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Parasitism and survival rate of *Diadegma fenestrale* (Hymenoptera: Ichneumonidae) and DfIV gene expression patterns in two lepidopteran hosts

Ju Il Kim [a, *], Min Kwon [a], Si Hyeock Lee [b], Yonggyun Kim [c]

[a] *Highland Agriculture Research Institute, Rural Development Administration, National Institute of Crop Science, Pyeong-chang 232-955, Republic of Korea*
[b] *School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*
[c] *Department of Bioresource Sciences, Andong National University, Andong 760-749, Republic of Korea*

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ABSTRACT

The genus *Diadegma* is a well-known parasitoid group and some are known to have symbiotic virus, polydnavirus (PDV). A novel IV was discovered from the calyx of *Diadegma fenestrale* female and sequenced its genome. *D. fenestrale* has more than two hosts, including potato tuber moth (PTM) and diamondback moth (DBM). *D. fenestrale* preferred PTM to DBM as hosts based on the oviposition and survival rate. Nevertheless, the developmental period and morphology of *D. fenestrale* were not significantly different between PTM and DBM. We compared DfIV gene expression patterns between PTM and DBM under various conditions to understand the phenomena. DfIV genes were more widely expressed in PTM with large numbers than in DBM after parasitized by *D. fenestrale*, particularly at the initial point. They showed differential expression patterns between two lepidopteran hosts. This DfIV gene expression plasticity showed a dependency on the lepidopteran host species and parasitization time, suggesting that it may contribute to increase the parasitoid survival rate. This might be one of the key elements that determine the symbiotic relationship between PDV and parasitoid.

1. Introduction

Parasitoids occur in seven holometabolous orders of insects, including Hymenoptera [1,2]. Complex procedures such as right host choose, overcome the host immune response and adopt to or regulate host physiology are involved in the successful parasitism by insect parasitoids for the sake of the larval parasitoid [3]. Hymenoptera possess the largest number of parasitoid among the six orders (Pennacchio and Strand, 2006). Certain parasitoids from the Braconidae and Ichneumonidae families have developed an extraordinary strategy to protect their egg and larva from the host's immune responses [4]. These parasitoids employ several factors that can regulate female reproductive system, including the venom, ovarian proteins, and symbiotic virus, PDV. PDVs were first discovered from some parasitoid calyx fluid using electron microscope and classified as a polydnaviridae 4 decades ago [5–8]. Previous studies reported that PDVs alone or in conjunction with other factors actively suppress host immunity [9,10]. That means that PDVs contribute to the survival of parasitoid in its hosts, such as lepidopteran caterpillar [11].

Diadegma fenestrale is known as a generalist [12]. *D. fenestrale* was initially collected from parasitized PTM infesting potato cultivation field in Jeju, Korea in May 2009. Moreover, it was also collected from parasitized DBM infesting cabbage nearby potato field. It has more than two lepidopteran hosts such as PTM and DBM in Korea [13]. In earlier study, *D. fenestrale* ichnovirus (DfIV) had already identified and sequenced its genome [14].

Nevertheless, the emergency rate of *D. fenestrale* in PTM larvae was two-fold higher than that of DBM [13]. Therefore, this finding led to ask following questions: why does *D. fenestrale* prefer PTM to DBM and why is the parasitism success rate higher in PTM? To understand this host preference, parasitism success rate of *D. fenestrale*, we characterized of PDV and its gene expression patterns.

The objective of this study were to compare the successful parasitism rate difference of *D. fenestrale* on two lepidopteran host;

Abbreviation: DfIV, *Diadegma fenestrale* Ichnovirus; PDV, polydnavirus; IV, ichnovirus; BV, bracovirus; PTM, potato tuber moth; DBM, diamondback moth; *rep*, repeat element protein gene; *cys-motif*, cysteine motif protein gene; *cys-rich*, cysteine rich protein gene; *vankyrin*, viral ankyrin; *vinnexin*, viral innexin.

* Corresponding author. Fax: +82 33 330 1519.
E-mail address: forweek@korea.kr (J.I. Kim).

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to investigate the progressive transcriptional profiles of DfIV and its hosts following parasitization; to compare the viral gene expression for parasitized or non-parasitized larval samples of PTM and DBM. This study would contribute to the understanding of host-specific gene expression patterns of PDV.

