Increased RNAi Efficiency by dsEGFP-Induced Up-Regulation of Two Core RNAi Pathway Genes (OfDicer2 and OfAgo2) in the Asian Corn Borer (Ostrinia furnacalis)

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Simple Summary: RNA interference (RNAi) has shown great potentials as a novel technology for insect pest management. However, numerous studies have shown that the efficiency of RNAi varies substantially among different insect species. For example, as a major insect pest of corn, the Asian corn borer (Ostrinia furnacalis) showed very low RNAi efficiency. Therefore, it is necessary to develop new strategies for enhancing RNAi efficiency in insects with low RNAi efficiency. In this study, six core RNAi pathway genes were identified and characterized from O. furnacalis transcriptome database. After dsEGFP was injected into O. furnacalis, the expression of the core RNAi pathway genes (OfDicer2 and OfAgo2) was significantly up-regulated in response to the exposure of dsEGFP. As a result, the RNAi efficiency against the target genes in certain tissues of O. furnacalis was significantly improved. These results suggest that RNAi efficiency can be improved by inducing the expression of key RNAi pathway genes in O. furnacalis.

Abstract: RNA interference (RNAi) is a sequence-specific gene silencing mechanism that holds great promise for effective management of agricultural pests. Previous studies have shown that the efficacy of RNAi varies among different insect species, which limits its wide spread application in the field of crop protection. In this study, we identified and characterized six core RNAi pathway genes including OfDicer1, OfDicer2, OfR2D2, OfAgo1, OfAgo2, and OfAgo3 from the transcriptomic database of the Asian corn borer (Ostrinia furnacalis). Domain analysis showed that the six deduced proteins contained the necessary functional domains. Insect developmental stage- and tissue-specific expression analysis showed that five genes were expressed in all the stages and tissues examined except OfAgo3, which showed low expression in larvae, and high expression in pupae and adults and in the midgut. RT-qPCR was performed to examine the response of these six genes to exogenous double-stranded RNA (dsRNA). Interestingly, the transcript levels of OfDicer2 and OfAgo2 were significantly enhanced after the injection of dsEGFP at different time points and tissues investigated. Consequently, the RNAi efficiency in targeting the insect endogenous genes can be greatly enhanced in the hemolymph or midgut. Taken together, our investigations suggest that RNAi efficiency can be enhanced by pre-injection of dsRNA to induce the RNAi core machinery genes, which could be a useful strategy to improving RNAi efficiency for studying gene functions under laboratory conditions.

Key Words: Core RNAi pathway gene; Ostrinia furnacalis; RNAi efficiency; siRNA-mediated pathway
1. Introduction

RNA interference (RNAi) has become a powerful tool for gene functional studies and next-generation insect pest control [1,2], since its discovery by Andrew Z. Fire and Craig C. Mello in 1998 [3]. Previously, a myriad of reports have shown robust and systemic RNAi responses in various insect species belonging to Coleoptera [2,4] and Orthoptera [1,5,6]. However, in some insect species of Diptera, Lepidoptera, and Hemiptera, RNAi efficiency appeared very poor [7–11]. This variability in RNAi efficiency among various insect species greatly limits the widespread use of RNAi technology in both basic research and field applications for pest management.

