.pestbp.2019.09.011

Pérez-López, E., Wist, T., Dumonceaux, T., Luna-Rodríguez, M., Nordin, D., Castro-Luna, A., Iglesias-Andreu, L., Olivier, C., 2018. Detection of Maize Bushy Stunt Phytoplasma in Leafhoppers Collected in Native Corn Crops Grown at High Elevations in Southeast Mexico. Florida Entomologist, 101(1), 12–19. https://doi.org/10.1653/024.101.0104

Perfecto, I., 1990. Indirect and direct effects in a tropical agroecosystem: the maize-pest-ant system in Nicaragua. Ecology, 71(6), 2125–2134.

Prentice, K., Smagghe, G., Gheysen, G., Christiaens, O., 2019. Nuclease activity decreases the RNAi response in the sweetpotato weevil *Cylas puncticollis*. Insect Biochemistry and Molecular Biology, 110, 80–89. https://doi.org/https://doi.org/10.1016/j.ibmb.2019.04.001

Pugsley, C. E., Isaac, R. E., Warren, N. J., Cayre, O. J., 2021. Recent Advances in Engineered Nanoparticles for RNAi-Mediated Crop Protection Against Insect Pests. Frontiers in Agronomy, 3. https://www.frontiersin.org/articles/10.3389/fagro.2021.652981

Rao, X., Huang, X., Zhou, Z., Lin, X., 2013. An improvement of the 2ˆ (–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostatistics, Bioinformatics and Biomathematics, 3(3), 71.

Remes Lenicov, A., Virla, E., 1993. Aportes al conocimiento de la biología de *Dalbulus maidis* (Homoptera-Cicadellidae) en condiciones de laboratorio. Neotrópica, 39(101–102), 103–109.

Ren, D., Cai, Z., Song, J., Wu, Z., Zhou, S., 2014. dsRNA uptake and persistence account for tissue-dependent susceptibility to RNA interference in the migratory locust, *Locusta migratoria*. Insect Molecular Biology, 23(2), 175–184. https://doi.org/https://doi.org/10.1111/imb.12074

Rozen, S., Skaletsky, H., 1999. Primer3 on the WWW for General Users and for Biologist Programmers. In S. Misener and S. A. Krawetz (Eds.), Bioinformatics Methods and Protocols (pp. 365–386). Humana Press. https://doi.org/10.1385/1-59259-192-2:365

Sharma, R., Nji, C., Taning, T., Smagghe, G., Christiaens, O., 2021. Silencing of Double-Stranded Ribonuclease Improves Oral RNAi Efficacy in Southern Green Stinkbug *Nezara viridula*. Insects, 12(2), 115. https://doi.org/10.3390/insects

Shukla, J. N., Kalsi, M., Sethi, A., Narva, K. E., Fishilevich, E., Singh, S., Mogilicherla, K., Palli, S. R., 2016. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. RNA Biology, 13(7), 656–669. https://doi.org/10.1080/15476286.2016.1191728

Sievers, F., Higgins, D. G., 2014. Clustal Omega, Accurate Alignment of Very Large Numbers of Sequences. In D. J. Russell (Ed.), Multiple Sequence Alignment Methods (pp. 105–116). Humana Press. https://doi.org/10.1007/978-1-62703-646-7_6

Silver, K., Cooper, A. M. W., Zhu, K. Y., 2021. Strategies for enhancing the efficiency of RNA interference in insects. Pest Management Science, 77(6), 2645–2658. https://doi.org/https://doi.org/10.1002/ps.6277

Singh, I. K., Singh, S., Mogilicherla, K., Shukla, J. N., Palli, S. R., 2017. Comparative analysis of double-stranded RNA degradation and processing in insects. Scientific Reports, 7(1). https://doi.org/10.1038/s41598-017-17134-2

Song, H., Fan, Y., Zhang, J., Cooper, A. M. W., Silver, K., Li, D., Li, T., Ma, E., Zhu, K. Y., Zhang, J., 2019. Contributions of dsRNases to differential RNAi efficiencies between the injection and oral delivery of dsRNA in *Locusta migratoria*. Pest Management Science, 75(6), 1707–1717. https://doi.org/https://doi.org/10.1002/ps.5291

Song, H., Zhang, J., Li, D., Cooper, A. M. W., Silver, K., Li, T., Liu, X., Ma, E., Zhu, K. Y., Zhang, J., 2017. A double-stranded RNA degrading enzyme reduces the efficiency of oral RNA interference in migratory locust. Insect Biochemistry and Molecular Biology, 86, 68–80. https://doi.org/https://doi.org/10.1016/j.ibmb.2017.05.008

Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G., Vanden Broeck, J., 2017. Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*. Insect Biochemistry and Molecular Biology, 81, 103–116. https://doi.org/https://doi.org/10.1016/j.ibmb.2017.01.004

Stothard, P., 2000. The Sequence Manipulation Suite: JavaScript Program for Analyzing and Formatting Protein and DNA Sequences. BioTechniques, 28(6), 1102–1104. https://doi.org/10.2144/00286ir01

Summers, C. G., Stapleton, J. J., 2002. Management of corn leafhopper (Homoptera: Cicadellidae) and corn stunt disease in sweet corn using reflective mulch. Journal of Economic Entomology, 95(2), 325–330.

Tayler, A., Heschuk, D., Giesbrecht, D., Park, J. Y., Whyard, S., 2019. Efficiency of RNA interference is improved by knockdown of dsRNA nucleases in tephritid fruit flies. Open Biology, 9(12), 190198. https://doi.org/10.1098/rsob.190198

Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., Winther, O., Brunak, S., von Heijne, G., Nielsen, H., 2022. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nature Biotechnology, 40(7), 1023–1025. https://doi.org/10.1038/s41587-021-01156-3

Tsai, J. H., Capinera, J. L., 2008. Corn leafhopper, *Dalbulus maidis* (Delong and Wolcott) (Hemiptera: Cicadellidae). Encyclopedia of Entomology; Capinera, J., Ed.; Springer: Dordrecht, The Netherlands, 1072–1074.

Tsai, J. H., Steinberg, B., Falk, B. W., 1990. Effectiveness and residual effects of seven insecticides on *Dalbulus maidis* (Homoptera: Cicadellidae) and *Peregrinus maidis* (Homoptera: Delphacidae). Journal of Entomological Science, 25(1), 106–111.

Wang, K., Peng, Y., Pu, J., Fu, W., Wang, J., Han, Z., 2016. Variation in RNAi efficacy among insect species is attributable to dsRNA degradation *in vivo*. Insect Biochemistry and Molecular Biology, 77, 1–9.

