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"Genome Sequencing and Annotation of *Toxoneuron nigriceps* Bracovirus"

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A THESIS FOR THE DEGREE OF PH. D. IN ENTOMOLOGY

"GENOME SEQUENCING AND ANNOTATION OF *TOXONEURON NIGRICEPS* BRACOVIRUS"

UNDER THE DIRECTION OF ADVISORS

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CONTENT

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Abstract

Polydnaviruses (PDVs) are virus-like particles associated with wasp species that parasitize lepidopteran larvae. PDV particles, injected into the host's body along with the parasitoid egg, express genes responsible for the alteration of the host physiology, including suppression of the immune response. These functional characteristics stimulated a considerable interest in PDVs, viewed as a natural source of factors with potential insecticidal or seriously detrimental activity towards pest insects. *Toxoneuron nigriceps* (Hymenoptera, Braconidae) is an endophagous larval parasitoid of the tobacco budworm, *Heliothis virescens* (Lepidoptera, Noctuidae). In this study, we completed the genome sequence of its associated *T. nigriceps* bracovirus (*TnBV*). This genome consists of 27 circles, ranging in size from 3.9kb to 13.9kb. 42 genes were identified by *in silico* analyses. Most of them were similar to genes also found in other bracoviruses, but four were unique to *TnBV*, These last genes coded for a putative aspartyl protease, a putative DNA helicase, a putative UDP glucose 6 phosphate dehydrogenase and a putative Major Facilitator Superfamily (MFS) secondary transporter. As already described for other bracoviruses, *TnBV* contained several members of two gene families, coding for protein tyrosine phosphatases (PTP) and ankyrin-repeat-containing proteins (ANK). Phylogenetic analyses aimed at establishing the evolutionary relationships between genes coding for these proteins in 4 different bracovirus species, indicated a clear clustering pattern of PTP proteins from viruses of the subfamily Microgastrinae (*CpBV, CcBV, and MdBV*). *TnBV* PTPs, on the other hand, mostly clustered together, but few of them were included in a clade that contained also members from the other bracovirus species, suggesting that they might have all derived from a common ancestral gene. Unlike PTPs, bracoviral ANK proteins did not indicate a clear evolutionary relationship. The annotation of the *TnBV* genome indicated that 78% of its sequences were non-coding. We report here the initial characterization of a set of partially overlapping noncoding RNA molecules transcribed from a single *TnBV* locus. These molecules displayed a complex splicing patterns as well as alternative polyadenylation sites. They were expressed in several tissues of parasitized larvae including hemocytes, fat body, gut and malpighian tubules and showed sequence complementarity with the 5' untranslated (5' UTR) region of a host RNA, named 102, that was expressed in the same tissues except malpighian tubules. These findings suggested that the identified *TnBV* non-coding RNAs might functionally control the 102 RNA by

direct interaction. Interestingly, the 102 gene was highly expressed in haemocytes upon immune challenge, indicating a possible role in immune response. The 102 gene is conserved throughout evolution. Two putative homologues were identified in the *Drosophila melanogaster* genome: only one of them was expressed in larval hemocytes. This last finding might open the way to functional analyses in a model system which offers a wide array of molecular genetics tools not available in other species.

Keywords: ncRNAs, endoparasitoids, immune challenge, hemocytes, polydnvirus

INTRODUCTION

Crop losses due to insect pests are a significant factor in limiting food production. The need for pest control strategies other than indiscriminate pesticide usage stimulated, since the second half of the last century, the development of integrated pest management programs including extensive exploitation of natural enemies. Among them, insect parasitoids gained substantial interest, not only as biological control agents, but also as sources of natural compounds which are harmful to the target host species. Actually, parasitic insects evolved sophisticated strategies to manipulate the physiology and the development of their hosts. These strategies rely on a set of molecular factors which may be suitable for developing effective and environmentally safe bioinsecticides (Beckage and Gelman, 2004). The term "bioinsecticide" is in general referred to any natural material, deriving from animals, plants, bacteria and viruses, that represents a valuable alternative to chemical pesticides. This is because bioinsecticides display several advantages over chemical pesticides:, they (1) are less toxic (2) affect only the target pest or closely related species, in contrast to traditional pesticides which have a wider spectrum of negative effects on all organisms, including humans (3) are usually used in smaller amount and decompose faster.

1. Parasitoid insects

Parasitic species can be classified based on several criteria. With respect to their behavior, parasitoids are divided into iodiobionts and koinobionts. Idiobionts block host development after parasitization, while koinobionts allow host growth till their own maturation is complete. Parasitoids can also be classified according to their lifestyle. In this case, they are distinguished into ectoparasitoids and endoparasitoids, which live outside or inside their host, respectively. Moreover, depending on the number of eggs successfully developing on a single host, parasitoids may be defined as solitary or gregarious.

Parasitoid insects are currently believed to comprise as much as one quarter of all insect species (Godfray, 1994). Although they belong to diverse insect orders (Diptera, Coleoptera, Lepidoptera, Trichoptera, Neuroptera, Strepsiptera) they are especially common in the Hymenoptera (Quicke, 1997). Phylogenetically the first hymenopteran

parasitoid appeared 160 million years ago, which is 60 million years after appearance of the first hymenopteran insect (Rasnitsyn, 1988; Whietfield, 1998). It is believed that the first hymenopteran parasitoid was an ectoparasitic idiobiont from which other developmental strategies came out (Pennacchio and Strand, 2006). Sometimes later, endoparasitism arose where some parasitoids remains idiobionts while some others became koinibionts (Wharton, 1993).

Some hymenopteran wasps belonging to families Braconidae and Ichneumonidae complete their egg and larval development within the hemocoel of another insect (Mackauer and Sequeria, 1993). For successful parasitization, the endoparasitoid wasp should overcome its host immune responses to avoid killing its eggs by the host immune system (Li and Webb, 1994; Webb and Strand, 2005; Ibrahim and Kim, 2006; Strand et al., 2006). They sometimes alter host larval period to complete their feeding stages (Lee and Kim, 2004). The above mentioned factors together comprise the host regulation as the parasitoid regulates the development and the immune system of the host for its success. To induce the host immunosuppression and developmental arrest, several parasitic factors are required and can be classified as maternal and embryonic factors (Theopold et al., 2000). Maternal factors, which are delivered by the adult female wasp during oviposition, include ovarian proteins (Webb and Luckhart, 1994), venoms (Richards and Edwards, 1999; Richards and Parkinson, 2000), and polydnaviruses (Strand and Pech, 1995a) while embryonic factors include teratocytes (a specific cell type derived from the embryonic serosal membrane) and parasitoid larva itself (Krell et al., 1982; Dahlman and Vinson, 1993; Jones and Coudron, 1993). Teratocytes have been found in six Hymenoptera families: Braconidae, Ichneumonidae, Platygastridae, Scelionidae, Trichogrammatidae and Aphelinidae (Basio and Kim, 2005), while polydnaviruses have been found only in two families, Braconidae and Ichneumonidae, and are classified into bracovirus and ichnovirus, respectively (Webb et al., 2000). Several braconid and ichneumonid wasps have symbiotic relationships with polydnaviruses including *Cotesia congregata* (Espagne et al., 2004), *Micropletis demolitor* (Webb et al., 2006), *Cotesia plutellae* (Choi et al., 2005), *Toxoneuron nigriceps* (Varricchio et al., unpublished data), *Glyptapanteles indiensis, Glyptapanteles flavicoxis*

(Desjardins et al., 2008), *Campoletis sonorensis* (Webb et al., 2006)*, Tranosema rostrale* and *Hyposoter fugitivus* (Xu and Stoltz, 1993; Tanaka et al., 2007).

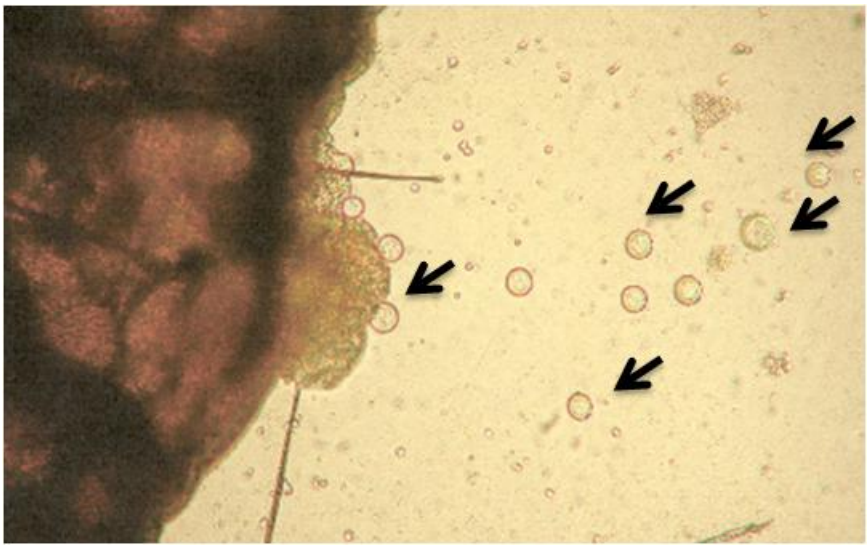

Fig. 1. Teratocytes of *Cotesia plutellae (*arrows). Ibrahim et al., unpublished data.

