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TRANSCRIPTION OF POLYDNAVIRUS GENES IN THE OVAIRES OF ICHNEUMONID *HYPOSOTER DIDYMATOR*

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A B S T R A C T

Some endoparasitic wasps like *Hyposoter didymator* lay eggs in lepidopteran larvae along with polydnaviruses which disturb the immune system and physiology of the host and thus allow successful parasitism. Polydnaviruses have been divided into two genera, *Ichnoviruses* (IV) and *Bracoviruses* (BV), found in *Ichneumonidae* and *Braconidae* respectively. *Ichnoviruses* contain some important genes families like the repeat element genes (rep genes) family. Different genes of this family have different levels of transcriptions. Thirty rep genes have been described in *H. didymator* IV (HdIV) up to now. Nine HdIV rep genes in *H. didymator* females were analyzed by real time PCR and found different levels of transcriptions of rep genes in the wasp's ovary while in the head and thorax the transcription was very low. All the 9 rep genes are transcribed in the wasps ovaries, one of them, rep8, being transcribed at very low level. This pattern of transcription in the wasp was compared with results obtained with the same 9 rep genes in Lepidoptera (Galibert *et al*., 2006). Comparison indicates that rep1 is transcribed in both Lepidoptera and Hymenoptera whereas rep2, rep4, rep7 seem to be preferentially transcribed in the wasps. These differences in transcription may be due to different functions of the genes in different tissues.

Keywords: *Hyposoter didymator,* polydnavirus, *repeat element* genes, transcription level, ichnoviruses, endoparasitic relation.

INTRODUCTION

Parasitic wasps and associated polydnaviruses: Parasitic life among insects is frequent, in particular in the Diptera and Hymenoptera orders. Up to now about 225,000 species of parasitoid wasps have been identified. Their host range is quite variable however they are more generally successful parasitizing one or a few host species. The work described below was conducted on a parasitic wasp, *Hyposoter didymator* from the family Ichneumonidae. This species develops in several lepidopteran larvae from the family Noctuidae (Glatz *et al*., 2004). The wasp *H. didymator* is a solitary endoparasitoid that lays its eggs into the lepidopteran larvae where wasp larval development takes place. Inside the lepidopteran host, the eggs are protected due to the presence of viruses produced in the parasitoid wasp ovaries that belong to the family *Polydnaviridae*. Indeed, if the eggs are laid in the caterpillar without these viruses, they will be encapsulated rapidly by the immune system of the lepidopteran host larva. Besides this protection from the immune defense, the polydnaviruses are also responsible of physiological modifications of the lepidopteran host and are necessary for successful parasitoid development. Thus, a mutual beneficial relationship exists between polydnaviruses and the associated wasp. At the end of larval development, the mature larvae exits from the caterpillar, spins its cocoon, pupates and new adults emerge. Polydnavirus virions are formed only in the calyx cells of the female reproductive tract. During polydnavirus morphogenesis in the reproductive tract of the female, more copies of its DNA are made and packaged with appropriate proteins and the nucleocapsid acquires a double coat: one membrane layer from the nucleus, another from the cell membrane. Replication starts at the end of pupal life and as the adult emerges, viruses particles are present in large amount within the calyx of *H. didymator* females (Volkoff *et al*., 1995). The DNA packaged in these virions consists of

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multiple molecules of circular double-strand DNA (Turnbull & Webb, 2002), hence the name of "polydnavirus". Virions are then released into the calyx fluid and injected into the caterpillar when the wasp lays its egg. Once in the lepidopteran larvae, the polydnaviruses do not replicate but the virions are capable of infecting cells of the caterpillar and specific subsets of viral genes are expressed in the larval tissues. By the expression of genes the viral particles alter the physiology, immune system and the development of the lepidopteran larvae (reviewed by Webb, 1998). Polydnaviruses are unique viruses as their DNA is integrated into the wasp DNA and thus transmitted with them (Beckage, 1998); the virus is therefore vertically transmitted to the entire wasp's offspring. Polydnaviruses are found in parasitic wasps belonging to the Ichneumoninae. Today, they have only been described in species that develop in lepidopteran larvae. They are divided into two genera, on the basis of their morphology and morphogenesis, the *Ichnoviruses* and the *Bracoviruses* which are specifically associated with wasps of the families Ichneumonidae and Braconidae respectively (Beckage, 1998). Polydnaviruses help the wasp by allowing the wasp eggs to avoid encapsulation (and hence the death) by the lepidopteran larvae's immune cells. Actually, two types of hosts can be found. Permissive hosts are those caterpillars in which the eggs escape detection and the wasp develops; by the effect of polydnavirus and parasistism-associated factors, the caterpillars normally die. On the other hand non permissive host are hosts that either do not provide something very important for the survival of wasp egg or in which the wasp egg is recognized as a foreign particle and is destroyed by the immune system.

Polydnavirus morphogenesis in the female ovaries: The polydnavirus genome is integrated in the wasp genome and thus is present in all cell of all individual, as well in males as in females. However, polydnavirus replication and virion formation take place in the calyx cells of the female reproductive tract and only in this tissue. The virus particles are visible by transmission electron microscopy only at the end of pupal stage (Norton & Vinson, 1983). Initiation of polydnavirus replication appears related to ecdysone titers in the wasp during pupal development (reviewed by Webb, 1998). The encapsidated polydnavirus genome is composed of several circular double-stranded DNA. The DNA molecules are therefore generated from the integrated linear form. The exact mechanism of replication of polydnavirus is still a mystery. In the braconid wasp *Chelonus inanitus*, it has been shown that at the beginning of pupal stage, the nuclei of calyx cells undergo polyploidy what leads to a first amplification of viral DNA together with the amplification of wasp cellular DNA. Then, viral DNA is specifically amplified what precedes excision and circularization (Marti *et al*., 2003).