Wilson, R. C., Doudna, J. A., 2013. Molecular Mechanisms of RNA Interference. Annual Review of Biophysics, 42(1), 217–239. https://doi.org/10.1146/annurev-biophys-083012-130404

Wynant, N., Santos, D., Verdonck, R., Spit, J., Van Wielendaele, P., Vanden Broeck, J., 2014. Identification, functional characterization and phylogenetic analysis of double stranded RNA degrading enzymes present in the gut of the desert locust, *Schistocerca gregaria*. Insect Biochemistry and Molecular Biology, 46, 1–8. https://doi.org/https://doi.org/10.1016/j.ibmb.2013.12.008

Yan, S., Ren, B. Y., Shen, J., 2021. Nanoparticle-mediated double-stranded RNA delivery system: A promising approach for sustainable pest management. Insect Science, 28(1), 21–34. https://doi.org/https://doi.org/10.1111/1744-7917.12822

Yoon, J. S., Ahn, S. J., Flinn, C. M., Choi, M. Y., 2021. Identification and functional analysis of dsRNases in spotted-wing drosophila, *Drosophila suzukii*. Archives of Insect Biochemistry and Physiology, 107(4), e21822. https://doi.org/https://doi.org/10.1002/arch.21822

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., Smagghe, G., 2013. Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Science, 20(1), 4–14. https://doi.org/https://doi.org/10.1111/j.1744-7917.2012.01534.x

Zamore, P. D., Tuschl, T., Sharp, P. A., Bartel, D. P., 2000. RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals. Cell, 101(1), 25–33. https://doi.org/https://doi.org/10.1016/S0092-8674(00)80620-0

Zhang, J., Khan, S. A., Heckel, D. G., Bock, R., 2017. Next-Generation Insect-Resistant Plants: RNAi-Mediated Crop Protection. In Trends in Biotechnology (Vol. 35, Issue 9, pp. 871–882). Elsevier Ltd. https://doi.org/10.1016/j.tibtech.2017.04.009

Zhang, T., Zhi, J. R., Li, D. Y., Liu, L., Zeng, G., 2022. Effect of different double-stranded RNA feeding solutions on the RNA interference of *V-ATPase-B* in *Frankliniella occidentalis*. Entomologia Experimentalis et Applicata, 170(5), 427–436. https://doi.org/https://doi.org/10.1111/eea.13153

Zhu, J. Q., Liu, S., Ma, Y., Zhang, J. Q., Qi, H. S., Wei, Z. J., Yao, Q., Zhang, W. Q., L. S., 2012. Improvement of Pest Resistance in Transgenic Tobacco Plants Expressing dsRNA of an Insect-Associated Gene *EcR*. PLOS ONE, 7(6), e38572-. https://doi.org/10.1371/journal.pone.0038572

Table legends

Table 1. *In silico* identification of *Dmai-dsRNases*. Annotations were predicted by sequence similarity searches. cDNA: transcript length; ORF: Open Reading Frame; EPL: Encoded Protein Length; best hit: the closest match found by local BLASTp search; % ID (percent identity), alignment length and e-value to the best hit. *Hvit*: *Homalodisca vitripennis* and *Ncin*: *Nephotettix cincticeps*.

Annotation	Transcript ID	cDNA (bp)	ORF	EPL (aa)	BLASTp			
					Best hit	% ID	Alignment length	E-value
Dmai-dsRNase-1	TRINITY_DN13786	1,73[illegible]	1,540-318	440	*Hvit* (010673.1)	79.51	444	0.0
Dmai-dsRNase-2	TRINITY_DN22752	1,6[illegible]14	1,461-37	474	*Ncin* (00007745-RA)	47.85	464	1.65e-143
Dmai-dsRNase-3	TRINITY_DN5008	1,738	1,539-304	411	*Hvit* (015154.1)	63.26	381	0.0

Figure legends

Fig. 1. *In silico* characterization of *Dmai-dsRNase* proteins. Upper panel: schematic diagram of the three predicted *Dmai-dsRNase* proteins, including their conserved domains and length (in brackets). The sequences of the signal peptides are also specified. Lower panel: partial alignment of the three predicted DNA/RNA non-specific endonuclease domains (PF01223). Asterisks mark active sites, triangles mark Mg^{++} binding sites and circles mark substrate binding sites. The amino acid and nucleotide sequences of *Dmai-dsRNases* ORFs are provided in Supplementary Materials (File S1).

Fig. 2. Phylogenetic tree of arthropod *dsRNases*. Amino acid sequences of *dsRNases* from different insect orders, including predicted *Dmai-dsRNases* (in bold), are grouped in different colors. Sequences of the crustaceans *Hirondellea gigas*, *Penaeus monodon* and *Trinorchestia longiramus* serve as a root. For each *dsRNase*, the access number and species name are given.

Fig. 3. Expression levels of *Dmai-dsRNases in D. maidis* tissues. Transcript levels of *Dmai-dsRNase-1* (**A**), *Dmai-dsRNase-2* (**B**), and *Dmai-dsRNase-3* (**C**) were measured in the salivary

glands, midgut, fat body, and the rest of body of adult females. Each bar represents the mean and standard error of three biological replicates mean (SEM) (ANOVA, Bonferroni test, α=0.05).

Fig. 4. Expression levels of *Dmai-dsRNase-2* and *Dmai-BicC* after *dsRNA^{RNase2}* injection. Transcript levels of *Dmai-dsRNase-2* and *Dmai-BicC* in salivary glands (**A**), midgut (**B**), and ovaries (**C**), as it corresponds. Expression was measured at one and seven days after completion of dsRNA administration. Each bar represents the mean and standard error of three biological replicates mean (SEM) (ANOVA, Bonferroni test, α=0.05)

Fig. 5. Survival of *D. maidis* females after dsRNA^{RNase2} injection. Kaplan–Meier curves representing the percentage survival of *D. maidis* females over time. T1: dsRNA^{RNase2} or dsRNA$^{\beta}$ injection; T2: female recovery on corn plants (48 h); T3: dsRNA^{BicC} or dsRNA$^{\beta}$ feeding days (72 h); T4: female and male introduction to the rearing cages; T5 and T7: dissection at one and seven days after the end of dsRNA administration, respectively; T6: female monitoring at four days after completion of dsRNA administration. According to the Log-rank test, survival curves were not significantly different (α=0.05).

Fig. 6. Survival of *D. maidis* females after dsRNA co-feeding. Kaplan–Meier curves representing the percentage survival of *D. maidis* females throughout the experiment. 1-3: dsRNA$^{RNase2/BicC}$, dsRNA$^{\beta/BicC}$ or dsRNA$^{\beta/\beta}$ feeding; 4: female and male introduction to the rearing cages; 4-22: oviposition and survival recording. Survival curves were not significantly different according to the Log-rank test (α=0.05).