Venom proteins start to be produced during the pupal stage (Jones and Wozniak, 1991). Their synthesis takes place in 2 specialized organs called venom glands which are connected to a reservoir used for venom collection and storage (Fig.2). The reservoir is directly attached to the terminal part of the oviduct where the venom is mixed with the calyx fluid, produced in the swallen base of the ovary, to be injected in the host's body at oviposition. The size range of venom proteins varies from 10 kDa to over 100 kDa (Leluk et al., 1989). An overwhelming number of them are enzymes with similarities to insect metabolic enzymes, suggesting their recruitment for expression in venom glands with modified functions. Other components include protease inhibitors, paralytic factors, and constituents that facilitate/enhance entry and expression of genes from symbiotic viruses or virus-like particles (Asgari and Rivers, 2010)

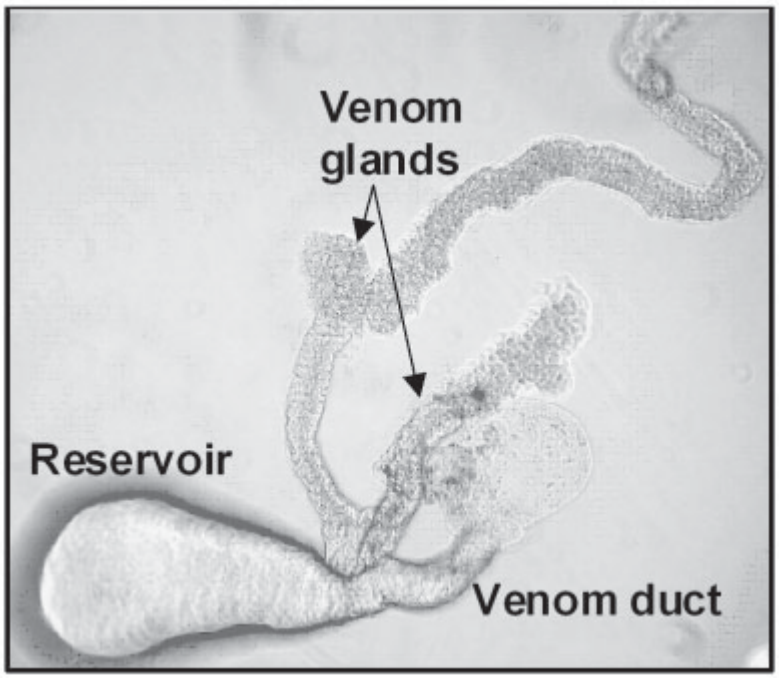

Fig. 2. Venom apparatus in *Cotesia rubecula (*Asgari, 1996).

It is believed that venom and ovarian proteins save the parasitoid eggs during the first few hours after parasitization before transferring this function to polydnavirus and teratocytes for long term preservation of the parasitoid egg.

2. Host regulation

As mentioned before, parasitic insects tend to regulate the physiology of the host for their success. In general, the immune system is the primary target of parasitization as regulation of this host system is critical for parasite development (Ibrahim and Kim, 2006). Furthermore they sometimes alter the patten of development in the host to increase a specific stage period in order to give the growing parasite enough time for its development inside the host (Pennacchio et al., 1992). Finally some parasites kill their host at the end of the parasitization period while other only block the host responses for the parasitization period. Usually, the last behavior is performed by ectoparasitoids while endoparasitoids tend to kill their host upon parasitoid eclusion.

Several weapons are used to introduce the host regulation including maternal and embryonic factors as described before. Among the maternal factors are venom, ovarian

proteins and polydnaviruses. Each of these components were shown to be able to induce immunological and developmental alteration when injected separately to healthy larvae of different host-parasite systems as in the case of *Plutella xylostella* parasitized by *Cotesia plutellae* (Yu et al., 2007; Nalini et al., 2009), *Spodoptera littoralis* parasitized by *Microplitis rufiventris* (Hegazi et al., 2005) and *Pseudaletia separata* parasitized by *Cotesia kariyai* (Nakamatsu et al., 2001). Even teratocytes alone can inhibit the host development and interfere with its normal development through changing the hormonal balance in the host (Pennacchio et al., 1992; Dahlman et al., 2003). Here I am going to stress the host regulation in terms of developmental alteration as the impact on the immune system will discussed in details in the next part.

Almost in all polydnavirus-containing host-parasitoid system, parasitized moth larvae take longer than nonparasitized larvae to develop until the wandering stage, and die after egression of the full grown wasp larvae. Developmental analysis using juvenile hormone and ecdysteroid analogs suggests that altering endocrine signals could induce the retardation of larval developmental rate in healthy larvae (Kwon et al., 2010). This occurs through reduction of JH Esterase (JHE) leading to accumulation of JH in the larvae as in the case of *Lymantria dispar* parasitized by *Glyptapanteles liparitis* (Schafellner et al., 2007). Down-regulation of JHE activity is primarily due to the injection of PDV/venom at the time of oviposition, with only very small additive effects of teratocytes and wasp larvae (Schafellner et al., 2007).

Using transient transfection of *CpBV* segment 27, it markedly interfered with the host larval development (Kwon et al., 2010). Genetic analysis of this segment revealed that it contains seven PTP genes. Mutations in these putative PTPs recovered the normal development in *P. xylostella* indicating possible implication of development during parasitization. Other PTP genes from *TnBV* were indirectly proved to block prothoracic gland function in *H. virescens* parasitized by *T. nigriceps* (Falabella et al., 2006). Similarly, Cys-motif proteins of *Completis sonorensis* ichnovirus capable of inducing significant reduction in growth and developmental delay in *H. virescens* larvae ingesting this protein (Fath-Goodin et al., 2006).

3. Insect immunity and immunosuppression by parasitoid insects

Insect immunity processes can be classified into cellular and humoral immune responses (Ratcliffe et al., 1985). This classification is not strictly correct because some humoral factors affect cellular immune functions and immune cells may in turn contribute to the synthesis/activation of some humoral factors. Humoral responses require several hours to be effective and involve the synthesis of antimicrobial peptides, the activation of the prophenoloxidase cascade and the production of reactive intermediates of oxygen and nitrogen. The cellular defense reactions are typically induced within minutes of infection and include phagocytosis, nodulation and encapsulation. Since parasitoid eggs usually elicit cellular immune reactions, that must be suppressed to allow parasitoid offspring survival, I am going to describe in more detail the different types of cellular immune responses carried out by different hemocyte types, indicating how these responses are elicited and what factors take part to their regulation.

3.1. Molecular events involved in cellular immune responses

Hemocyte immune functions entail a number of molecular events which take place in a sequential manner. They can be better described in the context of three different steps: recognition, mediation, and effectors responses.

3.1.1. Recognition

In order to be activated, hemocytes first need to recognize the target surface as nonself (foreign). In higher eukaryotes, discrimination of self from nonself can be achieved either by recognition molecules present in the blood or by recognition receptors located on the surface of blood cells (Aderem and Underhill, 1999). A similar situation is found in insects where some recognition molecules are freely circulating in the hemolymph, like lectins (Yu and Kanost, 1999, 2000; Yu et al., 2005), hemolin, lipopolysaccharides (LPS)-binding proteins, peptidoglycan recognition proteins (Ma and Kanost, 2000), and thioester-containing proteins (Christophides et al., 2002). These molecules were identified in several insects and called pattern recognition receptors (PRRs) (Bulet et al.,

1999; Schmidt et al., 2001). They can recognize microorganisms and act as opsonins, linking the hemocyte or fat body cell surface to the intruder. For example, the TEP1 protein of the mosquito *Anopheles gambiae* binds to Gram negative bacteria and acts as a recognition receptor needed for phagocytosis, as demonstrated by gene silencing experiments (Levashina et al., 2001).

3.1.2. Mediation

Insect cellular immunity responses are mediated by the synthesis of eicosanoids (Stanley et al., 1991; Miller et al. 1994), biogenic amines (Dunphy and Downer, 1994; Wiesner et al., 1996) cytokines (Lavine and Strand, 2002), and Hemocyte membrane receptors like Toll and Imd related genes found in *Drosophila* genome (Imler and Hoffmann, 2000) and integrin, which may play as nonself signal mediators.