Polydnaviruses function to disrupt the host immune system: Action of hemocytes constitutes the cellular immunity that leads to three defensive responses, phagocytosis, encapsulation and nodule formation. There are several types of hemocytes, among which granulocytes and plasmatocytes that cumulatively phagocytose and encapsulate the foreign particles or form nodules around them. Hemocytes also play an important role in the immune system by secreting some chemicals which help in poison detoxification and coagulation. Hemocytes are regulated by small peptides that induce spreading which is a pre-requisite for encapsulation of parasitoid's eggs. Humoral response and cellular immunity are closely related in immune system. The way of affecting the cellular immune system by the polydnaviruses varies among species. According to some reported data, polydnaviruses have important impact on hemocytes by disturbing or disrupting their functions. A general inhibition of hemocytes adhesion is usually observed in parasitized larvae and changes in hemocytes are often linked to effects on the cytoskeleton. In CsIV (*Campoletis sonorensis* ichnovirus) system, inhibition may be due to secretion of viral cys-motif proteins. These proteins are expressed in the hemocytes of host and start harming and damaging the network of hemocytes's actin cytoskeleton (Li & Webb, 1994). Almost all ichnoviruses have some impact on the cellular immune system of the host but surprisingly TrIV (*Tranosema rostrale* ichnovirus) have very low or no apparent impact on the immune system of the host (Cusson *et al*., 1998). In fact, TrIV particles cover the wasp's egg and save it from encapsulation by the lepidopteran host (Cusson *et al.*, 1998).

Shared and species-specific features among Ichnoviruses genomes: The genomes of three ichnoviruses (IVs) have been sequenced: CsIV (*Campoletis sonorensis* Ichnovirus), HfIV (*Hyposoter fugitivus* Ichnovirus) and TrIV (*Tranosema rostrale* Ichnovirus). All have low coding densities, strong A+T bias, large genome

size (Table 1). However, they have some differences, for example degree of sedimentation and gene frequency. In the same way when the bracoviruses (BVs) MdBV (*Microplitis demolitor* bracovirus) and CcBV (*Cotesia congregata* bracovirus) are compared on the basis of sequencing, they also showed some similarities and differences (Table 1). Both bracoviruses and ichnoviruses lack of genes that are required for the replication of DNA. IVs and BVs are both characterized by existence of gene families (Tanaka *et al*., 2007).

Table 1. Genome characteristics of sequenced polydnaviruses.

Virus	Family	Genome size	Nb segments	AT content (%)	Coding sequences	Coding density (%)
Cotesia congreata	BV	580	30 (5.0-41.6 kb)	66	156	27
Microplitis demolitor	BV	190	15 (3.6-34.3 kb)	66	61	17
Campoletis sonorensis	IV	247	24 (6.1-19.6 kb)	59	101	29
Hyposoter fugitivus	IV	246	56 (2.8-8.9 kb)	57	150	30
Tranosema rostrale	IV	200	+27 (4.1-10.1 kb)	58	86	22

The gene families conserved in ichnoviruses are the *cysteine-motif* genes, the *viral innexin* genes, the *viral ankyrin* genes, the *repeat element* genes, the *N* genes and the *polar residue rich protein* (PRRP) genes. The number of genes in each of these families is species specific but the most abundant one is with no doubt the *repeat element* gene family. All of these gene families have been also identified in HdIV. Besides these conserved gene families, the different ichnoviruses encode a certain number of specific genes. Polydnaviruses do not replicate in the parasitized lepidopteran host, they only express their genes in several host tissues. Polydnaviruses produce major physiological alterations in parasitized host such as immune disruption, developmental arrest, alteration of hormones and a decrease the hemolymph storage proteins (Webb, 1998). The evolution and origin of polydnaviruses have been discussed for over long time but no clear and firm results were found (Beckage, 1998). One hypothesis is that polydnaviruses originated from ancestral viruses. Indeed, they act like viruses and their mode of actions also resembles that of viruses. Conversely, they have unique features which normally differ from viruses. For example, most of the polydnavirus genes are closely related to insect (or eukaryotic) genes. Some authors thus described the polydnaviruses as analogous to transportable wasp organelles and maternal secretions (Glatz *et al*., 2004). The majority of the genes encoded by ichnoviruses, with the exception of the *viral innexin* and *viral ankyrin* genes have no similarity with known sequences. It is therefore difficult to hypothesize on their function in the parasitized lepidopteran host or in the parasitoid host.

The repeat element genes (rep genes): The repeat element genes (rep genes) constitute a family that is conserved in ichnoviruses, and which contains an important number of members. Indeed, 38, 30 and 17 rep genes were identified in the ichnoviruses associated with Hyposoter fugitivus, Campoletis sonorensis and Tranosema rostrale, respectively (Tanaka et al., 2007). In HdIV, 30 rep genes were identified to date. These characteristics suggest that ichnoviruses rep genes must have very important role in host-parasitoid interaction.