Fig. 7. Oviposition of *D. maidis* females after dsRNA co-feeding. Scatter plot representing the number of total (**A**) and embryonated eggs (**B**) per treatment. The black lines indicate the mean of each group. Values with the same letter are not significantly different according to contrasts in the DGC test (α=0.05). The treatment factor significantly affects the response variable (p<0.0001).

Fig. 8. Expression levels of *Dmai-dsRNase-2* and *Dmai-BicC* after dsRNA co-feeding. Transcript levels of *Dmai-dsRNase-2* and *Dmai-BicC* in the midgut (**A**), salivary glands (**B**), and ovaries (**C**), as it corresponds. Expression was measured at one and seven days after completion of dsRNA administration. All values were referred to as the *Dmai-dsRNase-2* or *Dmai-BicC* expression at 1-day post treatment in females treated with dsRNA$^{\beta/\beta}$. Each bar represents the mean and standard error of three biological replicates mean (SEM) (ANOVA, Bonferroni test, α=0.05).

Supplementary Materials:

Table S1. Primers used for dsRNA synthesis and RT-(q)PCR.

Table S2. Effect of dsRNA co-feeding on *D. maidis* female reproduction. (**A**) The number of total (dark gray rows) and embryonated (white rows) eggs was recorded every 48 h for 18 days. The percentage of embryonated eggs relative to the total is expressed in brackets. The "total" column provides the sum of ovipositions within each category during the experiment. The factors 'treatment' and 'time', as well as their interaction, significantly affect the response variable (p<0.0001 in all cases). (**B**) Percentage of females belonging to each class throughout the experiment. Females were organized into three categories according to their oviposition behavior: sterile (no egg laying), non-embryonated (mostly non-embryonated eggs), and embryonated (mostly embryonated eggs).

Fig. S1. Assessment of dsRNA stability in different tissues of *D. maidis*. Degradation of dsRNA at 1, 12, and 24 h after exposure to tissue homogenates, visualized on 1% agarose gels. L: 100 bp ladder; RB: rest of body; FB: fat body; M: midgut; SG: salivary glands; C: control (naked dsRNA).

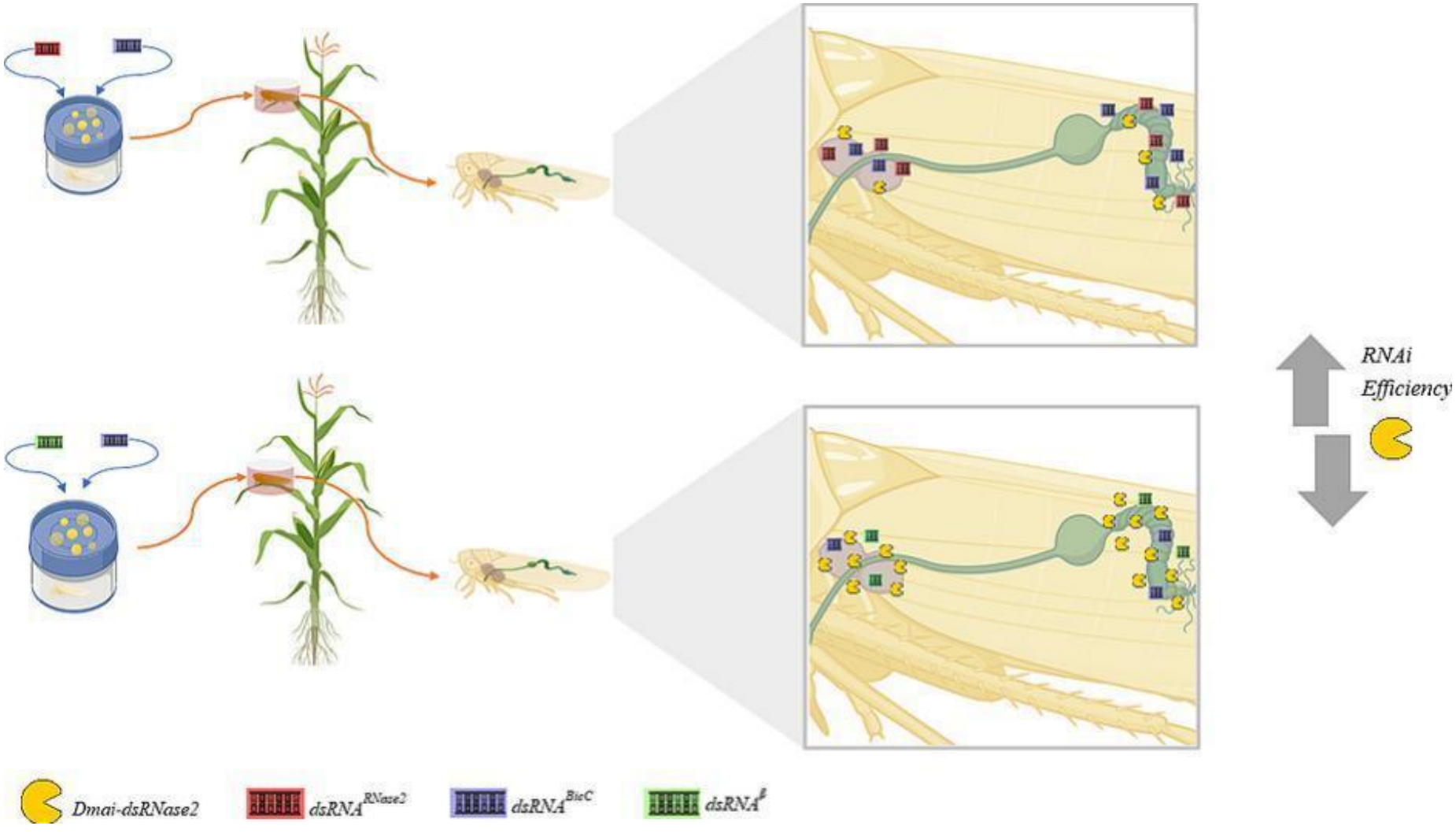
Fig. S2. Effect of dsRNA co-feeding on *D. maidis* female reproductive organs. Ovary morphology of control (**A**) and silenced (**B**) females under stereomicroscope. Silenced females have tubular-shaped ovarioles -germarium (G), oocytes (O) and pedicle (P)- as the controls. However, their ovarioles contain a greater number of immature oocytes. Scale bar: 500 µm. Ovipositions of control (**C**) and silenced (**D**) females under stereomicroscope. White arrowhead: red eye. Scale bar: 200 µm.

File S1. Amino acid and nucleotide sequences of *Dmai-dsRNases*.

Graphical Abstract

Highlights:

- *Dalbulus maidis* is the primary vector of Corn Stunt Spiroplasma (CSS).
- RNAi has emerged as a potent tool for the biological control of pests.
- *dsRNases* are conserved in the leafhopper, and show a specific expression profile.
- *Dmai-dsRNase-2* reduce oral RNAi efficiency in *D. maidis*.