There are several potential factors affecting RNAi efficiency [12]: (1) Stability of dsRNA. Degradation of dsRNA by nucleases (dsRNase or REase) has been documented to be the primary factor in reducing RNAi efficiency in many insects, whether in Orthoptera (susceptible to RNAi by injection) or in Lepidoptera (insusceptible to RNAi) [8,13–15]. Studies have shown that suppression of nuclease (dsRNase or REase) gene expression with RNAi [13,15] or application of liposomes or nanomaterials to protect dsRNA [16,17] could improve the stability of dsRNA. (2) Cellular uptake of dsRNA. Internalization of dsRNA into cells is an essential step to generate high RNAi efficiency. The approaches for overcoming limitations in dsRNA uptake in insects include expressing dsRNA in recombinant microbes for repeated oral delivery to insects [18], genetically engineering symbiotic bacteria to express and deliver dsRNA intracellularly [19], and enhancing clathrin-dependent endocytosis using arachidonic acid or hydrogen peroxide as was done for B. dorsalis [20,21]. (3) RNAi core machinery. Even if dsRNA is internalized and reaches the core machinery of the RNAi pathway in the cytoplasm (i.e., reaching the core RNAi enzymes), differences in the expression and function of the core RNAi enzymes could contribute to differences in RNAi efficiency among insects [22]. In Leptinotarsa decemlineata, expression of the core siRNA enzymes peaks in young larvae, when RNAi efficiency is the highest [23]. In contrast, a study did find a correlation between low RNAi efficiency in reproductive tissues of Schistocerca gregaria and low expression of Dicer2 and Ago2 [24].

The Asian corn borer (Ostrinia furnalalis) is one of the most destructive pests of corn in Asia, South-east Asia, and Oceania. In China, despite numerous control measures, this pest causes an estimated yield loss of 6–9 million tons per year. At present, its control relies mainly on synthetic insecticides and transgenic crops producing Bacillus thuringiensis (Bt) toxins. However, the heavy use of insecticides and Bt corn has resulted in some severe problems such as environmental pollutions and insecticide resistance in insect populations [25]. To hinder these catastrophic effects, there is an urgent need to find alternate approaches for insect pest management. In recent years, RNAi technology has emerged as a promising tool for pest management. However, RNAi has proven difficult to achieve in O. furnalalis [8]. Previously, studies have shown that degradation of dsRNA by OfREase and OfdsRNase2 is the reason for the low RNAi efficiency in O. furnalalis [8,13]. However, there has been no research on relationship between the expression levels of core RNAi pathway genes and RNAi efficiency in O. furnalalis. In this study, we identified six core RNAi pathway genes (OfDicer1, OfDicer2, OfR2D2, OfAgo1, OfAgo2, and OfAgo3) from the transcriptomic databases of O. furnacalis and examined the relationship between the up-regulation of these genes and the RNAi efficiency against target genes.

2. Materials and Methods

2.1. Insect Rearing

The eggs of O. furnacalis were purchased from Keyun, Henan Province, China, and were continuously reared in the laboratory at the Institute of Plant Protection, Shanxi Academy of Agricultural Sciences (Taiyuan, Shanxi, China) as described in Fan et al. [8].

2.2. Identification and Sequence Analysis of Core RNAi Pathway Genes

The candidate core RNAi pathway genes were identified in the transcriptomic databases of O. furnacalis through local BLAST using the sequences of Bombyx mori Dicer1 (XP_028040138.1)
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and Ago1 (NP_001095931.1), and Ostrinia nubilali OnDicer2 (MT921812), OnR2D2 (MT981255), OnAgo2 (MT524717), and Drosophila melanogaster DmAgo3 (EF211827) as query sequences based on annotation information of cDNA sequences. The identities of the candidate genes were further confirmed using BLAST searches against the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov, accessed on 30 November 2020. The obtained cDNAs were translated into protein sequences using the ExPASy-translational tool (https://web.expasy.org/translate/, accessed on 30 November 2020). SMART domain analysis tool (http://smart.embl-heidelberg.de/, accessed on 30 November 2020) was used to predict domain architecture.