The rep genes in ichnoviruses: To date, transcription studies for ichnoviruses rep genes have been carried out by Northern blot analysis (Volkoff et al., 2002; Theilmann & Summers, 1988) or by RT-PCR (Hilgarth & Webb, 2002). Results indicate that members of this gene family may be transcribed in both wasp and caterpillar hosts (Hilgarth & Webb, 2002; Theilmann & Summers, 1988) and in different tissues of the parasitized lepidopteran host (Volkoff et al., 2002; Theilmann & Summers, 1988). Variations in the number of transcripts during the first day after parasitism have also been suggested for members of this gene family by Northern-blot analysis (Theilmann & Summers, 1988). Altogether, these results seem to indicate that rep genes show a wide range of expression patterns, making it difficult to identify any putative physiological function. Based on the abundance of rep genes in ichnoviruses genomes, one might expect that they have diverged in their expression pattern, acquiring specificity for given tissues, hosts or development stages. The rep genes which have been described up to know are without intron and they encode protein with no predicted signal peptide (Hilgarth & Webb, 2002; Volkoff et al., 2002; Theilmann & Summers, 1988).

The rep genes in HdIV: Up to now 30 rep genes have been identified in HdIV. For nine of them, the transcription pattern was analyzed by qPCR in the

parasitized or HdIV-injected Spodoptera frugiperda larvae (Galibert et al., 2006). Different levels of transcription have been found for the different HdIV rep genes. Among the rep genes, rep1 showed the highest level of transcription and then rep7, rep3 and rep2 genes. Rep6 and rep11 have high degree of similarity in sequences and level of transcription is almost same. Rep7 also has similarity with rep6 and rep11. While the others rep4, rep8, rep5, rep12, have low level of transcription and the lowest level of transcription was found for rep12. The transcription of the HdIV rep genes has also been analyzed in H. didymator host. Transcripts were detected in the female wasp only, and rep1 transcripts at high level while the others at very low level. The transcription was very low in male wasps (200 folds less than in female wasps) (Galibert et al., 2006). The aim of the work conducted during my stage was to analyze the pattern of transcription of HdIV rep genes in Hyposoter didymator females in order to verify if some of the rep genes were or not specifically transcribed in the calyx cells. Indeed, one hypothesis was that some of the HdIV rep genes no or low transcribed in the lepidopteran host could be genes somehow involved in virus particles morphogenesis. To achieve this purpose, I analyzed 9 different HdIV rep genes, as well as three H. didymator housekeeping genes, in both replicative (ovaries) and in non-replicative tissues (head and thorax) as the negative control. I used quantitative PCR for having their patterns of transcription. The 9 genes that have been chosen for these studies are the rep genes that have been previously analyzed by Galibert et al. (2006). Thus, by the comparison I will see the difference among the H. didymator and the lepidopteran larvae regarding pattern of transcription of rep genes.

MATERIALS AND METHODS

Dissection: Experiments started with the dissection of female *Hyposoter didymator* under the microscope in PBS solution in order to collect the needed tissues: head and thorax (T, negative control), and ovaries (OV). The dissected samples were preserved in RLT RNA analysis buffer (Promega). Three biological replicas were collected, each consisting of 15 ovaries (OV1, OV2 and OV3) or 5 head and thorax (T1, T2 and T3).

Extraction of RNA: RNA was extracted using the "RNeasy kit" from Promega. There are several steps for RNA extraction. At first, 70% ethanol is added to the lysate, and the sample (up to 700 µl) is transferred in an RNeasy spin column and centrifuged for 15 sec at 10,000 rpm. After discarding the flow-through, 500 µl of RPE washing buffer is added in the column that is centrifuged for 15 sec at 10,000 rpm to wash the column. This step is repeated and the column is then transferred in a new 2 ml collection tube and centrifuged for 1 min at full speed. To elute the RNA, 30 µl of RNase-free water is added to the column that is centrifuged for 1 min at 10,000 rpm. After that, the collection tube contains RNA and may be some DNA.

Assessment of RNA quantity by spectrophotometer: To check the concentration of RNA in the sample, the OD (optical density) was measured using a spectrophotometer. The absorbance was measured at two different wavelengths, 260 nm (A_{260}) and 280 nm (A_{280}). Both dilutions 1/50 and 1/100 were used in the experiment. The same water that the one used for the dilutions was used to calibrate to zero the spectrophotometer, otherwise results may differ. RNA concentration was calculated based on the A_{260} value, according to the following formula.

Concentration of RNA = 40 µg/ml x A_{260} x dilution factor

Purity (protein contamination) of the RNA samples was evaluated by the ratio of the readings at λ=260 nm and λ=280 nm. Pure RNA should have an A_{260}/A_{280} ratio of 1, 9-2, 1.

Volume was calculated for having 8 µg of RNA in the sample because for further steps I needed at least 8 µg of RNA.

Assessment of RNA quality by gel electrophoresis: After having the concentrations of RNA in the samples, the RNA quality was checked by gel electrophoresis, in order to verify that it was degraded or not in the sample. A 1% agarose gel was made. To do that, 0.8 g of agarose powder was placed in a glass flask and 80 ml of TAE1X buffer were added. After heating the solution in a microwave, the mix was poured in the apparatus for making the gel. After 40-45 min at room temperature, the gel polymerizes and the comb can be retrieved (that allows formation of the cuvets for placing the samples in the gel). Then the samples were prepared for loading in the gel. 1 to 5 µl of RNA solution was taken in the 1.5 ml tube. 3 µl of loading buffer (containing glycerol and bromophenol blue) and 10µl of sample buffer (containing formamide and formaldehyde) were added in each sample and then the tubes were heated for 10 min at 65°C in a water-bath. After 10 min, the samples were putted gently in the wells of the gel side by side; we also placed the molecular weight marker, to verify the

length of band of RNA. After loading all the samples in the gel, TAE1X buffer was added in the apparatus containing the gel in such a way that the gel is covered by the buffer and allow the current to pass through. For migration, current was adjusted at 80 V for 1-2 hours. Then, to check the migration of RNA, the gel was taken in the dark room for verifying under the UV light. Gel was placed in the BET (ethidium bromide) for at least 15 min and then put for few min in the distilled water to clean the gel. After staining, the gel was placed on the UV plaque and we captured the image.