Eicosanoids are oxygenated metabolites of 20 carbon poly unsaturated fatty acids and can be classified into three categories including prostaglandins, various lipoxygenase products and epoxyeicosatrienoic acids (Stanley-Samuelson, 1994). The role of eicosanoids in cellular immune responses is supported by several literature data. For example, during the nodulation process, eicosanoids mediate the formation of hemocyte microaggregates (Miller et al., 1994; Miller and Stanley, 2001). Moreover, in the larvae of the wax moth, *Galleria melonella,* eicosanoids mediate cell spreading, which is known to be important for both phagocytosis and encapsulation (Mandato et al., 1997). Concerning the involvement of biogenic amines in cellular immune responses brought about by insect hemocytes, Wiesner et al. (1996) suggested that one biogenic amine may act as phagocytosis stimulating factor for the plasmatocytes of *Galleria mellonella*. Octopamine was also reported to modulate nodulation in *Galleria mellonella* larvae (Dunphy and Downer, 1994). Integrin is a well known cellular immunity mediator in insects (Bogaert et al., 1987). Integrin is a heterodimeric molecule formed by two subunits, α and β (Ruoslahti and Pierschbacher, 1987). In resting state, integrins are found on the surface of hemocytes, but their activation occurs only when they are exposed to foreign surface (Lavine and Strand, 2003). Upon non-self recognition, the activation of several signal transduction pathways cause integrin to change from unsticky

to sticky state. Integrins can recognize specific peptide sequence on the cell surface that mainly contains specific amino acid sequence, RGD (Arg-Gly-Asp) (Ruoslahti, 1996). Pech and Strand (1995b) reported that RGD-coated sepharose beads can be easily encapsulated by hemocytes of *Pseudoplusia includens*. Furthermore, soluble RGD inhibited granular cell and plasmatocyte spreading as well as the ability of these cells to encapsulate RGD coated beads. This suggests that change of hemocytes into adhesive state involves presence of adhesion molecules (integrins) containing RGD recognition sequence. Several studies (Giancotti and Ruoslahti, 1999; Lavine and Strand, 2003) reported that integrin mRNA level increases when hemocytes undergo capsule formation. This suggests that integrin is important molecule in mediation of cellular immunity and most especially in encapsulation. Integrin signal transduction still not yet understood in insects but well characterized in mammals. Several studies reported that the level of phosphorylation within the cell is a key factor in regulation of integrin activation (Schlaepfer et al., 1999). The phosphorylation state within the cell is controlled by two antagonistic enzymes, kinases and phosphatases, the latter are important genes in all bracoviruses and possibly they are integrin pathway targeting.

3.1.3. Effector responses

As mentioned above, three different effector responses are performed by the hemocytes in insect immunity: phagocytosis, nodulation, and encapsulation.

(A) Phagocytosis

Phagocytosis is the process in which hemocyte engulfs foreign bodies like bacteria, viruses and yeast or simply particles smaller than hemocytes in size. The major type of hemocytes reported to be phagocytic is varying among insects. For example, in *Drosophila*, phagocytosis is essentially performed by plasmatocytes (Elrod-Erickson et al., 2000) while in some lepidopteran insects like *P. includens* this task is carried out by

granular cells (Strand et al., 2006). In the case of *Plutella xylostella,* however, both granular cells and plasmatocytes are phagocytotic (Ibrahim and Kim, 2006). Several studies revealed the role of polydnavirus genes in blocking the ability of host hemocytes to phagocytose foreign bodies, especially by the direct action of viral PTPs (Ibrahim et al., 2008; Suderman et al., 2008).

(B) Nodulation

Nodules are aggregates of hemocytes that surround and trap microorganisms (Ratcliffe and Gagen, 1976). This is a particularly effective response to get rid of a large number of microorganisms at one time. After nonself recognition, granular cells trap the invading organism and then plasmatocytes aggregate around the forming nodule probably by the action of cytokine(s) released by the granular cells (Lavine and Strand, 2002). A cytokine isolated from the moth *Pseudopulsia includens* was shown to be released by granulocytes to stimulate plasmatocyte spreading. Parasitized larvae were unable to respond to this cytokine. It was demonstrated that transient expression of two members of the *CpBVPTP* gene family suppressed the ability of *P. xylostella* hemocytes to be activated by this cytokine and to perform their characteristic spreading behavior. Moreover, a lectin gene from the same virus was linked to the inactivation of the parasitized host nodule forming capacity (Ibrahim and Kim, 2008; Lee et al., 2008).

(C) Encapsulation

The encapsulation response is triggered by non-self targets whose mass largely exceed the hemocyte size, like protozoa, metazoan parasites and eggs or larvae of parasitoid wasp (Gillespie et al., 1997). In this immune response, hemocytes form a multiple layered capsule around the foreign body. In lepidopteran insects at least the two major hemocyte types, plasmatocytes and granular cells, participate in this response, while in *Drosophila* lamellocytes are the major cell type involved in encapsulation (Schmidt et al., 2001; Vass and Nappi, 2001). In the Lepidopteran species *P. includens*, capsule formation is initiated by granulocytes which surround the foreign body and recruit

plasmatocytes to build up several cell layers. Finally, granulocytes complete the capsule by assembling the outer most cell layer (Pech and Strand, 1996, 2000). This process has been well characterized from a morphological point of view showing that granular cells expell their granules content upon coming in contact with foreign surfaces (Pech and Strand, 1996; Gardiner and Strand, 1999). The expelled materials is believed to include cytokine molecules that change the behavior of plasmatocytes from nonadhesive, unspread state to adhesive spread state (Lavine and Strand, 2002). Plasmatocyte spreading is a very important step of cellular encapsulation in insects and can be used as indicator for this process. One insect cytokine is known so far to stimulate plasmatocyte spreading. It is a 23 amino acid peptide, named plasmatocyte spreading peptide (PSP), that binds to a 190 kDa cell receptor (Clark et al., 1997; Yu et al., 2001; Clark et al., 2004). Although this peptide was initially isolated from *P. includens* it can stimulate also the hemocytes of other lepidopteran insects such as the diamondback moth, *P. xylostella* (Ibrahim and Kim, 2006).

Following encapsulation, the nonself target may be killed by the toxic metabolites formed during melanin synthesis. This process is called melanization and is regulated by a serine protease cascade. Serine proteases are enzymes able to cut specific peptide bonds in a protein. A structural feature shared by serine proteases is the presence of the amino acid serine in their active site. In mammals serine proteases perform multiple physiological functions, participating to food digestion processes, blood coagulation and complement system activation. In all eukaryotic cells, serine protease regulation is performed by their inhibitors, Serpins, which mimic the three dimensional structure of the normal substrate and compete with it for enzyme binding (Hedstrom, 2002; Otlewski et al., 2005).

Serine protease cascades play multiple, essential roles in insect immune responses. A serine protease cascade is for example responsible for the activation of the Toll signaling pathway upon recognition of Gram-positive bacteria and fungi, leading to the synthesis of antimicrobial peptides (Nappi and Cristensen, 2005). Serine protease function in the melanization process was studied in several insect species including *Manduca sexta* and *Drosophila melanogaster* (Liu et al., 2007; Zhao et al., 2007). Thus, serine proteases were found to be responsible for activation of the Prophenoloxidase (ProPO) zymogen into the active Phenoloxidase (PO) enzyme which catalyzes the oxidation of phenols into

quinones, precursors of the melanin. This latter molecule is deposited on wounds or parasites (Nappi and Cristensen, 2005).

4. Polydnavirus

Polydnavirus is a unique group of viruses, which exist in obligatory mutual association with some hymenopteran wasp species (Krell et al., 1982). They have a segmented double stranded DNA genome), that is integrated in the wasp genome. This means that viral transmission does not occur by direct infection of cells but the virus is rather inherited through the germline. Replication of the viral genome and production of mature viral particles only occur in the cells of the ovarian calyx. It is regulated by hormonal changes occurring during the wasp development, as it starts in the late pupal stage and continues through adulthood (Webb and summers, 1992).

Two genera of polydnavirus are known, Bracovirus and Ichnovirus. This classification is based on two distinctive features, the first one being the mutualistic partner wasp family and the second one the morphology of the virus particles. Bracovirus are associated with braconid wasps and Ichnovirus with ichneumonid wasps (Stoltz et al., 1995). The morphological characteristics of these two groups were defined by transmission electron microscope studies. Ichnovirus particles are released from calyx cells through budding and this process does not damage the calyx cell (Volkoff et al., 1995). This is not the case for Bracoviruses. Here, virus replication occurs in the nuclei of the calyx cells, leading to nuclear swelling and invasion of the entire cell space. Finally, virus particles are released by cell destruction, which implies the need for continuous cell renewal (DeBuron and Beckage, 1992, Pasquier-Barre et al., 2002). Ichnovirus nucleocaspids are ellipsoid in shape and surrounded by two membranes while bracovirus caspids have rod shape and are surrounded by a single membrane (Webb, 1998) (Fig. 3).