2.3. Expression Analysis of Core RNAi Pathway Genes

Total RNA was isolated from the whole bodies of O. furnacalis at seven developmental stages (first to fifth instar larva, pupa and adult), and three tissues (hemolymph, integument, and midgut) dissected from 2-day-old fifth-instar larvae. We chose these three tissues because integument and midgut are the main exposed tissues when dsRNA is delivered by spray and feeding, respectively, whereas hemolymph is a key exposed tissue when dsRNA is delivered by injection. TRIzol reagent (Takara, Dalian, China) was used to homogenize the whole body and different tissues harvested from the larvae of O. furnacalis. Five biological replicates were made, and three tissues or individuals were pooled for each biological replicate. The quality and concentration of total RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µg of total RNA was taken for reverse-transcription using reagents and enzymes from Takara (Dalian, China). The synthesized cDNA samples were diluted ten-fold for use as template for reverse transcription quantitative PCR (RT-qPCR) analysis. RT-qPCR was performed using SYBR® Green Real-time PCR Master Mix (Promega, Madison, WI, USA) in a Light Cycler® 480II (Roche, Basel, Switzerland). The total volume of RT-qPCR reaction was 20 µL, containing 10 µL of 2 × SYBR mix, 0.8 µL of primers (F/R), 4 µL cDNA templates, and 4.4 µL ddH2O. The cycling conditions were: 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 31 s. Primers used for quantification of mRNA levels of core RNAi pathway genes were designed using primer Premier 5.0 software (Supplemental Table S1). Ribosomal protein S3 gene (OfRpS3, EU275206) was used as an internal control. OfRpS3 has been proven a suitable reference gene and widely applied in RNAi research of O. furnacalis [26,27]. Melting curves were analyzed to confirm the specificity of amplification. Relative mRNA levels were calculated as expression of the target gene relative to the mean of the reference gene using the 2−∆ΔCt method [28].

2.4. Expression of Core RNAi Pathway Genes after dsRNA Injection

Double-stranded RNA (dsRNA) was synthesized using T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). Primers for dsRNA synthesis were designed using the E-RNAi web service (http://www.dkfz.de/signaling/e-rna3/, accessed on 30 January 2020). The 2 × Taq Master Mix kit (Takara, Dalian, China) was used to synthesize the templates containing a T7 promoter sequence at each end. The amplified products were visualized by agarose gel electrophoresis (1%), and the PCR products were purified using the E.Z.N.A™ Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). PCR products were used directly as templates for the synthesis of dsRNA. The synthesized dsRNA was dissolved in nuclease-free water, and the final concentration of the dsRNA was adjusted to 2.5 µg/µL.

To investigate transcriptional responses of the core RNAi pathway genes at different times after injection of dsRNA, 2-day-old fifth-instar larvae (L5D2) of O. furnacalis were injected with dsEGFP at 5 µg/larva and then placed on diets. The larvae injected with ddH2O served as controls. Three individuals were collected as a sample at each of four time points (2, 4, 6, and 12 h) for expression analysis with RT-qPCR.

To investigate transcriptional responses of OfDicer2 and OfAgo2 in different tissues after the injection of dsRNA, each of L5D2 larvae was injected with 5 µg dsEGFP and then
placed on the artificial diet. Larvae injected with ddH2O were used as controls. After 2 h, hemolymph, integument, and midgut were dissected and collected in ice-cold 1.5-mL microcentrifuge tubes. Total RNA was extracted and RT-qPCR was performed to detect the expression of OfDicer2 and OfAgo2 as described above. Five biological replicates were made and three tissues were pooled as a biological replicate.