DNase treatment: After that, sample free from DNA was prepared. To do this RNase-free RQ1 DNase (Promega) was used. The volume necessary for 8 µg of RNA was calculated. And then the maximum volume from the sample was taken as a total volume of reaction. According to the protocols 8 µl of RNase-free RQ1 DNase was added, 8 µl of 10 X buffer and 1 µl of RNasin. I added these components in each tube of samples and put them in a water-bath for 3 hours at 37°C. After heating, the tubes were centrifuged. Then 8µl of RQ1 Dnase Stop solution was added in the samples to stop the reaction and the samples were put in a water-bath for 10 min at 65°C. Then 9 µl (0.1 vol) Acetate of Sodium 3 M ph 5.5 was added to precipitate the RNA from the sample. Then 250 µl (2.5 vol) of ETOH 100 was added and place at -20°C for 30 min. After that the samples were centrifuged at 4°C for 15 min at 14,000 rpm. Then ETOH 75% was added and samples were centrifuged 2 times for 15 min at 14,000 rpm. After centrifugation all the ETOH was eliminated by pipeting. The RNA pellet remaining in the tube was resuspended in 8 µl of RNase-free water for each sample tubes.

To verify the quality of the RNA after DNase treatment, an agarose gel was made following the same procedure as described above.

Verification of absence of contamination DNA by polymerase chain reactions (PCR): To verify that there is no contaminating DNA, a PCR analysis was done using the primers specific to the Elongation factor 1 alpha gene. The primer used were the CL23_Reverse primer (5'-TGT AAA TAG CTC GCG TAT TTT GG-3'; Tm=58) and the CL23C_Forward primer (5'- TCG ATC GTT CGA TAG CAG TG-3'; Tm= 58). The expected product size is 333bp. We also used a viral gene (viral innexin; with the primers 5'-Inx X41 (Forward); 5'- GAA TTA CTC AAC ATG CCG GAC-3' and 3'-Inx X41 (Reverse) 5'-TCA GTA ATA AAC TTA AGC GAC TC-3'). The expected product size is 1100 bp. A mixture had been prepared containing all the buffers, primers, water, RNA, enzymes, salts and nucleotides, according to the protocol described below.

Table 2. Elements used in the experiments and their respective concentrations (Total 25 µl).

Name of elements	For one tube
TP5X (BUFFER)	5 µl
Mgcl2 25 mM	2 µl
dNTP 10mM	0.5 µl
Primer (Forward) 10pmole	1 µl
Primer (reverse) 10pmole	1 µl
GoTaq enzyme	0.125 µl
Water	13.375 µl
RNA	2 µl

There are three different steps in PCR with specific temperature according to the requirement of the experiment; 95°C for denaturation, 72°C for hybridization, and 72°C for elongations were used. In the programme of PCR I used these cycles of temperatures and the total cycle were 30. PCR amplification products were controlled on agarose gel. There were no amplifications in the samples and it was sure that there was no DNA left in the samples.

Reverse transcription or cDNA synthesis: Reverse transcription was performed according to the protocol described below: 1 µl Oligo (dT) (500 µg/ml) and 1 µl of dNTP (10 mM) were mixed in one tube as a Mixture1. The mixture was incubated at 65°C for 5 min and then chill on ice. The following components were then added to the mixture: 4 µl (5 X First Strand Buffer), 1 µl (0.1 M DDT) and 1 µl Rnasin (40 U/µl). After mix, 1 µl (200 U) of Superscript III RT was added. The reaction was placed in a water-bath for 1 hour at 50°C, and then heat-inactivated at 70°C for 15 min. The concentration of cDNA in the samples was then assessed by the spectrophotometer and the quality by PCR amplification,

using the same procedure, conditions and cycles of temperatures as above. For the PCR, the primers that are designed to amplify a cellular gene (Elongation factor 1 alpha) and a viral gene (viral innexin) were used.

Quantitative polymerase chain reactions (qPCR): The quantitative PCR was performed using a total of 20 ng of cDNA in a final volume of reaction of 25 µl. We used 5 µl (4 ng/µl) of cDNA and 20 µl of qPCR mixture containing SYBR green, from Invitrogen Platinum SYBR Green qPCR SuperMix-UDG with ROX. qPCR were performed with three biological replicas of both head and thorax and ovaries dissected from *Hyposoter didymator* females. Two genes were analyzed in one 384-wells plate. Transcription pattern was analyzed for nine HdIV *repeat element* genes (*rep1*, *rep2*, *rep3*, *rep4*, *rep5*, *rep6*, *rep7* and *rep12*) and used three cellular genes for reference (ribosomal RNA gene 18S, cytochrome c oxidase, subunit VIIC and mitochondrial ribosomal protein L55) and water as a negative control. Primers were designed using Primer Express software in order to amplify gene specific fragments 51 bp in size (primer sequences are indicated in Table 3).

Table 2. List of the primers used in qPCR experiments.