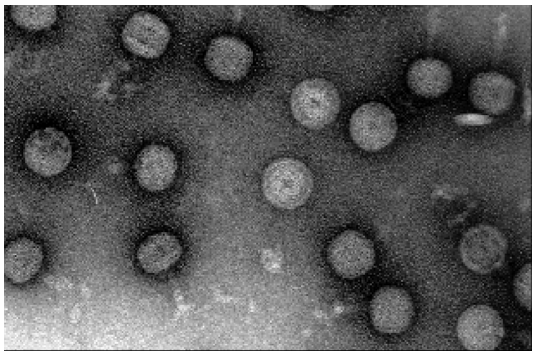

Fig.3 *Toxoneuron nigriceps* bracovirus particles as seen by Electron microscopy. Like other bracoviruses it has rod shape nucleocaspids surrounded by a single membrane.

At oviposition, the virus is delivered into the host hemocoel, where it expresses its genes but does not replicate (Fleming and Summers, 1991). These genes were proven to be the major immunosuppressive agents involved in the interaction between the host and the parasitoid (Ibrahim and Kim, 2008; Thoetkiattikul et al., 2005).

5. Polydnavirus genomes

Initial hypothesis of polydnavirus phylogeny suggested that they might be closely related to baculoviruses based on morphological similarity especially between bracovirus and baculovirus particles (Stoltz et al., 1976; Stoltz et al., 1981a; Stoltz et al., 1984), however, deep molecular and biochemical analysis didn't show close relationship between polydnavirus and baculovirus as in the case of protein tyrosine phosphatases which are of dual specific type in baculovirus and classical type in polydnavirus (Kim and Weaver, 1993). Several symbiotic viruses and virus like particles with hymenopteran braconid and ichneumonid wasps able to replicate inside their lepidopteran host like CmV2 virus which were described in *Cotesia melanoscela* (Stoltz and Faulkner, 1978) such kind of virus might be originating from lepidopteran pathogens rather than wasp due to their ability to replicate inside the host (Whitefield and Asgari, 2003). This is not the case in polydnavirus in which replication occur only in the wasp partner. Other symbiotic viruses, Ascovirus, are capable of replication in wasp tissues only and they are transmitted vertically through germ line (Federici et al., 1991, 2000) suggesting that they

might be ancestors for ichnoviruses (Whitefield and Asgari, 2003; Bigot et al., 2008). A recent study (Bezier et al., 2009) indicated that bracovirus-associated wasp species share genes coding for structural components of polydnavirus particles and that these genes are related to genes of nudivirus suggesting that polydnavirus ancestor is a nudivirus.

Several sequencing projects has been launched to obtain the entire genome sequence of several polydnaviruses, including *Cotesia congregata* bracovirus (*CcBV*) (Espagne et al., 2004), *Micropletis demolitor* bracovirus (*MdBV*) (Webb et al., 2006), *Cotesia plutellae* bracovirus (*CpBV*) (Choi et al., 2005), *Toxoneuron nigriceps* bracovirus (*TnBV*) (Varricchio et al., unpublished data), *Glyptapanteles indiensis* bracovirus (*GiBV*), *Glyptapanteles flavicoxis* bracovirus (*GfBV*) (Desjardins et al., 2008), *Campoletis sonorensis* ichnovirus (*CsIV*) (Webb et al., 2006), *Tranosema rostrale* (*TrIV*) and *Hyposoter fugitivus* ichnoviruses (*HfIV*) (Xu and Stoltz, 1993; Tanaka et al., 2007). The aggregate genome size range in polydnavirus varies among different members between 187 to 567 kb while the GC content varies from 34% to 43%.

Genome analysis of Polydnavirus members clearly show that they share 4 common characteristics in which they have low coding density ranging from 17% to 32%, their genes are not including virus structural proteins or virus-replication related genes, virus genes are closely related to wasp cellular proteins and with the exception of ankyrin genes, Ichnovirus and Bracovirus genes are unrelated (Lapointe et al., 2007). On the other hand several differences between Polydnavirus gene content were reported previously. The largest gene family in Bracovirus is the PTP family; this family is not yet detected in Ichnovirus genomes (Provost et al., 2004; Ibrahim et al., 2007). On the contrary, several gene families were detected in Ichnovirus only and not in Bracovirus including Repeated Element Protein (rep family) and Viral Innexin gene family (inx family). Twenty eight copies of rep genes were found in *CsIV* located on 10 segments while 4 genes of inx genes were reported on 3 different segments of *CsIV* (Kroemer and Webb, 2004). Polydnavirus genome sequencing revealed a great variability in their coding density ranging from 17% in *MdBV* to 32.3% in *CpBV* (Webb et al., 2006; Choi et al., 2009). In the next part I am going to summarize gene and gene families present in Bracovirus and give indication about functional analysis of Bracovirus genes done so far.

5.1. Coding sequences

Bioinformatics analysis of the polydnavirus genomic sequences allowed defining their gene content. Polydnaviral genes are mostly represented by multiple members of specific gene families, including genes coding for Protein Tyrosine Phosphatases (PTPs), Ankyrin repeat proteins (IkB), Single copy genes may be shared by two or more bracovirus genomes and this is for example the case of the H4 viral genes found in *Cotesia plutellae* bracovirus (Ibrahim et al, 2005), *Cotesia congregata* bracovirus (Espagne et al., 2004) and *Cotesia glomerata* bracovirus (Kim et al., 2006), but there are also cases in which they are restricted to a single genome, like the aspartyl protease gene found in *Toxoneuron nigriceps* bracovirus (Falabella et al., 2003). In the next part I am going to give a detailed description about the two gene families shared by all bracoviruses, those coding for tyrosine phosphatases and ankyrins.

SPECIES	No. of tyrosine phosphatases	No. of Ankyrin genes
CpBV	35	8
CcBV	23	5
MdBV	13	12
TnBV	16	4

Table 1 Number of ankyrin-repeat-containing proteins and tyrosine phosphatases encoded in the genome of 4 species of polydnavirus

5.1.1 Main gene families

(A) Protein tyrosine phosphatases (PTPs)

Tyrosine phosphorylation plays a critical role in most cell signaling pathways (Ostman et al., 1994). In general, once a given signal has caused an increase in the intracellular level of phosphotyrosine, it must return to a baseline in order for the cell to respond to subsequent signals either positively or negatively (Cote et al., 1998). This balance is ensured by the opposing action of two different classes of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), the positive and negative regulators of the cell phosphorylation state, respectively (Fig.4). The *Drosophila* genome contains 38 PTPs as well as 32 PTKs genes (Morrison et al., 2000).

PTPs are found in all eukaryotes and are characterized by the (I/V) HCSxGxGR(S/T)G catalytic motif. They are grouped in a superfamily that consists of classical PTPs that specifically dephosphorylate tyrosine residues, dual specificity phosphatases that facilitate dephosphorylation of tyrosine, serine and threonine residues, and low molecular weight PTPs (Ramponi et al., 1989).

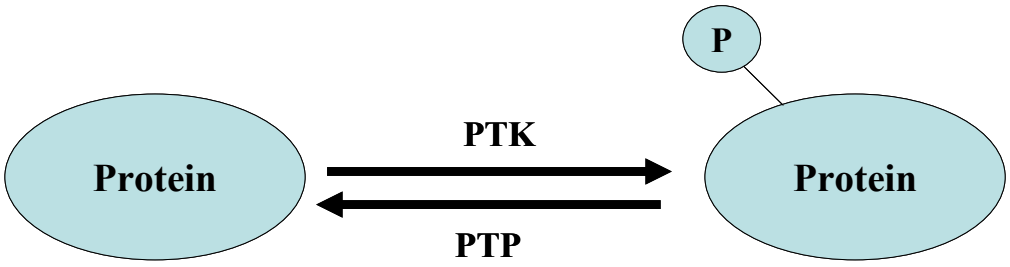

Fig. 4. Diagrammatic representation showing the antagonistic action of protein tyrosine phosphatase (PTP) and protein tyrosine kinase (PTK) in regulation of the cell phosphorylation state.

Classical PTPs are characterized by a catalytic domain consisting of 10 conserved motifs with long conserved noncatalytic region that can only regulate the enzyme activity. They can be grouped into two structurally distinct classes: receptor-like proteins that span the

membrane and soluble cytosolic enzymes. While receptor-like protein tyrosine phosphatases (RPTPs) consist of intracellular, transmembrane, and extracellular domains. The extracellular domains are extremely diverse in size, ranging from very short to very long. The cytoplasmic domains are characterized by the presence of two active sites and it was suggested that the membrane-proximal catalytic domain is catalytically active, whereas the membrane-distal catalytic domain has no measurable enzymatic activity but may have regulatory functions (Wu et al., 1996; Nam et al., 1999). Cytosolic PTPs (CPTPs) have only the intracellular domain and are characterized by the presence of only one active site.