Graphics Abstract

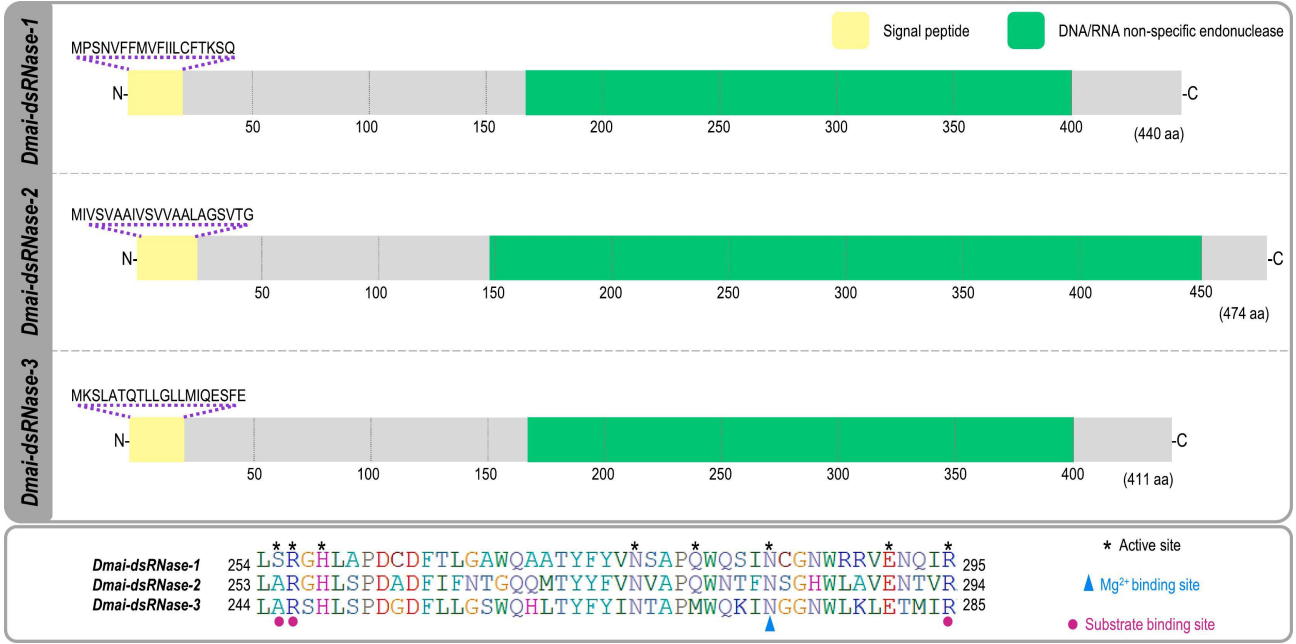

Signal peptide
DNA/RNA non-specific endonuclease
Dmai-dsRNase-1
MPSNVFFMVFIILCFTKSQ
N-
-C
50
100
150
200
250
300
350
400
(440 aa)
Dmai-dsRNase-2
MIVSVAAIVSVVAALAGSVTG
N-
-C
50
100
150
200
250
300
350
400
450
(474 aa)
Dmai-dsRNase-3
MKSLATQTLLGLLMIQESFE
N-
-C
50
100
150
200
250
300
350
400
(411 aa)
Dmai-dsRNase-1 254 LSRGHLAPDCDFTLGAWQAATYFYVNSAPQWQSINCGNWRRVENQIR 295
Dmai-dsRNase-2 253 LARGHLSPDADFIFNTGQQMTYYFVNVAPQWNTFNSGHWLAVENTVR 294
Dmai-dsRNase-3 244 LARSHLSPDGDFLLGSWQHLTYFYINTAPMWQKINGGNWLKLETMIR 285
Active site
Mg2+ binding site
Substrate binding site