Genes	Forward	Reverse
Rep 1	AACGTGGAAACTTTGTCGCC	CGTTCCTGGAGGGACTACCC
Rep 2	TCGGTGTGCTGATTGTGAGC	TCATGCCCAAGTCACACGG
Rep 3	GCCCCTGCCATTTGAAAAAT	TCGCGAATGCAGTAGCACTG
Rep 4	CGGCGTGTCACAAACTGTTG	GCTTCAAGATGTTGCCCCATT
Rep 5	GGAAGACCGCCTGCTTATCA	CCTCCGAATAAAGGCGTCAGT
Rep 6	AAGGCCAGAAGAAGATCGCC	AGAGGCATGAGCCAGTCCC
Rep7	TCGTATCGTTCCACCGGGTA	CAGCCAGATGGTGGAAGCTC
Rep 8	GTTTTGCCCCAATGGTGATG	TGCCACAGTTTTGCTCGAAC
Rep 11	AAGGCCAGAAGAAGATCGCC	AGAGGCATGAGCCAGTCCC
Rep 12	GGGTCGCAATGAAGGTGCTA	CTGGCGAGTGTGTTTGCAAT
CL25 L55	TCAACGTGGATTATTGCGAGC	TGTCCAAAGGCAGAGCAATG
CL34 Cytochrome	GCGAGCTTCTCACGATCATG	TCGAATGGCAAATTGCCAC
18 S	CATCGTGGTGCTCTTCATTGA	CAAAGTAAACGTACCGGCCC

Tm=58-60°C for all these primers and product length is 51 bp. Notes: 1- Rep 11 is similar to rep 6. 2- L 55 is mitochondrial ribosomal protein L55 CG14283-PA [Drosophila melanogaster] 3- Cytochrome is gi|108872170|gb|EAT36395.1| cytochrome c oxidase, subunit VIIC, putative [*Aedes aegypti*] 4- 18S is >gi|40806442|gb|AY433942.1| *Hyposoter didymator* 18S ribosomal RNA gene, partial sequence.

Cycle parameters: The real-time instrument (ABI 7700) was programmed by adjusting these temperatures in a specific manner.

50°C for 2 min (UDG incubation).

95°C for 2 min.

40 cycles of: 95°C for 15 sec, 60°C for 30 sec.

RESULTS AND DISCUSSIONS

RNA extraction and cDNA synthesis

RNA extraction: The RNA was extracted from the ovaries (replicative tissue) and from the head and thorax (negative control) of pools of female H. didymator. The concentration of nucleic acid was then calculated based on the optical densities measured with a spectrophotometer (Table 1). We obtained a total amount ranging from 13.25 to 24.84 µg in the different samples (Table 4).

Table 4. Concentration and total quantity of nucleic acid in the solutions "OV" corresponds to the ovary samples and "T" to the head and thorax samples. Data is given for the three biological replicas.

	Wavelength		Ratio	Concentration	Total quantity
	260	280		(ug/ul)	ug
OV1	0, 42	0, 23	1, 86	0, 84	19, 32
T1	0, 15	0, 07	2, 10	0, 31	24, 60
OV2	0, 54	0, 26	2, 09	1, 08	24, 84
T2	0, 11	0, 06	2, 00	0, 23	15, 63
OV3	0, 38	0, 20	1, 94	0, 76	17, 48
T3	0, 12	0, 07	1, 84	0, 25	13, 25

An aliquot of each sample (5 µl for ovary samples and 15 µl for head and thorax samples) was then run by electrophoresis in order to check the quality of the material that has been extracted (Figure 1).

After ensuring the good quality (no degradation), a DNAse treatment was done to eliminate the DNA from the samples. Eight µg of total RNA was used in this step. However, in order to control that material was not lost during this step, the concentration of the samples were measured again (Table 5). The absence of degradation following this treatment in the samples was again controlled by electrophoresis (Figure 2).

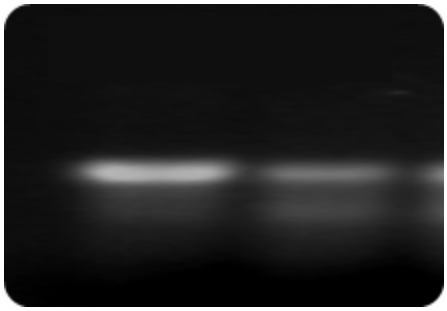

Figure 1. Extracted genetic material of *H. didymator* run on the agarose gel. In this figure, an example of an ovarian (OV) and a head and thorax (T) samples are shown.

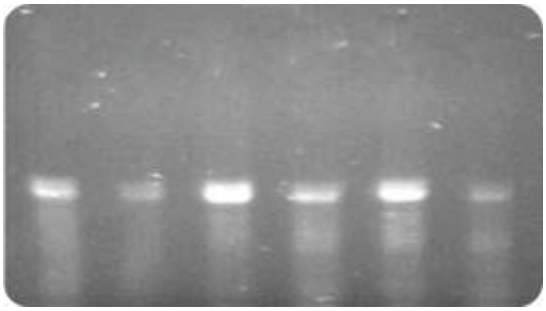

Figure 2. Electrophoretic profile of RNA extracted from *H. didymator* ovaries (OV) and head and thorax (T). The three biological replicas collected are shown separated and run on the agarose gel.

Table 3. Concentration and total quantities of RNA in the samples.

	Wavelength		Ratio	Concentration	Total quantity
	260	280		(ug/ul)	ug
OV1	0, 18	0, 117	1, 54	0, 72	7, 92
T1	0, 111	0, 079	1, 41	0, 444	4, 88
OV2	0, 084	0, 243	1, 95	1, 336	3, 70
T2	0, 097	0, 05	1, 94	0, 388	4, 27
OV3	0, 094	0, 044	2, 14	0, 376	4, 14
T3	0, 066	0, 033	2	0, 264	2, 90

Then a PCR was done using the RNA samples as template to verify that there was no contaminating DNA in the samples. Indeed, the Taq DNA polymerase used in PCR reactions is a DNA-dependant enzyme.