The prototype of PTPs is human PTP1B, a 321 residues long cytosolic protein, whose structure and mechanism of action have been extensively studied (Jia et al., 1995). it is composed of 8 α helices and 12 β strands. Ten mixed β sheets form a twist that spans the entire length of the protein. The catalytic site is located at the base of a 11A˚ deep cleft, and contains the nucleophilic cysteinyl residue (Jia et al., 1995). The side chain of active site Arg 221 positions the substrate phosphor close to the sulphur of the thiolate side chain of the cysteinyl residue, which then performs a nucleophilic attack on the substrate phosphor. The tyrosyl leaving group becomes protonated by Asp 181 acting as a general acid, formation of a cysteinyl-phosphate intermediate occurs (Jia et al., 1995). Gln 262 coordinates a water molecule that forms hydrogen bonds with the amide side chain of Gln266, the amide nitrogen of Phe 182 and the bound phosphate group to stabilize the closed conformation. Subsquent action of Asp 181 as a general base induces hydrolysis of the catalytic intermediate followed by release of the phosphate (Jia et al., 1995) (Fig. 5).

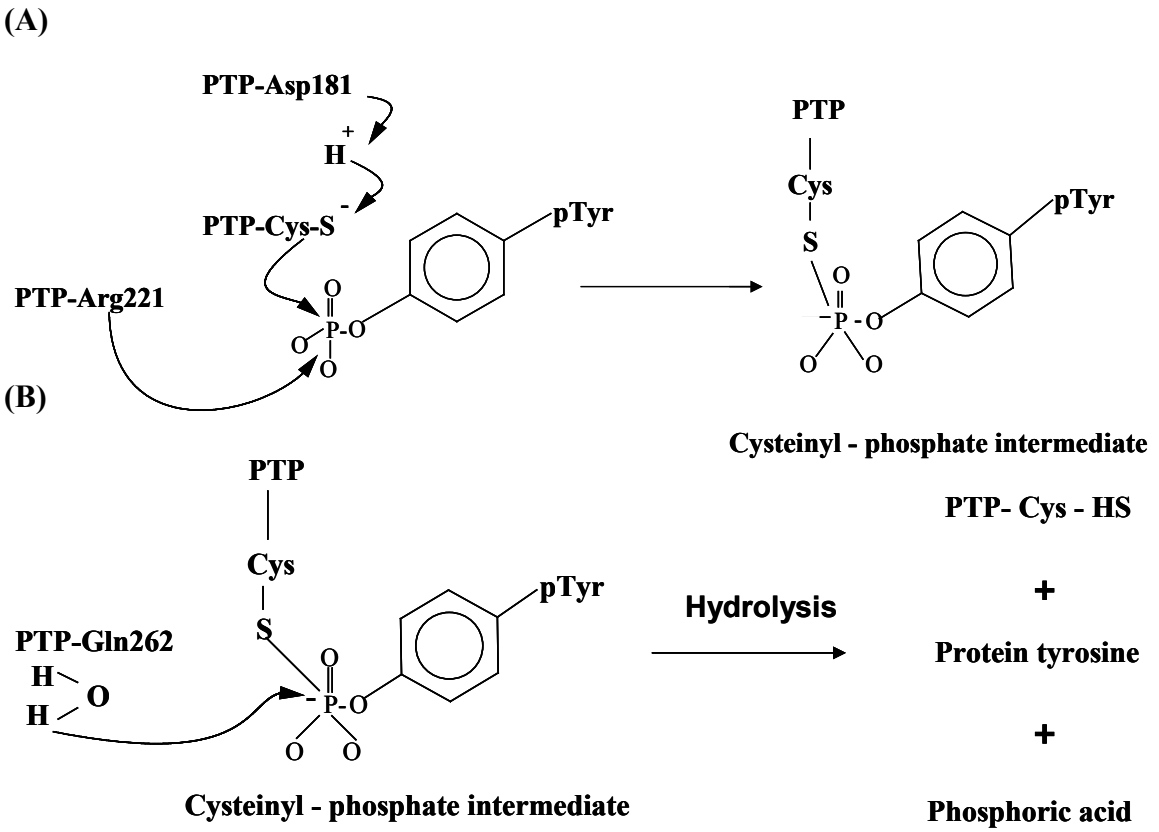

Fig. 5. Catalytic action of protein tyrosine phosphatase (PTP). (A) Formation of enzyme phosphate complex. PTP binds to phosphate group in tyrosine residue of cellular protein by the aid of arginine 221 residue of the enzyme. (B) Hydrolysis of the complex and release of the phosphate group from the tyrosine residue.

(B) Ankyrin-repeat-containing proteins

Vertebrate ankyrins are a family of adaptor proteins which mediate the attachment of integral membrane proteins to the spectrin-actin based membrane skeleton (Bennet and Baines, 2001). They have binding sites for both classes of proteins which is necessary to act as a linkage between them. This linkage is required to maintain the integrity of the plasma membrane and to anchor specific ion channels, ion exchangers and ion transporters in the plasma membrane. Amkyrins have 4 domains which are, N-terminal

domain having 24 ankyrin repeats, central domain which binds spectrin, death domain attaching apoptosis inducing proteins and C-terminal regulatory domain (Bennet and Baines, 2001). Vertebrate ankyrins fall into three classes (R, B and G), each containing multiple variants generated by alternative splicing of a unique gene (Mohler et al., 2002). Ankyrin-R proteins derive their name from their restricted distribution, as they were initially found only in erythrocytes, and are encoded by the *Ank1* gene. Ankyrin-B proteins, named after their broad expression pattern and encoded by the Ank2 gene, were characterized in the brain, but then found in most cell types. Finally, Ankyrin-G proteins**,** which are also expressed in most cell types even if they were first identified in the nervous system, got their name, Giant, because of their 480 kDa size.

Ankyrin repeat is a 33 residue motif which mediates protein-protein interactions and present in large number of proteins involved in diverse functions including transcription initiation, regulation of cell cycle and signal transducers (Mosavi et al., 2004). Polydnavirus has a gene family consisting of ankyrin repeats and showing high homology to members of IkB protein family which act as inhibitor of NF-kB signaling pathways in insects and invertebrate (Silverman and Maniatis, 2001). Polydnavirus IkB lack specific regulatory domains regulating signal-induced degradation (Falabella et al., 2007).

NF-kB/Rel proteins comprise a family of structurally-related eukaryotic transcription factors that are involved in the control of a large number of physiological processes, including immune response, development, cellular growth and apoptosis (Dea and Hoffman, 2010). In Drosophila, NF-kB/Rel proteins participate in embryonic dorso ventral patterning and antimicrobial response (Bergmann et al., 1996; Roth et al., 1991; De Gregorio et al., 2001; Hoffmann, 2003).

Several IkB proteins (IkBα, IkBβ, IkBα, IkBe, IkBz, P105, P100 and Bcl-3) have been identified so far, all sharing specific functional features. They are in fact capable of (1) binding NF-kB/Rel dimers, retaining them in a latent, inactive state, and (2) allowing for NF-kB activation by undergoing stimulus-induced proteolysis. The canonical signaling pathway leading to NF-kB activation involves the phosphorylation of IkB by a specific protein kinase complex, which tags it for ubiquitination and degradation via the proteasome. Several other IkB-like ankyrin-repeat containing NF-kB binding proteins have been reported to modulate nuclear NF-kB transcriptional activity on a subset of

genes (Yamamoto et al., 2004) or, when over expressed, prevent nuclear localization of NF-kB (Hatada et al., 1992; Inoue et al., 1992; Naumann et al., 1993).

Polydnavirus ankyrin-repeat-containing proteins are structurally related to IkB proteins, their ankyrin domains comprise four ankyrin repeats which show similarity to the ankyrin domains 3-6 of Drosophila and human IkB proteins (Fig. 6.). However, polydnavirus ankyrins lack the regulatory motifs present in their eukaryotic counterparts. Polydnavirus IkB lack N-terminal IKK target motif (IkB kinase phosphorylation motif) which mediate degradation of cactus. In vertebrates and Drosophila, IKK phosphorylates IkB which binds NF-kB and block its function. Phosphorylated IkB is degraded via upiquitination pathway, leaving NF-kB which then enters the cell allowing activation of various genes involved in immune response. It doesn't contain also PEST domain in the C-terminal. PEST domain is a signal peptide for protein degradation.

The predicted mode of action in polydnavirus IkB protein may be through competition with endogenous IkB. These proteins doesn't respond to signals produced in the host as they lack the phosphorylation sites for IKK which is critical for Cactus/IkB phosphorylation and degradation in response to immune challenge. Absence of PEST domain in polydnavirus IkB proteins might be involved in increasing the half life of these proteins during parasitization (Falabella et al., 2007).