2. Materials and methods

2.1. Parasitoid and lepidopteran hosts

The field collected *D. fenestrale* was reared and maintained in the HARI insect rearing room. *D. fenestrale* was reared on PTM and DBM hosts in plastic cages under the laboratory conditions. Third instar PTM or DBM larvae (5 and 3 days from the egg hatching, respectively) were exposed to *D. fenestrale* in cage for 24 h and parasitized hosts were reared in the same laboratory condition until adult parasitoid emergence. The emerged *D. fenestrale* adults were collected every day and allowed to mate for 24 h before use for parasitization. The PTM were also maintained in an open-type cylindrical plastic cage along with a filter paper on the top for oviposition. The PTM eggs attached to the filter paper were transferred to plastic with potato tuber or plant. PTM was reared in the same cage until adult stage. The third instar larva was separated to use as a parasitoid host.

The DBM larvae were collected from cabbage fields in Daegwallyeong, Korea in 2007 and maintained in the laboratory condition. DBM Larval was reared in Napa cabbage. DBM pupae were collected and held in an open-type cylindrical plastic cage with crumpled aluminum foil treated with cabbage extract solution for oviposition.

2.2. Development and morphology of D. fenestrale in two lepidopteran hosts

PTM and DBM 3rd instar larvae were exposed to *D. fenestrale* for 24 h by placing the larvae into the cages. After parasitization, > 30 larvae of PTM and DBM were collected and reared in a single individual dish to check the individual developmental period until adult emergence. The developmental periods and morphology of *D. fenestrale* were observed after dissection of PTM and DBM larva under stereomicroscope (Leica M205C) and photographed with a DFC450 camera system (Leica, Wetzlar, Germany) on a daily basis. All experiments were replicated three times for each PTM and DBM. Developmental time between the treatments were compared by T-test ($P < 0.05$ SAS 9.1, SAS institute Inc., Cary, NC, USA).

2.3. Comparisons of parasitism and survival rate of D. fenestrale

Comparisons of parasitism and survival rate of *D. fenestrale* between PTM and DBM was performed as wasp rearing methods as described above. In brief, 100 individual of 3rd instar larvae of PTM and DBM were exposed to *D. fenestrale* (30 pairs) for 24 h into the cages respectively. After parasitization, 30 larvae were randomly collected and dissected under a stereomicroscope (Leica M205C) for analysis of parasitism rate. Similarly, 30 larvae were also randomly picked up and reared in each cages to check the survival rate until *D. fenestrale* emergence. All experiments were replicated for three times.

2.4. DfIV gene expression comparisons between two lepidopteran hosts

After parasitization period (24 h after adult emergence), >70 larvae of parasitized, DfIV injected by parasitoid without parasitization, parasitized and unparasitized lepidopteran hosts were randomly selected 6 days after parasitization and dissected lepidopteran host to collect tissues only on slide glass, not parasitoid egg or larva. Lepidopteran larval stage was divided into six categories based on the parasitoid development. Sample larvae named with 1–6 denoted that *D. fenestrale* stages as 1 – egg, 2 – 1st instar, 3 – 2nd instar, 4 – 3rd instar, 5 – early 4th instar and 6 – 4th instar. The samples were prepared from all the larval instars were sampled from three treatment groups: unparasitized, parasitized and un-parasitized but DfIV injected by *D. fenestrale*. In total, 18 RNA samples were prepared from larval stage (6 steps based on *D. fenestrale* developmental stages) each host with 3 treatments. Additionally a pupa and adult samples were prepared from two treatments; unparasitized and un-parasitized but DfIV injected by *D. fenestrale*. Totally 22 RNA samples were prepared from each host. For qrtPCR, 45 samples were used. Finally, respective host samples were prepared, total RNA was extracted from these samples with suitable volume of TRI reagent (MRC, Cincinnati, OH, USA) according to the manufacture's protocols. Gene expression analysis was performed by DELTAgene™ assays system (Fluidigm, South San Francisco, CA, USA) with qrtPCR primer sets (Supplementary Table 1). Quantitative analysis was conducted by relative quantification method modified from the original concept of $2^{-\Delta\Delta Ct}$ methods [15]. All samples were independently prepared three times and DELTAgene™ assays data was analyzed Fluidigm real time PCR analysis program (Fluidigm).