2.5. RNAi Efficiency Detection after Injection of dsRNA

The experimental design consisted of six different treatments, including ddH2O+dsEGFP, dsEGFP+dsEGFP, ddH2O+dsOEF1a, dsEGFP+dsOEF1a, ddH2O+dsOfCTP8, and dsEGFP+dsOfCTP8. A total of 90 L5D2 larvae of O. furnacalis were collected and divided into six groups with an average of 15 larvae per group. Each larva was first injected with a dose of 5 µg of dsEGFP (concentration: 2.5 µg/µL) or ddH2O. After 2 h of first injection each larva was reinjected with a dose of 10 µg (concentration: 4 µg/µL) of dsRNA. Twenty-four hours after the second injection, each larva was dissected, and hemolymph, integument, and midgut tissues were collected in ice-cold 1.5-mL microcentrifuge tubes for the extraction of total RNA. First-strand cDNA was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (Takara, Dalian, China). RT-qPCR was then performed to detect the relative expression of target genes. Specifically, the transcript levels of OfEF1α were determined in the treatments of ddH2O+dsEGFP, ddH2O+dsOfEF1a, dsEGFP+dsEGFP, and dsEGFP+dsOfEF1a, whereas the transcript levels of OfCTP8 were determined in the treatments of ddH2O+dsEGFP, ddH2O+dsOfCTP8, dsEGFP+dsEGFP, and dsEGFP+dsOfCTP8.

2.6. Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey’s test for multiple comparisons when the data from more than two groups were compared. However, data were analyzed with a Student’s t-test when the data from two groups were compared. A p value < 0.05 was considered statistically significant (*p < 0.05, **p < 0.01).

3. Results

3.1. Identification and Domain Analysis of Core RNAi Pathway Genes

A total of six core RNAi pathway genes, including two OfDicers (OfDicer1 and OfDicer2), one OfR2D2 and three OfAgos (OfAgo1, OfAgo2, and OfAgo3), were identified through local BLAST in the transcriptomic database of O. furnacalis (Table 1).

Table 1. Characteristics of core RNAi pathway transcripts and their deduced proteins from O. furnacalis.

| GenBank Accession Number | ORF (bp) | Protein (aa) | Mass (kDa) | Isoelectric Point (pI) |
|--------------------------|----------|-------------|------------|-----------------------|
| OfAgo1                   | –        | 2287        | 762        | 86.55                 | 9.48                  |
| OfAgo2                   | XP_028167628 | 3153        | 1050       | 117.56                | 9.47                  |
| OfAgo3                   | XM_028315439 | 2763        | 920        | 103.93                | 9.12                  |
| OfDicer1                 | XP_028160127 | 3519        | 1172       | 132.57                | 5.06                  |
| OfDicer2                 | XM_028314437 | 5073        | 1690       | 193.19                | 6.51                  |
| OfR2D2                   | MT981255  | 1158        | 385        | 42.49                 | 9.01                  |

Domain analysis showed that almost all predicted OfAgo proteins contained common domains including DUF, PAZ, and PIWI. OfDicer2 contained each of DEXDc, HELICc, PAZ, RNase IIIa, RNase IIIb, and dsRBD domains, whereas the DEXDc and HELICc domains are absent in OfDicer1, probably due to the partial sequences in the database. In contrast, OfR2D2 contained three dsRBD domains (Figure 1). The PAZ domain are specialized to bind RNA ends, especially duplex ends with short (approximately 2 nt) 3’ overhangs in Ago proteins, and the PIWI domain is unique to the Argonaute superfamily. The PAZ and
RNase III domains play central roles in excising siRNAs preferentially from ends of dsRNA molecules in Dicer proteins. The function of dsRBD is to bind dsRNA [29].

Figure 1. Schematic diagram of deduced domains of Dicer1, Dicer2 (A), R2D2 (B), Ago1, Ago2, and Ago3 (C). Blank lines represent linker regions; yellow hexagons represent PAZ domains; pink quadrilaterals represent RNase IIIa domain; purple quadrilaterals represent RNase IIIb domain; green ellipses represent dsRBD domain; pink diamond represents DEXDc domain; purple pentagon represents HELICc domain; blue pentagons represent DUF domains and green quadrilaterals represent PIWI domain.