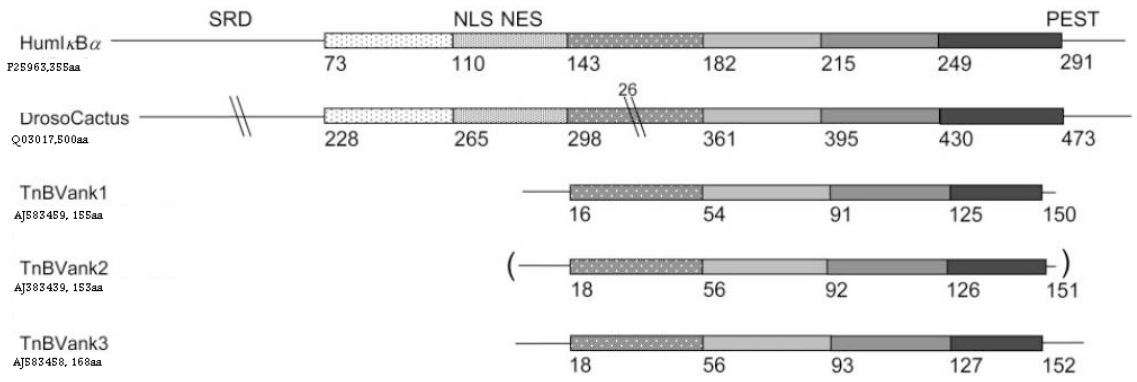

Fig. 6 Schematic representation showing the structural features of the proteins encoded by *TnBV*-IkB genes compared to human and Drosophila homologous proteins. Accession numbers and amino acid number are indicated under the name. Motifs are marked with different colors in the scheme and motif position in each protein is marked as numbers below each color. HumIkBa regulatory regions: SRD, signal-receiving domain mediating phosphorylation and ubiquitination; PEST, PEST region responsible for rapid protein turnover; NES, leucine-rich nuclear-export sequences, NLS, nuclear-localization signal (Falabella et al., 2007).

5.1.2 Functional analysis of polydnavirus gene products

Several proteins were analyzed either in *TnBV* or other members of bracoviruses. A number of functional studies focused on bracovirus protein tyrosine phosphatases. It is well known that signal transduction pathways involved in immune response are regulated by reversible phosphorylation of key tyrosine residues and that several bacterial and viral pathogens evade host immune reactions by altering the cellular phosphorylation status (Bliska and Black, 1995, Castandet et al., 2005). Some bracoviral PTPs may act in a similar manner. Two tyrosine phosphatases (PTP1 and PTP5) encoded by *Cotesia plutellae* bracovirus inhibited the ability of diamondback moth hemocytes to either phagocytose FITC labeled *E. coli o*r encapsulate chromatographic beads (Ibrahim and Kim, 2008). An anti-phagocytic function was also described for *MdBV* PTP-H1 and PTP-H3, which localize to focal adhesions (Pruijssers and Strand, 2007). Moreover, MdBV PTP-H2 induced apoptosis in insect cells (Suderman et al., 2008). Based on their expression pattern, which includes several different tissues and cell types, bracoviral PTPs might be involved in host physiological alterations other than immunosuppression

For instance, Falabella et al., suggested a potential role for *TnBV*PTPs in disruption of prothoracic gland function in *H. virescens* larvae parasitized by *T. nigriceps* (Falabella et al., 2006).

Functional studies of bracoviral ankyrins strongly supported early hypotheses based on their structural characteristics. As reported above, these proteins lack regulatory elements, which are responsible for signal-induced degradation and rapid protein turnover. It was therefore proposed that bracovirus ankyrins may bind their NF-kB targets irreversibly, leading to their permanent inhibition (Thoetkiattikul et al., 2005, Falabella et al, 2007). These transcription factors are implicated in mammalian and insect immune responses, as well as in regulation of development (Dushay et al., 1996; Engstrom et al., 1993; Kappler et al., 1993). A likely consequence of their inhibition by bracoviral ankyrins would be host immunosuppression. Actually, two *MdBV* ankyrins were able to inhibit antimicrobial peptides production (Thoetkiattikul et al., 2005). In addition, transfection experiments in human HeLa cells demonstrated that the TnBV ank1 gene product reduced the efficiency of the TNF-alpha-induced expression of a reporter gene under NF-kappaB transcriptional control (Falabella et al, 2007). However, bracoviral ankyrin genes may play additional functions, as suggested by the impact of *TnBV*ank1 gene expression on the microtubule network in a heterologous system (Duchi et al., 2010). Moreover, an ichnovirus ank gene was reported to have anti-apoptotic action (Fath-Goodin et al., 2009).

Several polydnavirus-encoded proteins other than PTPs and ankyrins were functionally analysed. *TnBV*1, a protein encoded by *T. nigriceps* BV causes apoptosis like programmed cell death in lepidopteran cells (Lapointe et al., 2005). A histone gene encoded by *CpBV* suppress the expression of host histone H4 leading to suppression of host immunity (Gad and Kim, 2008; Gad and Kim, 2009) and a putative protein translation inhibitory factor encoded by the same virus inhibits plasmatocyte spreading in response to plasmatocyte spreading peptide PSP1 (Nalini and Kim, 2007). Strand et al. (2006) showed that the inhibitory factor came from the polydnavirus of the endoparasitoid because *M. demolitor* bracovirus infection inhibited the hemocyte phagocytosis of *P. includens,* and proved that a mucin-like polydnaviral protein, Glc1.8, is an inhibitory factor in the phagocytosis. It hinders the functional interaction between

the hemocyte receptor and foreign ligand by depressing the inducible expression of selected α- and β-integrin or by forming a physical barrier. VHV1.1 gene of *Campoletis sonorensis* ichnovirus and CrV1 gene of *C. rubecula* bracovirus interrupt a normal cytoskeletal rearrangement in response to pathogen infection in parasitized host (Li and Webb, 1994; Asgari et al., 1996). A bracovirus lectin gene was identified in *Cotesia plutellae* bracovirus. It encodes for a protein with homology of about 80% to *C. ruficru*s bracovirus gene. This protein was proven to act as immunosuppressive agent against host immune responses (Lee et al., 2008). Functional analysis of PDV conserved hypothetical protein didn't take strong attention like those of eukaryotic like proteins. A recent study, (Park and Kim, 2010) shows that transient transfection of bracovirus hypothetical protein containing BEN domain induces immunosuppression of nonparasitized host in the form of reduction of hemocyte population as well as impairment in nodule formation.

5.2. Noncoding sequences

A large fraction of bracoviral genomes is apparently devoid of genes. Whether this huge amount of non-coding sequences has a functional relevance, is a fully unexplored issue. However, by analogy with other viral genomes, it can be hypothesized that also polydnaviral genomes contain DNA sequences that give rise to non-coding RNA molecules playing regulatory roles of the viral host physiology (Nair and Zavolan, 2006). Up to now, the only report concerning the synthesis of non-coding RNAs in a host-parasitoid association is related to the induction of host microRNA species in response to parasitic action in the *Lymantria dispar*/*Glyptapanteles flavicoxis* system. In this study, 27 miRNA species were found to be up regulated as a consequence of parasitization (Gundersen-Rindal and Pedroni, 2010).

In our lab, in the frame of a functional study of the *TnBV* genomic circle containing the *TnBV*ank1 gene, a non-coding sequence with a putative functional role in host regulation was identified. Since the characterization of this sequence was part of my PhD thesis work, a general discussion of the main structural and functional features of non-coding RNAs is reported in the next section.

Noncoding RNAs

Noncoding RNAs (ncRNAs) are functional RNA molecules which are not translated into proteins. Non-coding RNA genes include highly expressed RNAs (house keeping RNAs), such as transfer RNA (tRNA) and ribosomal RNA (rRNA), as well as regulatory RNAs. This latter group of ncRNAs comprises small ncRNAs, such as small nuclear RNAs (snoRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi associated RNAs (piRNAs) and long ncRNAs (lnRNA) (Fig. 7).

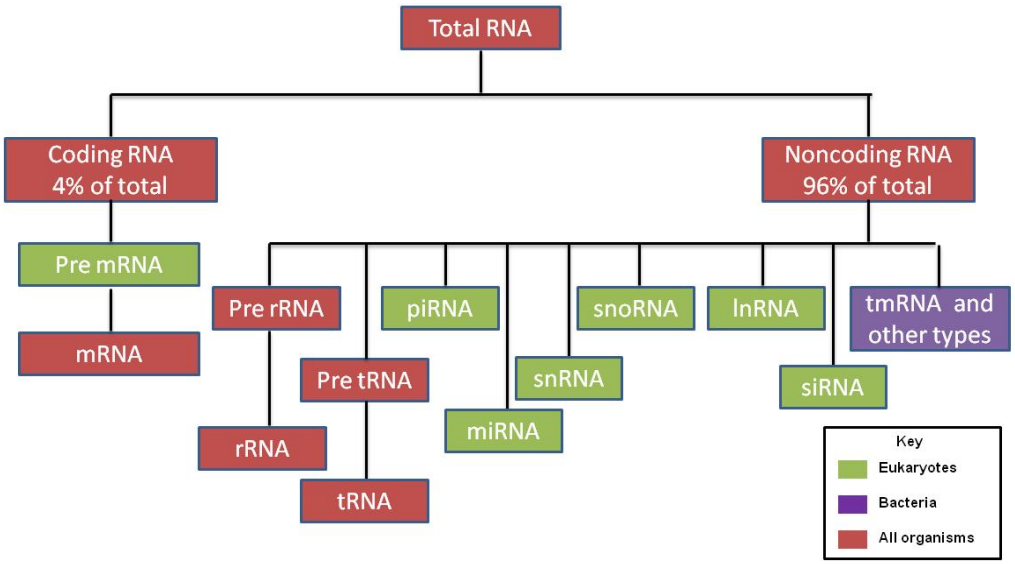

Fig. 7 Schematic representation of cellular RNA content. This scheme shows the different types of RNA existing in different organisms (eukaryotes, bacteria and archaea) and those categories found only in eukaryotic or bacterial cells. The non-coding RNAs of archaea have not yet been fully characterized and it is not clear which types are present in addition to rRNA and tRNA (modified from Brown, 2002).