Figure 1

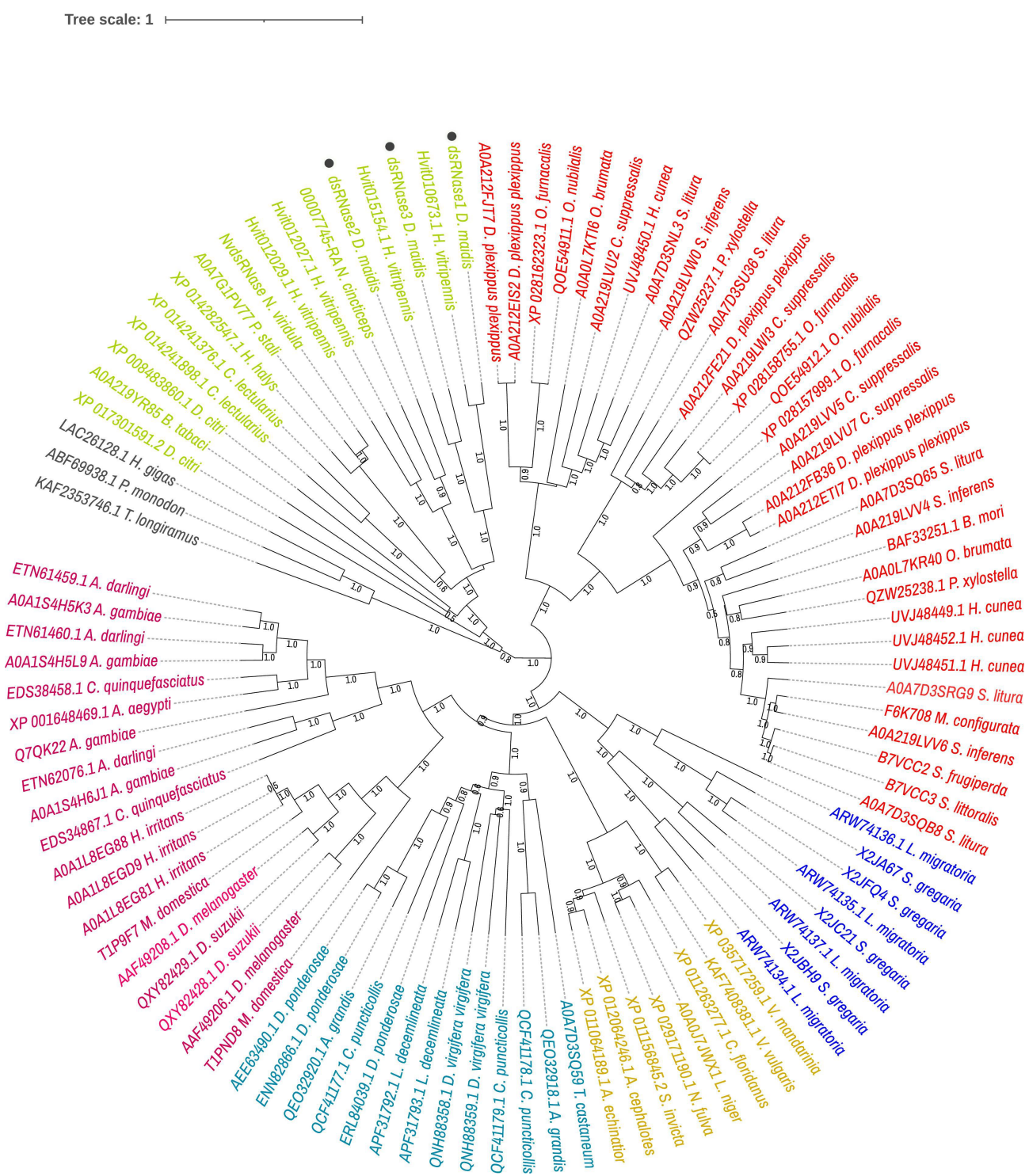

Tree scale: 1
dsRNase1 D. maidis
dsRNase3 D. maidis
dsRNase2 D. maidis
00007745-RA N. cincticeps
Hvit012027.1 H. vitripennis
Hvit012029.1 H. vitripennis
Hvit015154.1 H. vitripennis
Hvit010673.1 H. vitripennis
NvdsRNase N. viridula
A0A7G1PV77 P. stali
XP 014282547.1 H. halys
XP 014241376.1 C. lectularius
XP 014241898.1 C. lectularius
XP 008483860.1 D. citri
A0A219YR85 B. tabaci
XP 017301591.2 D. citri
LAC26128.1 H. gigas
ABF69938.1 P. monodon
KAF2353746.1 T. longiramus
A0A212FJT7 D. plexippus plexippus
A0A212EIS2 D. plexippus plexippus
XP 028162323.1 O. furnacalis
QOE54911.1 O. nubilalis
A0A0L7KTI6 O. brumata
A0A219LVU2 C. suppressalis
UVJ48450.1 H. cunea
A0A7D3SNL3 S. litura
A0A219LVW0 S. inferens
QZW25237.1 P. xylostella
A0A7D3SU36 S. litura
A0A219LWI3 C. suppressalis
A0A212FE21 D. plexippus plexippus
XP 028158755.1 O. furnacalis
QOE54912.1 O. nubilalis
XP 028157999.1 O. furnacalis
A0A219LVV5 C. suppressalis
A0A219LVU7 C. suppressalis
A0A212FB36 D. plexippus plexippus
A0A212ETI7 D. plexippus plexippus
A0A7D3SQ65 S. litura
A0A219LVV4 S. inferens
BAF33251.1 B. mori
A0A0L7KR40 O. brumata
QZW25238.1 P. xylostella
UVJ48449.1 H. cunea
UVJ48452.1 H. cunea
UVJ48451.1 H. cunea
A0A7D3SRG9 S. litura
F6K708 M. configurata
A0A219LVV6 S. inferens
B7VCC2 S. frugiperda
B7VCC3 S. littoralis
A0A7D3SQB8 S. litura
ARW74136.1 L. migratoria
X2JA67 S. gregaria
X2JFQ4 S. gregaria
ARW74135.1 L. migratoria
X2JC21 S. gregaria
ARW74137.1 L. migratoria
X2JBH9 S. gregaria
ARW74134.1 L. migratoria
XP 035717259.1 V. mandarinia
KAF7408381.1 V. vulgaris
A0A0J7JWX1 L. niger
XP 011263277.1 C. floridanus
XP 029171190.1 N. fulva
XP 011568452.1 S. invicta
XP 012064246.1 A. cephalotes
XP 011106189.1 A. echinatior
A0A7D3SQ59 T. castaneum
QEO32918.1 A. grandis
QCF41178.1 C. puncticollis
QCF41179.1 C. puncticollis
QNH88359.1 D. virgifera virgifera
QNH88358.1 D. virgifera virgifera
APF31793.1 L. decemlineata
APF31792.1 L. decemlineata
ERL84039.1 D. ponderosae
QCF41177.1 C. puncticollis
QEO32920.1 A. grandis
ENN82886.1 D. ponderosae
AEE63490.1 D. ponderosae
T1PND8 M. domestica
AAF49206.1 D. melanogaster
QXY82428.1 D. suzukii
QXY82429.1 D. suzukii
AAF49208.1 D. melanogaster
T1P9F7 M. domestica
A0A1L8EGD1 H. irritans
A0A1L8EG81 H. irritans
A0A1L8EG88 H. irritans
EDS34867.1 C. quinquefasciatus
A0A1S4H6J1 A. gambiae
ETN62076.1 A. darlingi
Q7QK22 A. gambiae
XP 001648469.1 A. aegypti
EDS38458.1 C. quinquefasciatus
A0A1S4H5L9 A. gambiae
ETN61460.1 A. darlingi
A0A1S4H5K3 A. gambiae
ETN61459.1 A. darlingi

Figure 2

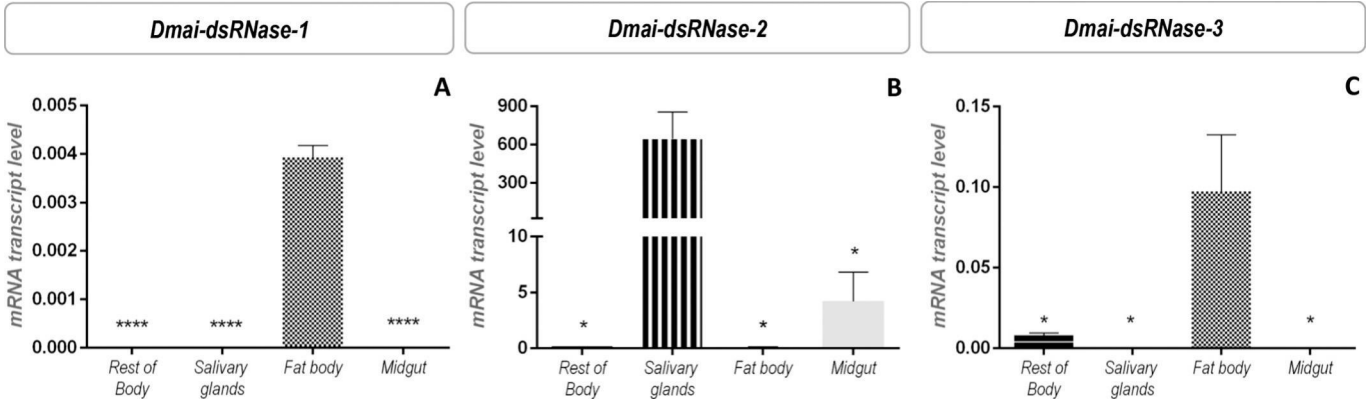

Dmai-dsRNase-1
Dmai-dsRNase-2
Dmai-dsRNase-3
A
B
C
mRNA transcript level
0.005
0.004
0.003
0.002
0.001
0.000
****
****
****
900
600
300
10
5
0
*
*
*
*
0.15
0.10
0.05
0.00
*
*
*
Rest of Body
Salivary glands
Fat body
Midgut