3. Results

3.1. Developmental period of D. fenestrale in two lepidopteran hosts

The rate of parasitism was 10–30 % against PTM in the field condition when surveyed in the potato fields, Jeju, Korea, from 2010 to 2012 but that of DBM was lower than 10%. The parasitism rates were dramatically increased in a laboratory condition up to 70% in both cases of PTM and DBM. This was likely to be due to the provided optimal oviposition time point the parasitoid to lepidopteran hosts (5 and 3 days after hatching, respectively) [13].

Even though, the developmental periods of *D. fenestrale* were significantly different ($p > 0.05$, *T*-test) between PTM and DBM during larval stage. However, sum of developmental periods in larval and pupal stages were almost identical (Fig. 1). That means,

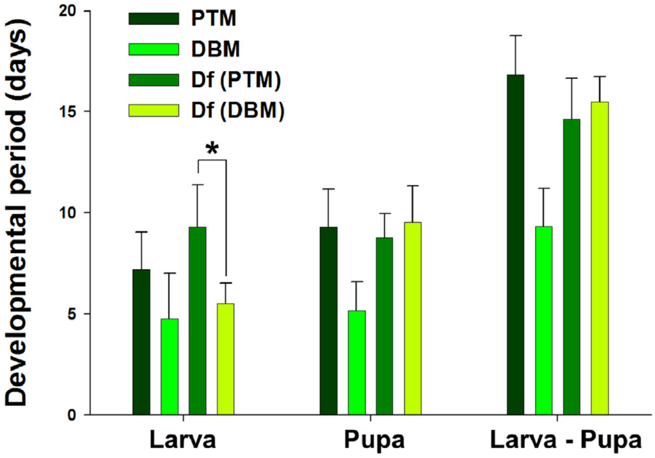

Fig. 1. Developmental periods two lepidopteran hosts, *P. xylostella* (DBM) and *P. operculella* (PTM) which parasite or not from *D. fenestrale* (Df). Larva: parasitoid oviposition point (early 3rd instar larva) to cocoon forming, Pupa: cocoon forming to emergence and Larva - Pupa: sum of two stage periods. All experiments were replicated three times in both PTM and DBM ($n = 30$ in each replicate). Error bar means standard deviation. '*' represents significantly difference ($P > 0.05$, *T*-test, SAS).

PTM was extended few days in larval stage otherwise, pupal stage in DBM after parasitization.

3.2. Morphology, parasitism and survival rate of D. fenestrale

D. fenestrale was dissected from two hosts and their developmental and morphological characteristics were compared for better understanding of *D. fenestrale* developmental physiology in two hosts. Nevertheless, there was no difference in morphology and developmental periods (Fig. 1) of *D. fenestrale* regardless of its hosts. Newly deposited eggs were white and arcuate (Fig. 2A). After maturation with segmentation (Fig. 2B), about two days after oviposition, 1st instar larva was hatched from egg (Fig. 2C). In this study, four larval instars were recognizable, which is consistent with the results of *Diadegma semiclausum* [16]. Larva had three thoracic segments, and 10 abdominal segments. In the first three instars, they had an enlarged head with tapered body and cauda (Fig. 2D–F). The body was colorless and transparent in the 1st instar, with only some trachea visible in white (Fig. 2D). In the 2nd instar, the tracheal system was visible through the integument; the gut was visible, and its color turned from yellow (Fig. 2E). In the 3rd instar, body size increased and the gut was filled with digested host tissue. Simultaneously, the cauda shortened but still significantly remained (Fig. 2F). The 2nd and 3rd instars also could be distinguished by their head shape; only 2nd instar had a node in their head. At the 4th instar stage, the spindle-shaped body was dramatically enlarged and the cauda was almost undetectable (Fig. 2G–I). At the 4th instar stage, parasitoid larvae consumed all the organs and tissues of the host except the cuticle. Pupal stage was divided into three stages. Approximately 8–9 days after oviposition the late 4th instar larva began to spin (Fig. 2J–K). Body became crumpled and turned yellow in the 1st pupal stage (Fig. 2L). Eyes were observed and body cocoon color changed in the 2nd pupal stage (Fig. 2M), where typical shape of wasp was observed (Fig. 2N).