Figure 3. Samples run on the agarose gel to verify that there is no more DNA in the samples with the specific molecular weight markers (lane MW). A positive control using HdIV DNA as a template was used (lane HdIV). Only in the positive control (HdIV) a band of expected size was obtained. The primers used here are specific to the viral innexin gene (expected size for the amplification product is 1100 bp).

Thus, we expect no amplification in the RNA samples if there is no more contaminating DNA. As shown in Figure 3, no amplification was obtained in the samples conversely to the positive control, where HdIV DNA was used as a template for the PCR amplification. Therefore, our RNA samples were free of contaminating DNA.

cDNA synthesis: After DNAse treatment, reverse transcriptase was done using 5 µg of RNA. After reverse transcription, a PCR was done to verify that cDNA has been synthesized. As shown in Figure 4, an amplification band of the expected size was obtained for all the samples analyzed, indicating successful reverse transcription and cDNA synthesis.

Real time PCRs: The cDNAs obtained above were used to study the patterns of transcription of the 9 rep genes in H. didymator by qPCR. The results were analyzed using the LinReg PCR program (Ramakers et al., 2003), using the Rn values. This approach gives the initial number of molecules present in the sample (N0 value).

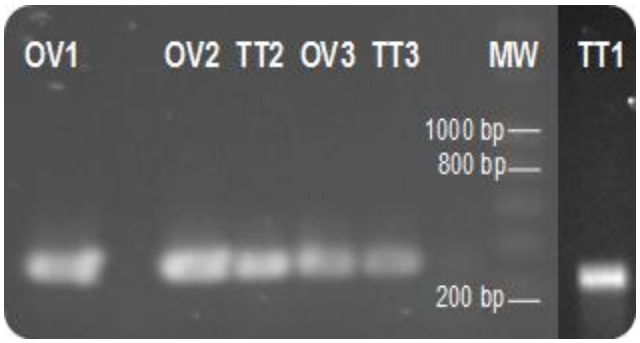

Figure 4. Electrophoretic profile of the PCR amplification products using cDNA as template and primers specific to the ELF-1 Hyposoter didymator gene. The amplification band of 333 nt was obtained for each sample.

Choice of the house-keeping genes for normalization: 3 house-keeping genes, cytochrome oxydase, 18S ribosomal RNA and ribosomal L55 protein were tested. Analysis of the N0 values (Figure 5) indicates a large variability among the samples and the genes. The 18S gene shows a behavior that is different from the other two genes, and the ribosomal L55 genes appear to be more variable than the cytochrome c gene. Cytochrome c was selected as the gene to use for normalization.

The concentrations of cDNA in the samples were then measured (Table 6) to ensure that a similar amount of material is used in the following steps.

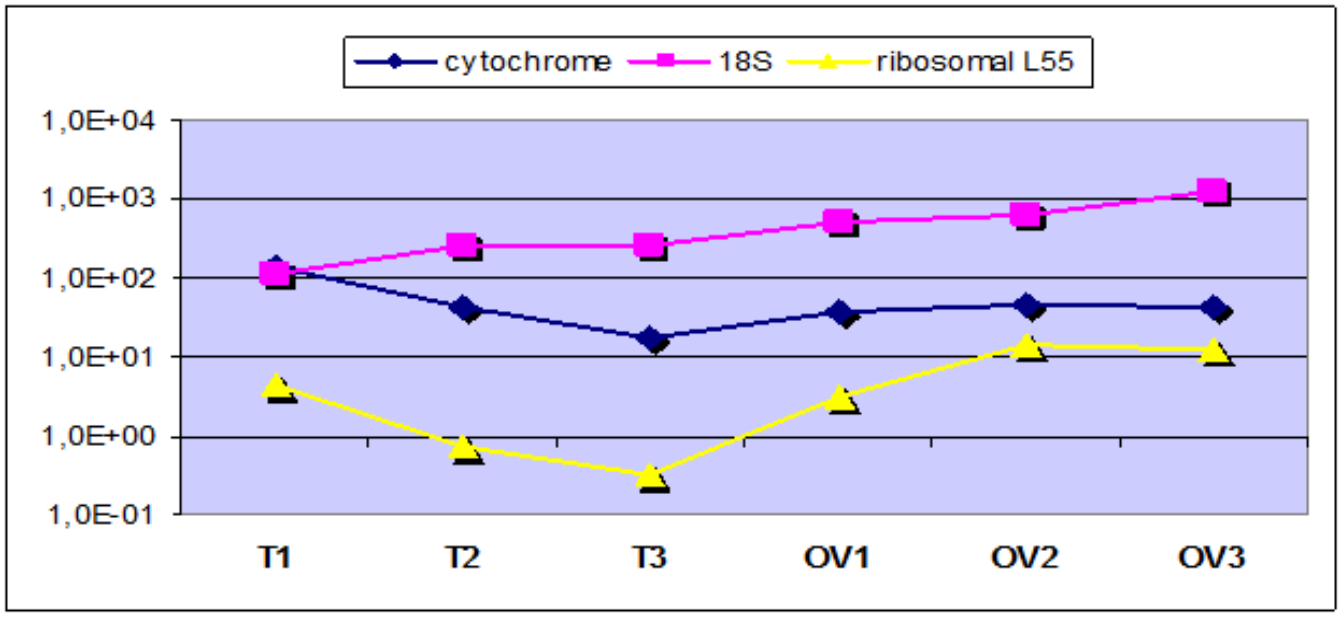

Figure 5. Graph representing the N0 values calculated for the three house-keeping genes in the different *H. didymator* samples while OV represent overy and T represent thorax of *H. didymator*.