3.2. Expression Patterns of Core RNAi Pathway Genes

To examine the transcription levels of core RNAi pathway genes, RT-qPCR was performed in seven developmental stages of O. furnacalis. The expression profiles showed that almost all the candidate genes were expressed in all the developmental stages examined except for OfAgo3 which showed the expression only in pupal and adult stages, but not in the larval stage. Generally, the expression of almost all the candidate genes was lowest in the larval stage, and gradually increased in the rest of developmental stages. Four candidate genes, including OfDicer1, OfDicer2, OfAgo1, and OfAgo2, displayed higher expression in pupal stage. However, almost all the candidate genes showed the highest expression in the adult stage (Figure 2A).
were highly expressed in the midgut, whereas OfAgo3 (Figure 4). The results demonstrated that there was no significant change in the relative expression levels of the respective core RNAi pathway genes were evaluated using RT-qPCR. Increased in the hemolymph, integument, and midgut by 2.8-, 2.0-, and 1.5-fold, respectively, whereas the expression level of OfAgo3 increased by 9.7-, 11.0-, and 13.5-fold, respectively, whereas the expression level of OfAgo3 showed that the expression level for different tissues including hemolymph, integument, and midgut by RT-qPCR. Our results injection of dsRNA injection. To our surprise, the expression levels of OfDicer2, OfDicer1, and OfAgo2 were up-regulated for long time after dsRNA injection. However, OfAgo1 was up-regulated only at 2 h after dsRNA injection. To our surprise, the expression levels of OfAgo3 and OfR2D2 were significantly increased after the injection of dsEGFP compared with the injection of ddH2O as control at each time interval (Figure 3).

3.3. Transcriptional Responses of Core RNAi Pathway Genes to Injection of dsEGFP

After introduction of dsEGFP into O. furnacalis, the response of RNAi pathway genes was investigated under two different experimental conditions. In the first experiment, larvae injected with 5 μg of dsEGFP in each were collected at 2, 4, 6, and 12 h and the expression levels of the respective core RNAi pathway genes were evaluated using RT-qPCR. The results demonstrated that there was no significant change in the relative expression of OfAgo3, OfDicer2, and OfR2D2 at any of these time points. However, OfAgo1 was up-regulated only at 2 h after dsRNA injection. To our surprise, the expression levels of OfAgo2 and OfDicer2 were significantly increased after the injection of dsEGFP compared with the injection of ddH2O as control at each time interval (Figure 3).

Since the expression of OfDicer2 and OfAgo2 was up-regulated for a long time after injection of dsEGFP, we evaluated the expression levels of OfDicer2 and OfAgo2 in three different tissues including hemolymph, integument, and midgut by RT-qPCR. Our results showed that the expression level for OfDicer2 in the hemolymph, integument, and midgut increased by 9.7-, 11.0-, and 13.5-fold, respectively, whereas the expression level of OfAgo2 increased in the hemolymph, integument, and midgut by 2.8-, 2.0-, and 1.5-fold, respectively (Figure 4).
To ascertain whether an increase in the transcript levels of OfDicer2 and OfAgo2 could lead to an increase in the RNAi efficiency, two target genes, OfEF1α (elongation factor 1α) and OfCTP8 (chymotrypsins 8) were selected as marker genes. In the control group, L5D2 larvae were first injected with ddH2O. After 2 h, dsEGFP or dsRNA of each of the target genes was injected into the larvae. Twenty-four hours later, RT-qPCR was carried out to analyze the relative expression levels of the target genes. Our results demonstrated that the transcript levels for OfEF1α remained constant and no significant change was observed.
in the hemolymph, integument, and midgut, while the expression level for OfCTP8 was efficiently silenced (81.6%) only in the integument, but not in the hemolymph and the midgut. When L5D2 larvae in the treatment group were injected with dsEGFP and then with the dsRNA of the target genes, the transcript level of OfEF1α was suppressed by 46.9% and 44.1% in the hemolymph and the midgut, respectively. In contrast, the transcript level for OfCTP8 was suppressed by 91.9% and 80.0% in the hemolymph and the integument, respectively (Figure 5). These results suggest that RNAi efficiency can be improved in some tissues of O. furnacalis by pre-injecting dsEGFP to induce the expression of RNAi core machinery genes.