D. fenestrale was parasitized 91.8% in PTM and 73.3% in DBM, in average (Fig. 3). *D. fenestrale* was survived 83.3% in PTM and 46.7% in DBM, respectively. Oviposition rates were 3.4 ± 0.7 and 1.4 ± 0.4 eggs/larva in PTM and DBM, respectively. Therefore, PTM was a better host in *D. fenestrale* survival.

3.3. DfIV gene expression comparisons between two lepidopteran hosts

Various lepidopteran host samples with different developmental stages were used and 49 genes were selected from the 120 DfIV genes (i.e., 28 *reps*, 8 *cys-motifs*, 7 *vankyrins* and 6 *vinnexins*). These genes were selected based on either their nature of relative over-expression after parasitization (e.g., *vankyrin*) or their well/partially known expression patterns from other PDVs (e.g., *rep, vankyrin* and *vinnexin*) [17–19]. All tested genes were not amplified in unparasitized samples. Therefore, these samples were excluded from the relative transcription analysis (Fig. 4). DfIV genes were typically more expressed in PTM at the beginning of parasitization and then their expression diminished. In contrast, some genes, such as *rep* 11 and *vankyrin* 1, were continuously expressed throughout the entire period of parasitization, particularly, in DBM larval stage. *Reps* were differentially expressed in two hosts, particularly at 1 day post-parasitization. Most *reps* were expressed in PTM, but only some *reps* were expressed in DBM. In PTM, the expression levels of *reps* decreased sequentially after parasitization except *rep 7*. Contrast to PTM, however, there was low clear correlation between the *rep* expression level and the time course of parasitization in DBM. *Cys-motif* genes, particularly 1and 2 were also highly expressed in PTM, but only *cys-motif 1*and *2* were lowly expressed in DBM at one day after parasitization. In PTM, *cys-motifs*' expression levels were sequentially decreased after parasitization but *cys-motif* 4, were highly expressed in DBM at late larval stages. Among the 7 *vankyrins*, *vankyrin 1* to *5* were continuously expressed over parasitization. In particular, *vankyrin 1* was mainly expressed in both hosts. Among the 6 *vinnexins*, *vinnexins 1* to *5* were constantly expressed in PTM. Especially *vinnexin 2* was mainly expressed in both hosts. *Vinnexin 2* was about 4 folds more expressed in PTM at 1 day post-parasitization than that of DBM.

From these results, it was clear that most of DfIV genes are predominantly expressed at the initial stage of parasitization in PTM. On the other hands, few genes were expressed at the initial stage of parasitization in DBM and lower numbers of genes were expressed rather continuously or at the late stage of parasitization in DBM.

4. Discussion

Most parasitoid species identified as generalists are actually complexes of closely related and relatively specialized taxa [20]. *D. fenestrale* (Hymenoptera: Ichneumonidae: Campopleginae) appears to be a true generalist by parasitizing the PTM larvae as well as DBM as hosts in both open field and laboratory condition [13,21]. Even though *D. fenestrale* can parasitize both PTM and DBM, they are individually grouped from comprehensive phylogenetic tree in Lepidoptera [22,23]. PTM and DBM were classified in Ditrysia and divided in superfamily level, Gelechioidea and Yponomeutoidea, respectively [24]. Evolutionary studies in Tachinidae (the most species rich group of parasitic fly, Diptera) conclude that the evolutionary flow in host ranges showed generalist to specialist [20]. *D. fenestrale* parasitize both lepidopteran hosts, but their parasitic rate (91.7% in PTM and 73.3% in DBM) and survival rate (83.3% in PTM and 46.7% in DBM) were different two hosts. Here, I have two questions. First, how *D. fenestrale* could be adopted in different environmental condition inside of the host? Second, what is the main factor that makes the difference in survival rate in two lepidopteran hosts?

First, *D. fenestrale* could normally grow in two different hosts but the host larval period after parasitization was extended for some days in larval or pupal stages in PTM and DBM, respectively. Therefore, this finding indicates that *D. fenestrale* can regulate the developmental period of lepidopteran host for its own survival and their maturation. These host development regulations were controlled by Juvenile hormone (JH) synthesis from parasitoid [25,26] and/or JH esterase (JHE) overexpression from PDV [27]. Until now, we did not analyzed JH and JHE concentration and activity, but JH and/or JHE could be contribute to control the lepidopteran host development for parasitoid.