Table 4. concentrations after reverse transcriptase.

	Wavelength		Ratio	Concentration
	260	280		(ug/ul)
OV1	0, 288	0, 183	1, 57	1, 44
T1	0, 314	0, 195	1, 61	1, 57
OV2	0, 322	0, 186	1, 73	1, 61
T2	0, 295	0, 166	1, 78	1, 475
OV3	0, 301	0, 166	1, 81	1, 505
T3	0, 25	0, 151	1, 66	1, 25

Analysis of rep genes transcription: The aim of the work was to analyze the transcription pattern of the HdIV rep genes in the H. didymator ovaries. Results, illustrated in Figure 5, indicate that most of the HdIV rep genes are transcribed in this tissue, except the rep8 gene for which transcripts were detected at low level.If the ratio between ovaries and other tissues (head and thorax) is considered, 4 genes seem to be more specifically transcribed in the ovaries, which are *rep1*, *rep2*, *rep4* and *rep7* (Figure 7).

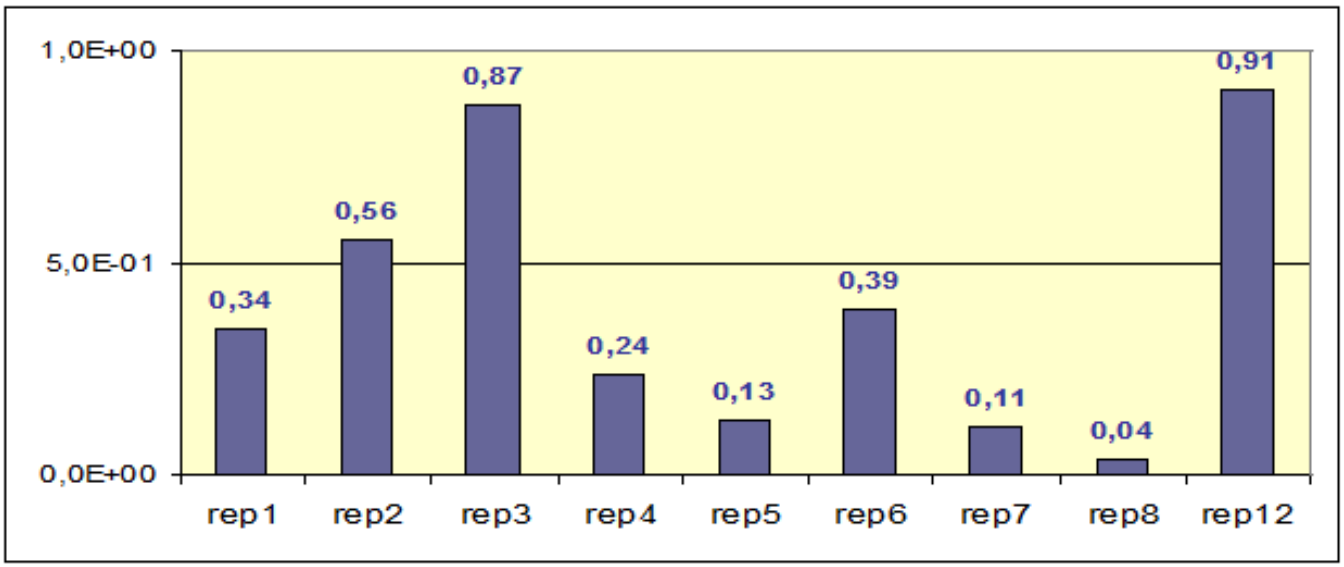

Figure 6. Graph representing the normalized average N0 values of *repeat element* transcripts in *H. didymator* ovaries.