Figure 5. Enhanced RNAi efficiency by pre-injection of dsEGFP in different tissues of O. furnacalis. (A) Overview of the experimental setup. ddH2O or dsEGFP was pre-injected into larvae (L5D2). After 2 h, dsEGFP or dsOfEF1α/dsOfCTP8 was injected into the larvae again. After 24 more hours, RT-qPCR was performed to examine the expression of OfEF1α or OfCTP8 in different tissues of O. furnacalis. (B) Different combinations of dsRNA or ddH2O injection were performed for this assay. The statistical significance between control and treatment results was assessed by Student’s t-test. Bars show mean ± SE (* p < 0.05; ** p < 0.01; N.S., nonsignificant) (n = 5).
4. Discussion

RNAi is a highly conserved, post-transcriptional gene-silencing mechanism in which small RNA molecules are utilized by RNAi core machinery that involves several core RNAi pathway genes to degrade complementary RNA molecules in a sequence-specific nature [12]. Core RNAi pathway genes are critical to RNAi efficiency. A previous study conducted by Tomoyasu et al. [30] has shown that two Ago2 (TcAgo2a and TcAgo2b) genes identified from the T. castaneum genome are involved in systemic RNAi response of RNAi. Similarly, both LmAgo2a and LmAgo2b have been found to contribute to the robust RNAi response in L. migratoria [31]. In L. decemlineata, two Dicer2 genes (Dicer2a and Dicer2b) and two Ago2 genes (Ago2a and Ago2b) were identified in L. decemlineata transcriptome database [23], which support the hypothesis that the number of core RNAi pathway genes may contribute to the variability of RNAi efficiency in insects. However, in the present study, only a single transcript of OfDicer2 and OfAgo2 was identified in O. furnacalis. It is speculated that this may be one of the reasons for the low RNAi efficiency in O. furnacalis.

In the present study, our results showed that the six core RNAi pathway genes (OfDicer1, OfDicer2, OfR2D2, OfAgo1, OfAgo2, and OfAgo3) were expressed in almost all the developmental stages and different tissues examined. In particular, the highest transcript level for the core RNAi pathway genes was observed at pupae and adult stages and in the midgut. Our results are in agreement with Cooper et al. [28] and Xie et al. [32] which showed that the expression patterns for Dicer2, R2D2, and Ago2 in O. nubilalis and B. dorsalis. Nevertheless, little has been known about differential expressions of core RNAi pathway genes during different developmental stages and in different tissues in relation to RNAi efficiency in insects. Because induced expression of core RNAi pathway genes can increase RNAi efficiency as we showed in this study, we hypothesize that RNAi-mediated suppression of a target gene is relatively easy to achieve when and where the core RNAi pathway genes are highly expressed. This information provides the fundamental basis to investigate the mechanism of RNA interference in various developmental stages and tissues of O. furnacalis.

Many previous studies have reported that RNAi acts as an RNA-based immune system against viral infections in insects, and plays a crucial role in regulating endogenous genes when insects are infected with a virus. To combat the viral infection, the RNAi machinery’s activity can enhance gradually by up-regulating some core RNAi components such as Dicer2 and Ago2 [32–34]. Similarly, non-specific dsRNAs have also been found in implicating as a trigger to induce the RNAi activity by up-regulating the expression of core RNAi pathway genes in certain insect species [23,35,36]. For example, it was found that dsLdSAHase-induced suppression of LdSAHase and the associated larval mortalities were influenced by pre-exposure to dsEGFP in Leptinotarsa decemlineata [23]. Similarly, pre-exposure of Acyrthosiphon pisum to dsGFP (600 ng) led to significant silencing of hunchback gene when the aphid was exposed to 60 ng of dsHunchback, a dose which cannot lead to the suppression of hunchback expression without pre-exposure of the aphid to dsGFP [35].