Second, the parasitoid survival rate is the result of complicated mechanism. There cases were reported in *Campoletis sonorensis* with CsIV [28,29]. Host cellular immune responses to parasitoid eggs appear to be important factor determining the level of success of parasitism and restricting host range. For example, generalist *C. sonorensis* parasitizes as many as 27 different lepidopteran species [30]. However, the level of success for parasitism varies among host species. *C. sonorensis* adults oviposit in lepidopteran larvae of several species including those in which parasitoid development is not successful. Hosts that do not support their development are considered non-permissive to parasitism. The molecular basis for successful parasitism or determination of host-range for most parasitoids is not well understood. However, some cases were reported that PDVs participate in host range determination. The one of the *cys-rich* CsIV VHv1.4 was differentially expressed in their lepidopteran hosts. Successful parasitism of *C. sonorensis* depends on the CsIV VHv1.4 expression level and durability [29]. Therefore,

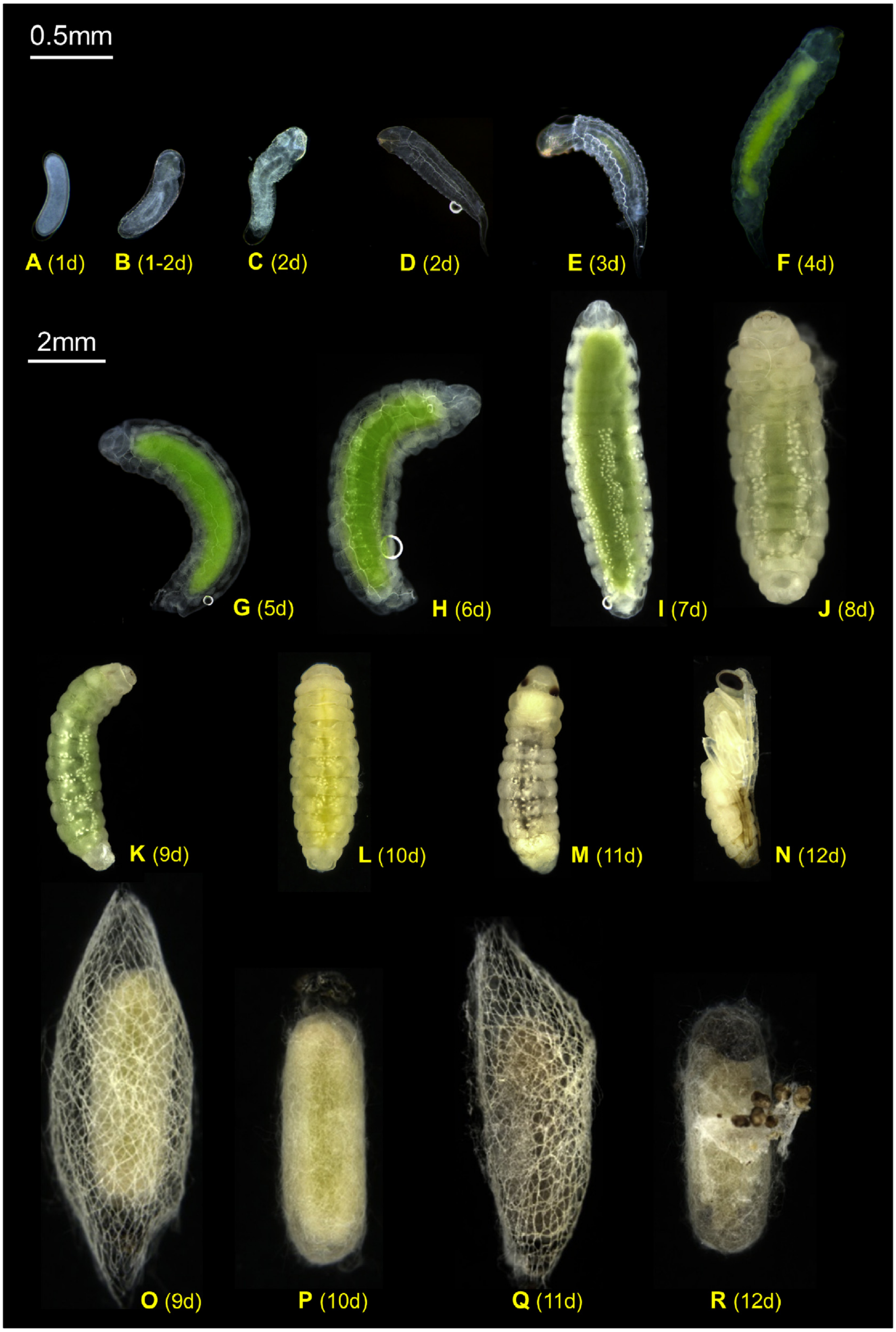

Fig. 2. External morphology of egg, larva, pupa and cocoon of *D. fenestrale*. Egg; 24 h after oviposition (A, 1 day), before hatching (B, 1–2 days) hatching (C, 2 days), 1st instar larva (D, 2 days), 2nd instar larva (E, 3days), 3rd instar larva (F, 4 days), 4th instar larva (G–I, 5–7 days), 1st pupal stage (J–L, 8–10 days), 2nd pupal stage (M, 11 days), 3rd pupal stage (*N*, 12 days after oviposition, respectively) dissected from parasitized DBM at different time points or cocoon. *D. fenestrale* cocoon was formed inside of pupal silk from DBM (O and R). After remove the pupal silk, cocoon shape was identical to another *D. fenestrale* which grown from PTM. Scale bar = 0.05 mm (A–F) and 2 mm (G–K).

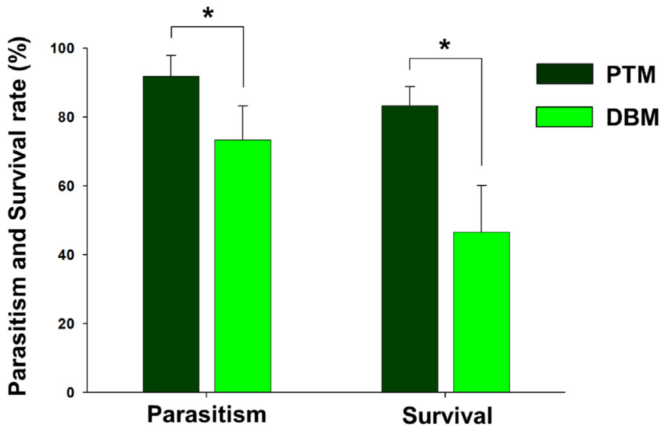