Figure 3

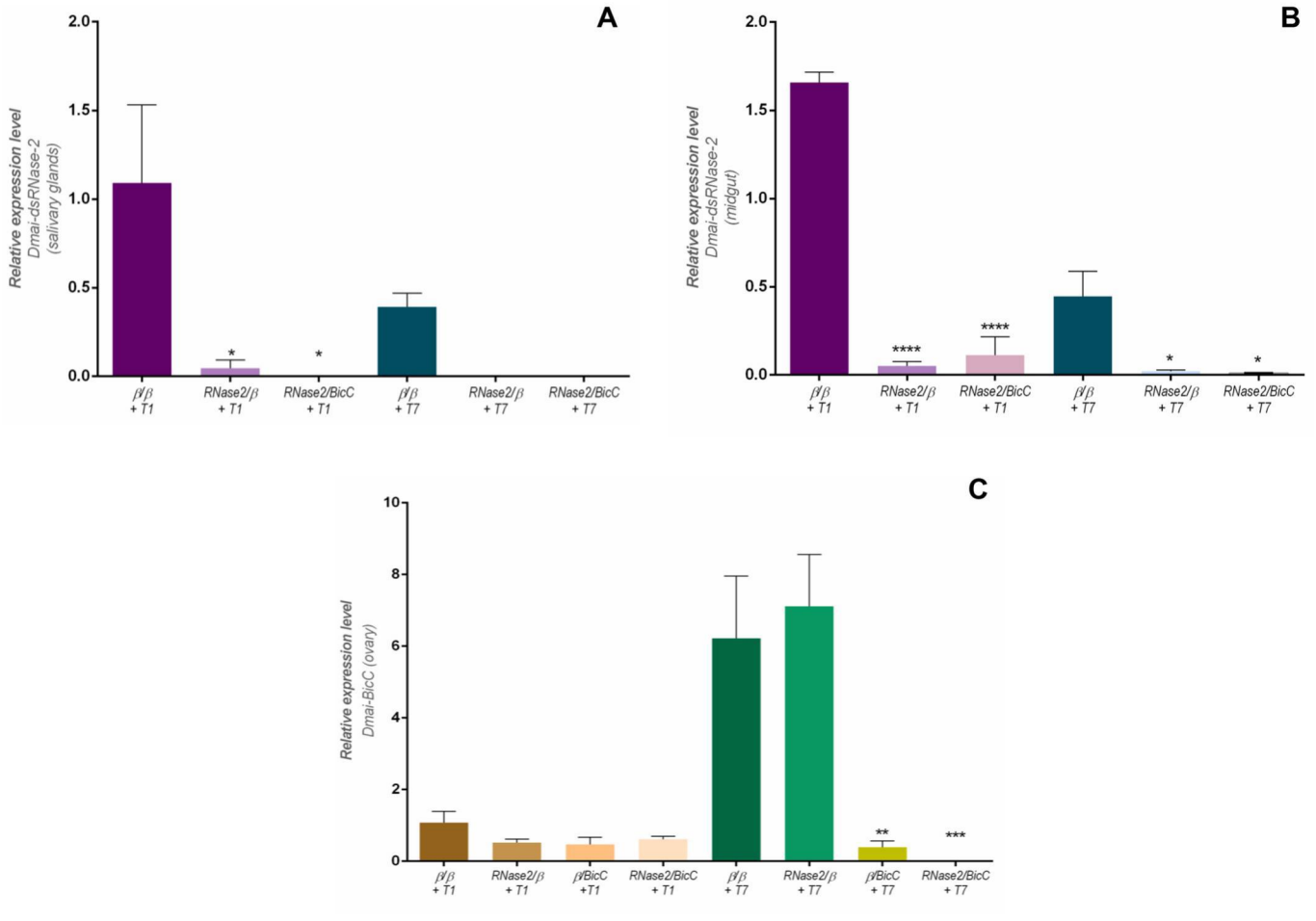

A
Relative expression level
Dmai-dsRNase-2
(salivary glands)
2.0
1.5
1.0
0.5
0.0
*
*
β/β + T1
RNase2/β + T1
RNase2/BicC + T1
β/β + T7
RNase2/β + T7
RNase2/BicC + T7
B
Relative expression level
Dmai-dsRNase-2
(midgut)
2.0
1.5
1.0
0.5
0.0
****
****
*
*
β/β + T1
RNase2/β + T1
RNase2/BicC + T1
β/β + T7
RNase2/β + T7
RNase2/BicC + T7
C
Relative expression level
Dmai-BicC (ovary)
10
8
6
4
2
0
**
***
β/β + T1
RNase2/β + T1
β/BicC +T1
RNase2/BicC + T1
β/β + T7
RNase2/β + T7
β/BicC + T7
RNase2/BicC + T7