In this study, we first investigated the possible association between RNAi efficiency and the expression levels of core RNAi pathway genes in O. furnacalis. Our result showed the mRNA levels of OfDicer2 and OfAgo2 were high at all of the insect developmental stages, particularly in the late larval (L5D2) stage, pupal and adult stages, and as well as in the tissues, particularly the midgut. Because Dicer2 and Ago2 are believed to be involved in the siRNA pathway as shown in Drosophila [37], high expression of OfDicer2 and OfAgo2 may be related to RNAi efficiency during these stages. Indeed, several studies have shown that sufficient silencing efficiency can be achieved during the wandering or pupal stages of lepidopterans, such as B. mori and Spodoptera litura [38,39], which may be related to high expression of Dicer2 and Ago2 during these stages. Similarly, high expression of the core RNAi pathway genes in certain tissues, particularly in the midgut, may also imply strong RNAi machinery in these tissues. However, up-regulation of OfAgo1 was observed 2 h post-injection of dsEGFP, which implies that OfAgo1 could also be involved in siRNA pathway at an early stage of dsRNA exposure in O. furnacalis. These results are
similar to the functions of Ago1 in L. migratoria, which has been shown to be involved in siRNA-mediated RNAi pathway [31]. However, further research is needed to clarify the role of OfAgo1 in siRNA-mediated RNAi pathway.

Nevertheless, our major focus of this study was on possible induced effects caused by injection of exogenous dsRNA (i.e., dsEGFP) on RNAi efficiency for attacking target genes in O. furnacalis. Since injection of dsEGFP could induce a strong response of OfDicer2 and OfAgo2, OfEF1α and OfCTP8 were selected as two target genes to determine whether the silencing efficiency against the two target genes could be improved by pre-injection of dsEGFP. Previous studies have shown that OfEF1α can be significantly silenced by tissue culture RNAi in O. furnacalis [40], and OfCTP8 can be significantly knocked down by injection of dsOfCTP8 in O. furnacalis [13]. As expected, OfEF1α could not be significantly silenced in any tissues (hemolymph, integument, and midgut) when O. furnacalis larvae were pre-injected with ddH2O. However, OfEF1α can be significantly silenced in the hemolymph and midgut, but not in the integument, when O. furnacalis larvae were pre-injected with dsEGFP. Furthermore, RNAi efficiency targeting OfCTP8 could be significantly improved in the hemolymph by pre-injection of dsEGFP compared with pre-injection of ddH2O. Although our results are highly encouraging for improving RNAi efficiency by inducing the activity of the core machinery of the siRNA-mediated RNAi pathway through the pre-injection of dsEGFP, the level of the improvement for different target genes and in different tissues still varies considerably. Presumably, many other factors may contribute to such variations, including dsRNA sequence difference, different efficiencies in dsRNA uptake in different tissues, and different intensities of responses of the RNAi core machinery genes (i.e., OfDicer2 and OfAgo2) to the injection of dsEGFP in O. furnacalis. Therefore, further study is needed to pinpoint the exact mechanisms causing such variations.

5. Conclusions

In the current study, we identified six core RNAi pathway genes (OfDicer1, OfDicer2, OfAgo1, OfAgo2, OfAgo3, and OfR2D2) in O. furnacalis. Expression profiles indicated that five genes were expressed in all the developmental stages and tissues examined except OfAgo3, which showed low expression in larvae. However, only OfDicer2 and OfAgo2 were upregulated in response to dsEGFP exposure at all four time points and tissues investigated. In addition, the RNAi efficiency of target genes can be significantly enhanced in the hemolymph or midgut by pre-injection of dsEGFP. Thus, this study provides new insights into developing useful strategies for improving RNAi efficiency in RNAi-refractory insect species, particularly in laboratory studies, where pre-exposure to an exogenous dsRNA in insects is feasible.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13030274/s1, Table S1: Primers for PCR amplification, dsRNA synthesis and RT-qPCR analysis.

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