(A) Small noncoding RNA

small non-coding RNA include microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs), and they are 18–31 nt in length and can affect diverse cellular pathways (Plasterk, 2006).

MiRNAs are 18–25 nt, small non-coding RNAs that are able to regulate protein

translation of target messenger RNA (mRNA) molecules. In mammals, most of endogenous miRNA genes are transcribed initially as primary transcripts (pri-miRNAs) that range from hundreds to thousands of nucleotides in length and contain one or more extended hairpin structures (Du and Zamore, 2005). The biogenesis of miRNA involves first RNAse cleavage, using two enzymes (Dorsha and DGCR8), of both strands near the base of the primary stem-loop and yields the precursor miRNA (pre-miRNA) (Fig. 8). This is followed by exportation of the pre-miRNA to the cytoplasm and Dicer cleavage in the presence of TAR RNA-binding protein (TRBP). After cleavage by Dicer and unwinding by RNA helicase, one strand of the miRNA/miRNA is then preferentially incorporated into the RNA-induced silencing complex (RISC), whereas the other strand is degraded (Fig. 8). The RISC uses the guide RNA to find complementary mRNA sequences via Watson–Crick base pairing, which leads to post-transcriptional gene silencing through inhibition of either translation initiation or elongation (Du and Zamore, 2005).

Piwi small RNA are a 24-31 nt Piwi protein interacting RNA. This protein is required also for PiwiRNA biogenesis in a Dicer dependent manner and they are believed to play a regulatory role in controlling gene expression in both mammals and Drosophila (Shuang et al., 2009). Short interfering siRNA are short 20-25 nt RNA molecules and they first discovered in RNA interference in *C. elegans*. Short interfering RNAs regulate gene expression through duplex RNA formation leading to RNA degradation using Dicer pathway (Elbashir et al., 2001).

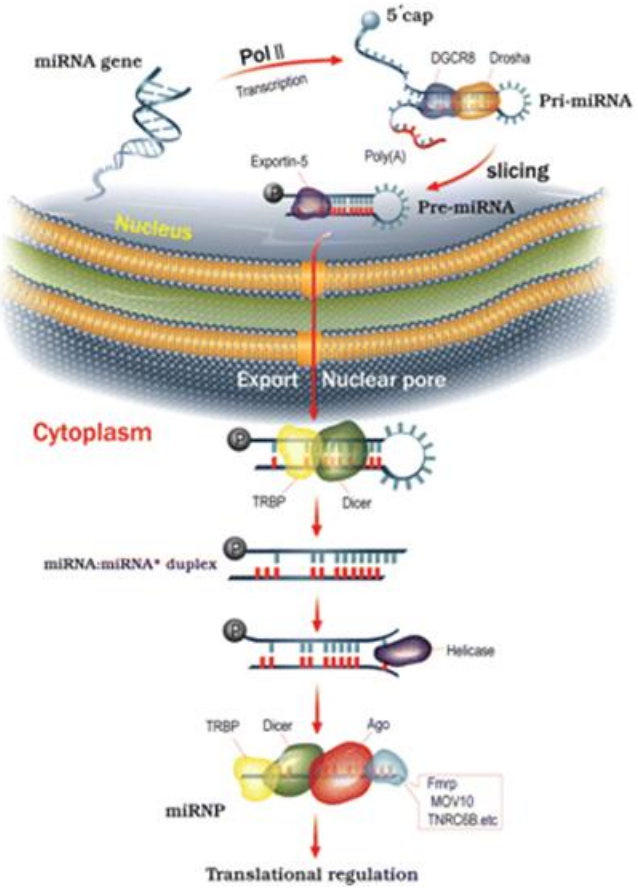

Fig. 8 MiRNA biogenesis. Genes encoding miRNAs are initially transcribed by RNA polymerase II or III to generate the pri-miRNA transcripts within the nucleus. The stem-loop structure of the pri-miRNA is recognized and cleaved on both strands by a microprocessor complex, which consists of the nuclear RNase III enzyme Drosha and an RNA-binding protein, DGCR8, to yield a pre-miRNA 60–70 nt in length. The pre-miRNA is then exported from the nucleus through a nuclear pore by exportin-5 in a Ran-GTP-dependent manner and processed in the cytoplasm by the RNase III Dicer–TRBP. Sliced RNA strands are further unwound by an RNA helicase. One strand of the miRNA/miRNA* or siRNA duplex (the antisense, or guide strand) is then preferentially incorporated into the RISC (or miRNP for miRNAs) and will guide the miRNP to a target mRNA in a sequence-specific manner. Once directed to a target mRNA, the RISC can mediate translational regulation by inhibiting the initiation or elongation step or through destabilization of the target mRNA. Alternatively, miRNAs may also up regulate translation of target mRNAs under certain conditions (Shuang et al., 2009).

(B) Long noncoding RNA

Long noncoding RNAs are transcripts longer than 200 nucleotides. Up to now, most research efforts focused on ncRNAs have been directed to the study of small ncRNA species, however an increasing interest on longer transcripts which do not give rise to proteins developed in the last years. Long ncRNAs may be located in the nucleus or in the cytoplasm, may or may not be polyadenylated and may be transcribed from one or both DNA strands (Birney et al., 2007; Carninci et al., 2005).

Unlike mRNA or miRNA the functions performed by long ncRNAs can not be predicted based on the sequence and this makes their study particularly difficult, also because they even lack conservation among related species. Several long ncRNAs were functionally characterized, for example a nitric oxide synthase (NOS) pseudogene is expressed in the CNS of the snail *Lymnaea stagnalis*. The pseudo-NOS transcript includes a region of significant antisense homology to a previously reported neuronal NOS (nNOS)-encoding mRNA. This suggested that the pseudo-NOS transcript acts as a natural antisense regulator of nNOS protein synthesis. Furthermore a stable hybrid was detected in vivo between sense and antisense transcripts. In vitro translation of nNOS mRNA in the presence of the antisense region of the pseudogene revealed significant inhibition in the nNOS protein (Korneev et al., 1999).

Long ncRNAs can be classified into five broad categories based on their structural relationship with a second, protein coding, transcript. According to this classification a long ncRNA may be: (1) sense or (2) antisense when it matches with one or more exons of another transcript deriving from a different portion of the genome, either on the same strand (sense) or on the opposite strand (antisense) (3) bidirectional when it is transcribed from the opposite strand of a coding transcript (4) intronic when it is derived from an intron of another transcript or (5) intergenic when it lies within the genomic distance between two adjacent genes (Ponting et al., 2009).

A diagrammatic representation summarizing how long ncRNAs work is shown in Figure 9 (Wilusz et al., 2009).