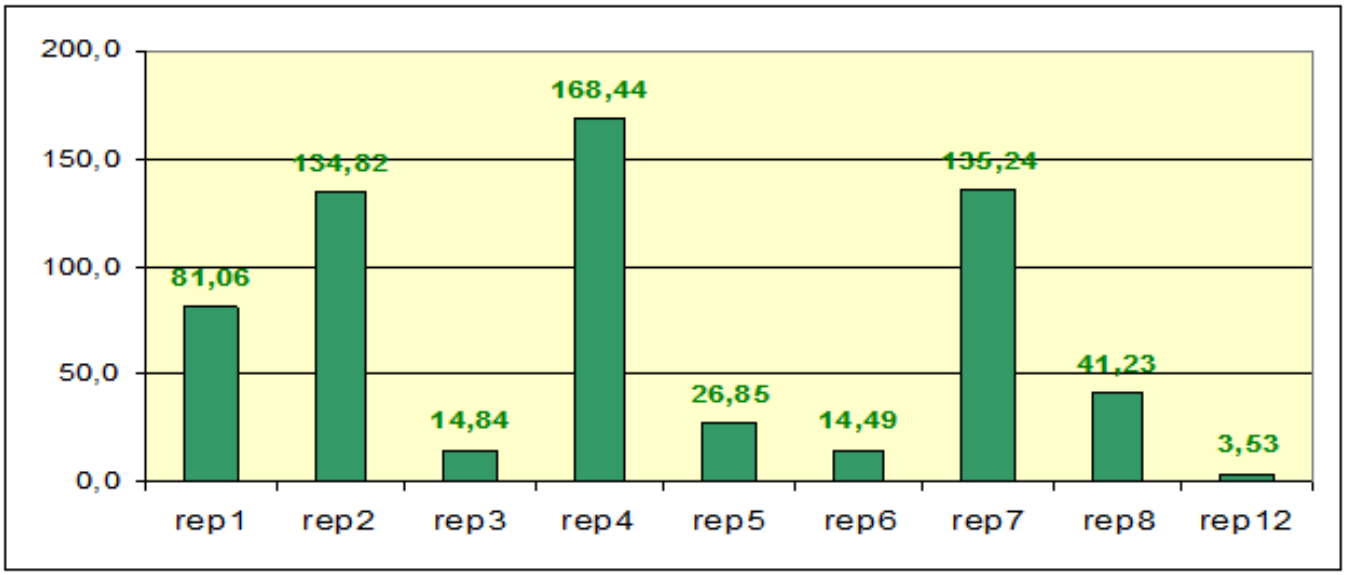

Figure 7. Graph representing the ratios of N0 values between ovary and head and thorax.

DISCUSSION

The aim of this work was to check if the HdIV *repeat element* genes were transcribed in the wasp female, and in particular in the ovaries, tissue where HdIV replication occurs. Indeed the *repeat element* genes form a gene family that is conserved among the ichnoviruses and presents an important number of members. For example, 38 genes have been described in *Hyposoter fugitivus* IV and already more than 30 *rep* genes have been identified in HdIV. This data set suggests an important role of members of this gene family in the host-parasitoid interaction. Moreover, such an abundant number of genes may be related to different functions of the different genes. Previous studies on the *rep* genes from *Campoletis sonorensis* IV have indicated that members of this gene family could be transcribed in both Lepidoptera and Hymenoptera hosts (Hilgarth & Webb, 2002; Theilmann & Summers, 1988). More recently, a work was conducted on HdIV that has shown that in this biological model also, the *rep* genes are transcribed in both hosts (Galibert *et al*., 2006). Galibert *et al* (2006) found that the *rep* genes are transcribed in the female wasp but at very low level with the exception of *rep1* gene. However, in this work, the entire female wasp has been analyzed. They also narrated that obtained data showed the early phases of infection (24 hours), HdIV rep genes each display different levels of transcripts in parasitized 2nd instar or HdIV-injected last instar *S.*

frugiperda larvae. Only rep1 is significantly transcribed in female wasps. Transcript levels of the HdIV rep genes were found as not correlated to their copy number in HdIV genome. HdIV rep genes display different tissue specificity, and that they are primarily transcribed in *S. frugiperda* fat body and cuticular epithelium. In the present work I therefore wanted to verify if transcription was or not specific of the replicative tissue, i.e. the calyx cells. According to obtained results the *rep* genes were transcribed in the ovarian cells of adult female wasps, as shown in the graph on Figure 5. The *rep8* gene presents N0 values that are very low, suggesting that this gene is low transcribed in calyx cells. The N0 values that are given in this graph are indicative as these experiments do not allow an absolute quantification. In LinReg program, the N0 values depend on the slope corresponding to the PCR efficiency, which is not the same for all the primers. If we compare the N0 values for each *rep* gene between ovaries (where there is viral replication) and other tissues such as heads and thoraxes where there is no virus replication, we found that all *rep* genes that have been analyzed, except *rep12*, show higher level of transcripts in the replicative tissue. Here again, there is a bias of the numbers obtained since the N0 values in the negative control (head and thorax) are very low (ranging from 0.0009 for *rep8* to 0.26 for *rep12*). Quantitative PCR allowed us to demonstrate that a number of HdIV rep genes are not transcribed at the same levels in the parasitized lepidopteran host. Even if transcript levels do not account for protein activity and needs, hypotheses can be made to explain the low transcript levels seen for some of the rep genes (rep4, rep5, rep8, rep12). Firstly, rep genes could be involved in host range for *H. didymator* wasp and those genes could be more transcribed inside other hosts. Another possibility is that these low transcribed rep genes have become pseudogenes, through genomic rearrangement in the wasp DNA (Galibert *et al*., 2006). Thus, these results indicate different patterns of transcription of the different HdIV rep genes in the two hosts, suggesting therefore that they may have different functions. However, these results will need to confirm by further absolute quantification of the level of transcription for each specific *rep* gene.

CONCLUSION

The results indicate different level of transcriptions of *rep* genes in *H. didymator* females. If we consider the ratio between ovaries and non-replicative tissues, the genes that are the most transcribed in the replicative tissue are *rep1*, *rep2*, *rep4* and *rep7*. In the lepidopteran host, the most transcribed genes are *rep1* and *rep6* (Galibert *et al*., 2006). Thus these results show that some of the *rep* genes, such as *rep1*, appear to be transcribed in both hymenoptera and Lepidoptera whereas others seem to be specific of a given host. For example, *rep6* is preferentially transcribed in the lepidopteran host; conversely, *rep2*, *rep4* and *rep7* are preferentially transcribed in the wasp host. One hypothesis would be that these different genes have different functions. Those expressed in the Lepidoptera would have a role in the host suitability for parasitoid development whereas those expressed in the wasp ovaries would have a role on virus morphogenesis. The exact function of *rep* genes remains therefore to be investigated, and in the future, when the functions would be elucidated, the solid reason of the different levels of transcription may be found as well as why different *rep* genes behave differently in different tissues of host. This strict parasitoid association with polydnaviruses can be utilized in biological control of some lepidopteran pests and also for new biopesticides development as these ichnoviruses harm strongly the immune system and physiology of the larvae. By identification of those genes which transcribe at high level, if they can be isolated, they could be used more effectively for the biological control.

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