Fig. 3. Comparisons of parasitism and survival rate of *D. fenestrale* in two lepidopteran hosts, *P. xylostella* (DBM) and *P. operculella* (PTM). After parasitization, parasitism rate was calculated based on existence or absence of egg in lepidopteran host and survival rate also deduced by parasitoid emergence rate/parasitism rate equation. All experiments were replicated three times in both PTM and DBM ($n = 30$ in each replicate). Error bar means standard deviation. '*' represents significantly difference ($P > 0.05$, *T*-test, SAS).

we focused on the PDV gene expression patterns particularly four main gene families (*rep, cys-motif, vankyrin and vinnexin*) which contribute to defense in lepidopteran host's immune response or replication. The function of the *rep* is not well known but it was predicted to play an important role in viral cycles [17], *cys-motif* is known to inhibit the host's cellular immune system in CsIV [31] and *vankyrin* is known to inhibit the lepidopteran host's transcription [32]. *Vinnexin* was known to create gap junctions in invertebrates (*innexin*) and IVs [33,34]. To identify the relationship between the survival rate and host preference of *D. fenestrale* and the DfIV expression patterns in two lepidopteran hosts, gene expression comparisons were conducted using two lepidopteran host samples of various hosts' conditions. Based on the expression quantification methods, DfIV gene expression levels were indirectly compared in all tested conditions between PTM and DBM. Among these genes, most DfIV genes were more expressed in PTM than DBM especially within a day after parasitized. These initial responses were very important to determine the success or fail of parasitism [35] and also permissive or non-permissive host discrimination [36]. Taken together, most of DfIV genes more expressed in PTM and these expressed genes contribute to increase the survival rate. This is one of the evidence that they have co-relationship between parasitoids and PDVs.