Figure 4

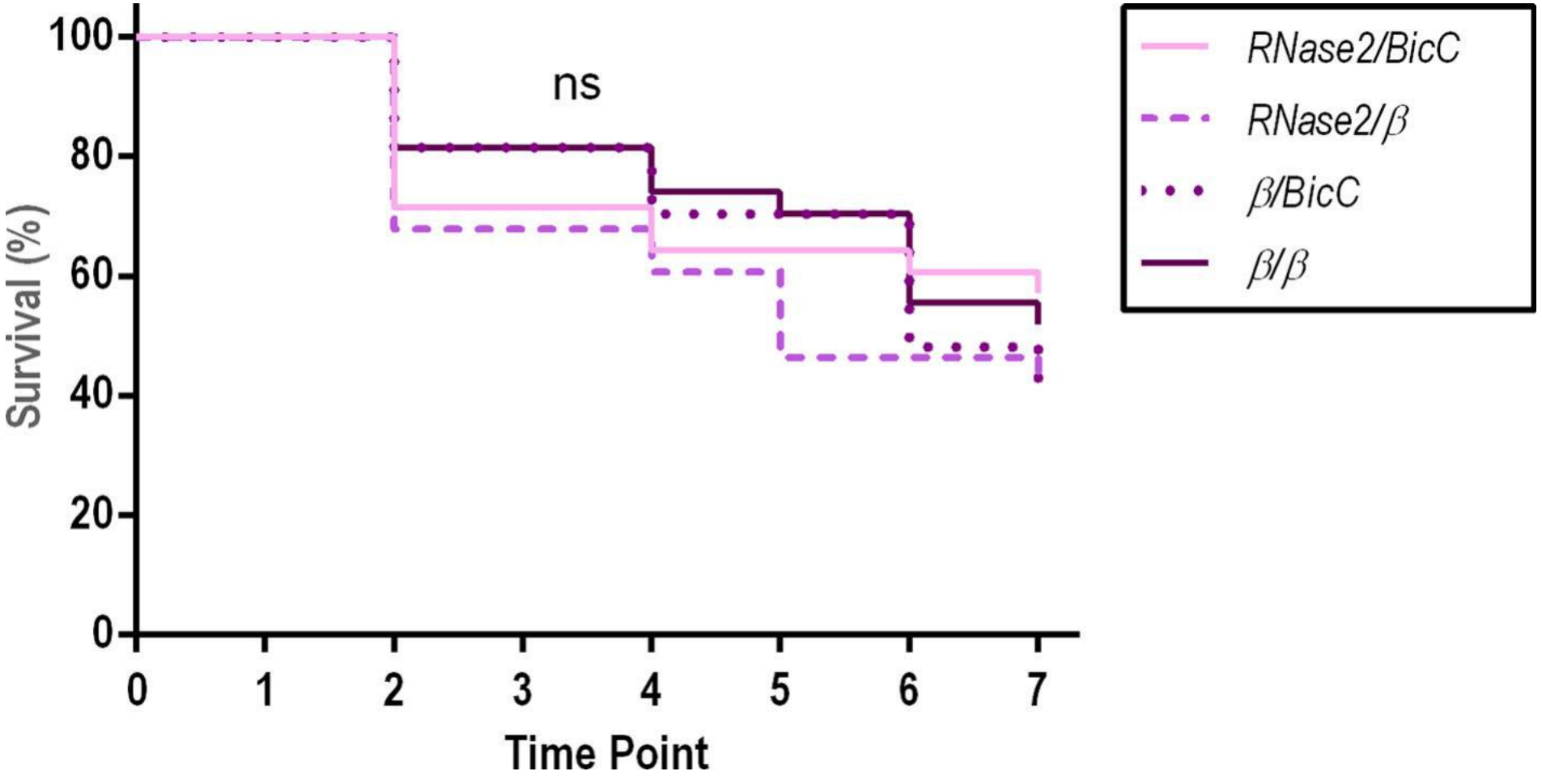

100
80
60
40
20
0
Survival (%)
ns
0
1
2
3
4
5
6
7
Time Point
RNase2/BicC
RNase2/β
β/BicC
β/β

Figure 5

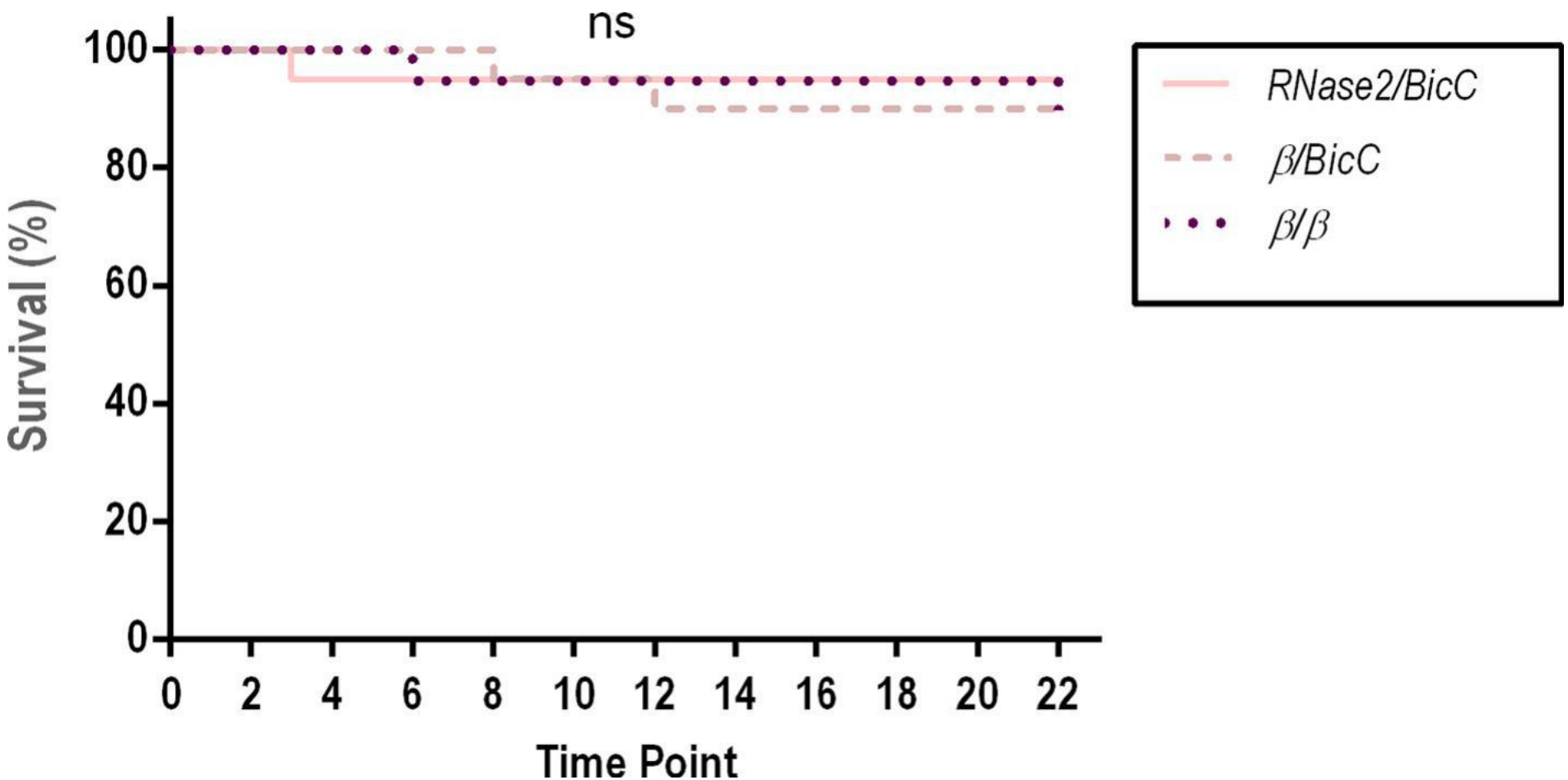

ns
100
80
60
40
20
0
Survival (%)
0
2
4
6
8
10
12
14
16
18
20
22
Time Point
RNase2/BicC
β/BicC
β/β