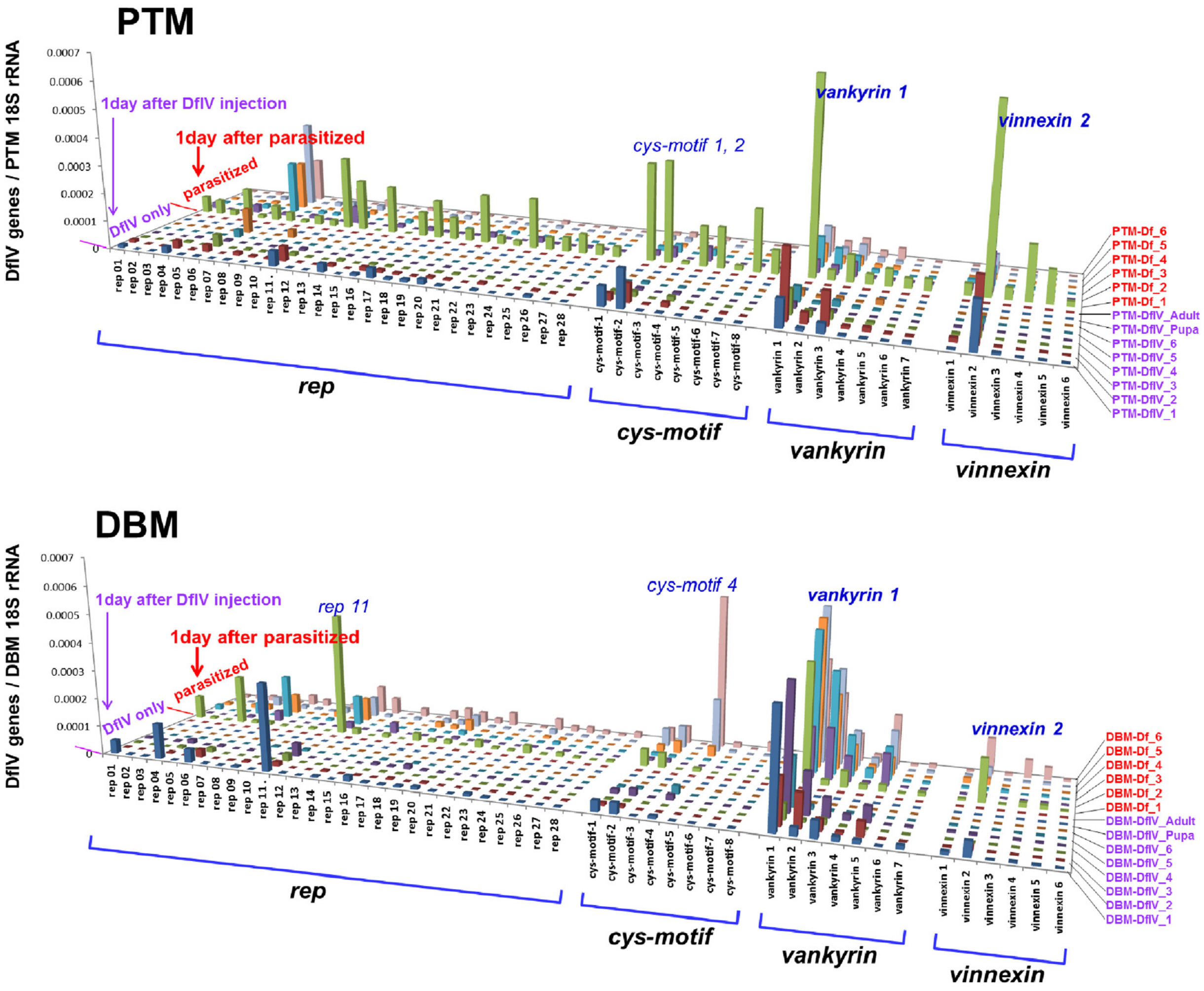

Fig. 4. qrtPCR results are shown in the relative transcript levels of DfIV *rep, cys-motif, vankyrin* and *vinnexin* gene families. Two groups (DfIV only, unparasitized but DfIV injected by *D. fenestrale*; and parasitized) with six different larval samples (i.e., larva 1 to 6 stand for the *D. fenestrale* developmental stages of egg, 1st, 2nd, 3rd, early and middle 4th instar, respectively, in each lepipdoptran host except the unparasitized group. Initial expression levels of DfIV genes were higher in PTM than that of DBM such as *cys-motif 1* and *2*, *vankyrin 1* and *vinnexin 2*. Quantitative analysis was conducted by relative quantification method modified from the original concept of $2^{-\Delta\Delta Ct}$ methods.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.150.

Transparency document

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