Figure 6

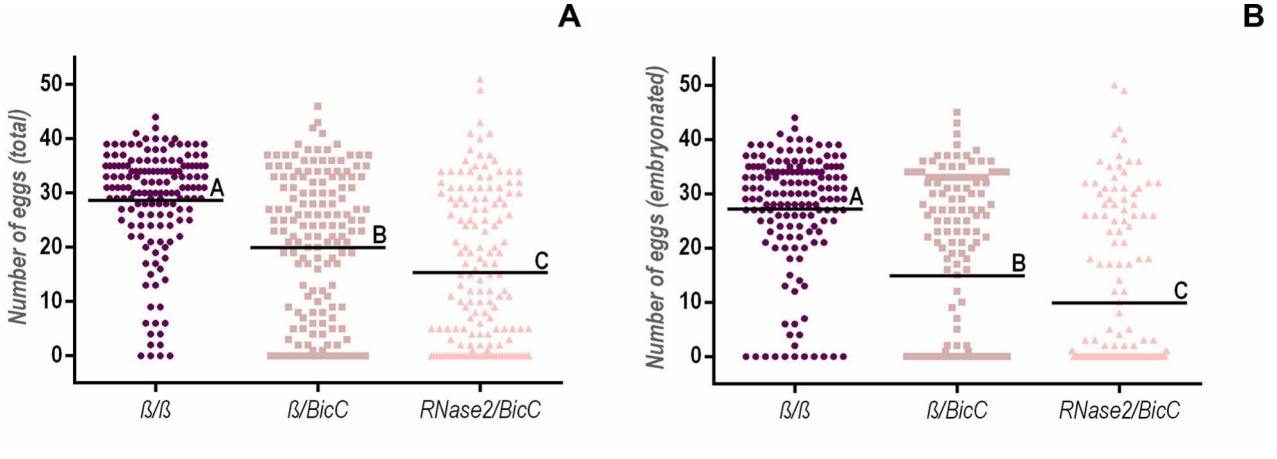

A
B
Number of eggs (total)
Number of eggs (embryonated)
0
10
20
30
40
50
A
B
C
ß/ß
ß/BicC
RNase2/BicC

Figure 7

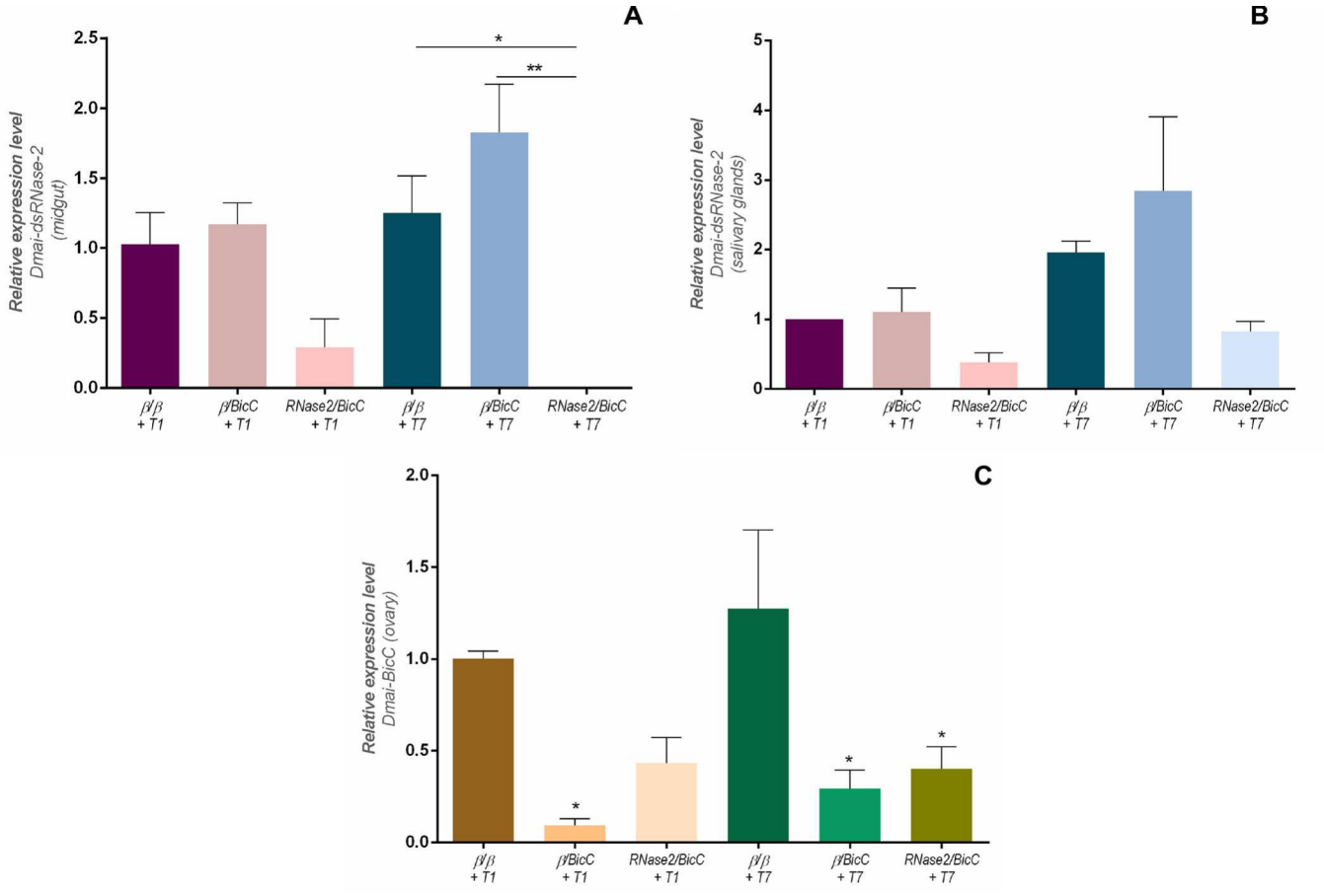

A
Relative expression level
Dmai-dsRNase-2
(midgut)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
β/β + T1
β/BicC + T1
RNase2/BicC + T1
β/β + T7
β/BicC + T7
RNase2/BicC + T7
B
Relative expression level
Dmai-dsRNase-2
(salivary glands)
5
4
3
2
1
0
β/β + T1
β/BicC + T1
RNase2/BicC + T1
β/β + T7
β/BicC + T7
RNase2/BicC + T7
C
Relative expression level
Dmai-BicC (ovary)
2.0
1.5
1.0
0.5
0.0
*
*
*
β/β + T1
β/BicC + T1
RNase2/BicC + T1
β/β + T7
β/BicC + T7
RNase2/BicC + T7

Figure 8