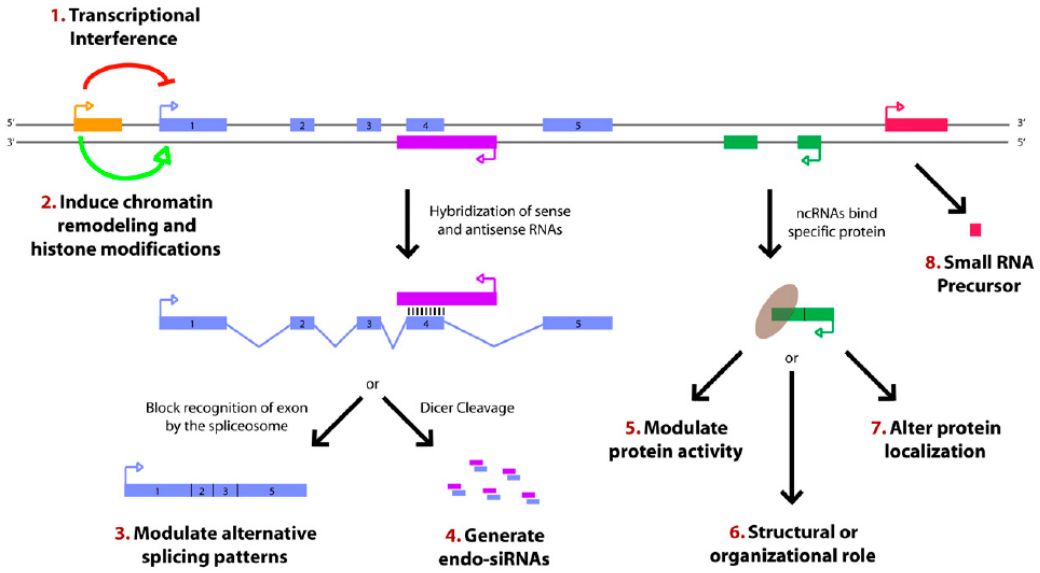

Fig. 9 Diagrammatic representation of long ncRNAs functional mechanisms. Transcription from an upstream noncoding promoter (orange) can negatively (1) or positively (2) affect expression of the downstream gene (blue) by inhibiting RNA polymerase II recruitment or inducing chromatin remodeling, respectively. (3) An antisense transcript (purple) is able to hybridize to the overlapping sense transcript (blue) forming a RNA-RNA hybrid and blocking recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript. (4) Alternatively, hybridization of the sense and antisense transcripts can allow Dicer to generate endogenous siRNAs to mimic the action of RNA interference. By binding to specific protein partners, a noncoding transcript (green) can modulate the activity of the protein (5), serve as a structural component that allows a larger RNA–protein complex to form (6), or alter where the protein localizes in the cell (7). (8) Long ncRNAs (pink) can be processed to yield small RNAs, such as miRNAs, piRNAs, and other less well-characterized classes of small transcripts (Wilusz et al., 2009).

6. The host-parasitoid association *Toxoneuron nigriceps-Heliothis virescens*

Toxoneuron nigriceps (Hymenoptera: Braconidae) is a solitary braconid endoparasitoid wasp that parasitizes the larval stages of the tobacco budworm, *Heliothis virescens* (Lepidoptera, Noctuidae). Parasitized *H. virescens* larvae display a complex array of physiological alterations, mainly affecting developmental, immune response and neuroendocrine processes (Falabella et al., 2006). Several lines of evidence support the idea that *Toxoneuron nigriceps* bracovirus (*TnBV*), the polydnavirus associated with this host-parasitoid system, plays a central role in the pathological effects induced by parasitization, especially in the immune suppression (Malva et al., 2004). Initial studies of the *TnBV* genome allowed the identification of PTP and IkB gene family members, shared, as discussed above, with the other bracovirus genomes, as well as few *TnBV* unique genes, such as an aspartyl protease gene (Falabella et al., 2003; Provost et al., 2004; lapointe et al., 2005; Falabella et al., 2007).

This work is divided into two parts. The first part includes full characterization and annotation of the *TnBV* genome, which was achieved by combining partial sequence information already available, with newly generated sequence data. Phylogenetic studies aimed at establishing the evolutionary relationships between *TnBV* proteins and homologous proteins encoded by different bracovirus genomes are also reported. The second part of the work is focused on the characterization of several *TnBV* noncoding RNA molecules, which are transcribed in several tissues of parasitized *H. virescens* larvae, including the hemocytes, and may be able to interact with a host transcript possibly involved in the immune response.

Fig. 10 *Toxoneuron nigriceps* female parasitizes *Heliothis virescens* larva.

MATERIAL AND METHODS

1. Insects and parasitization

T. nigriceps and *H. virescens* were cultured in laboratory condition as described before (Vinson et al., 1973; Pennacchio et al., 1998). Briefly, *H. virescens* larvae were fed on artificial diet at 29°C. Late third instar larvae were parasitized by adult *T. nigriceps* at a ratio 1: 1. Larvae were considered parasitized when oviposition was seen by eye. Parasitized larvae were cultured at 25°C and washed with 10% sodium hypochlorite in order to maintain sterilized condition to avoid secondary infection before parasitoid eclusion. After emergence, parasitoid males and females were kept for mating for 24 h at room temperature at a ratio of (3:1)(male: female). Adult *T. nigriceps* were fed on 10% honey and cultured at 18°C to be used for parasitization or viral DNA extraction.

2. Virus DNA sequencing and *TnBV* gene prediction

Sequencing of *TnBV* genome was done by construction of three different genomic libraries. The first two libraries contained viral DNA fragments, obtained by EcoRI or HindIII restriction enzyme digestion, ranging in size between 500bps and 7kb, cloned into the pGEM-3Z. The third library was done by mechanical fragmentation of the *TnBV* genome to generate a number of shorter supercontigs which were cloned and sequenced as described for the first two libraries. Merging data coming from the above mentioned sequencing projects successfully closed some circles of *TnBV* genome, however many other sequences remained unassembled. These sequences were subject to long polymerase chain reaction. In this method phusion high fidelity DNA polymerase (Finnzymes, Thermofisher, MA, USA) was used to close *TnBV* circles or identify the junction between two pieces of the genome using primers designed on either sides of a *TnBV* sequence. The reaction condition used in PCR was almost the same with initial denaturation of 98°C for 30 sec, 35 cycles of 98°C for 10 sec, annealing at X°C for 15 sec and extension at 72°C for 30sec/kb followed by 10 minutes final extension at 70°C. PCR products were purified from 1% agarose gel using SV gel and PCR cleaning

system (Promega, Madison, USA) according to manufacturer instructions. Purified DNA was quantified using nanodrop 1000 (Thermo Scientific, DE, USA) and checked for integrity on 1% agarose gel. Sequencing of PCR products was performed by Primm (Milan, Italy). *TnBV* encoded genes were predicted using several gene finding programs including Gene scan (http://genes.mit.edu/GENSCAN.html), FGENESV which is designed mainly for finding genes in viral genomes (http://linux1.softberry.com/berry.phtml?topic=index&group=programs&subgroup=gfindv) and FGENESH (http://linux1.softberry.com/berry.phtml?topic=index&group=programs&subgroup=gfind) which can predict genes in Eukaryotes. Predicted exons and open reading frames were subject to Blast analysis using the basic local alignment tool of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Collection of tissues and RNA extraction

Hemolymph was collected from larvae by cutting the 3rd proleg and collecting the solution in Mead buffer. Hemocytes were pelleted by spin down at 200g for 10 minutes. RNA was extracted from hemocytes, fat body, gut, malpighian tubules and epidermal cells of either parasitized or nonparasitized *H. virescens* or from hemocytes of *Drosophila melanogaster* using TRI reagent (Sigma-Aldrich, MO, USA) according to manufacturer instructions. Briefly, tissues were lysed in TRI reagent for 5 minutes at room temperature and RNA was extracted in 20% chloroform solution. RNA was precipitated using 0.7 volume isopropanol and the resulting pellet was washed in 70% ethanol and resuspended in DEPC treated H_20 and quantified using nanodrop 1000 (Thermo Scientific, DE, USA).

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4. DNAse treatment and cDNA synthesis

To confirm absence of DNA contamination of RNA preparation, RNA Samples were subject to DNAse treatment using RQI DNAse (Promega, WE, USA) according to manufacturer instructions where one enzyme unit was used to digest DNA from one microgram RNA for 30 minutes at 37°C. DNAse digestion of RNA samples was followed by 3 phenol-chloroform extractions and ethanol precipitation, this step was done to remove the DNAse from the RNA preparation. One μg RNA was used as template for cDNA synthesis using M-MLV reverse transcriptase (Ambion, Austin, TX, USA) according to manufacturer instructions. The reaction was placed at 42°C for 1 hour and the enzyme was destroyed for 10 minutes at 95°C. Synthesis of the first cDNA strand was performed using oligodT (5' TTT TTT TTT TTT TTT TTT TTT T 3') (MWG Biotech AG, Ebersberg, Germany).

5. Noncoding RNA cloning and sequencing

The sequences of the first two members of *TnBV*ncRNAs were identified during screening of cDNA library from hemocytes of parasitized *H. virescens* larvae. Two gene specific primers were designed and used in RT-PCR under the following condition (3 minutes of initial denaturation at 95°C and 35 cycles of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, 1 minute of extension at 72°C followed by 10 minutes of final extension at 72°C using Euroclone taq polymerase (Euroclone, UK) with forward (5' GTGCATGTACAATGAGGAAACAAGA 3') and reverse (5' GTGCATGTACAATGAGGAAACAAGA 3') primers. Amplified PCR fragments of *TnBV*ncRNAs were cloned into PCR2.1 cloning vector (Invitrogen, CA, USA) and transformed into DH5α chemically competent cells (Invitrogen, CA, USA) according to manufacturer instructions. Plasmids DNA were purified by Qiagen midiprep kit (Qiagen, CA, USA). Sequencing of the clones was performed by Primm (Milano, Italy).

6. Semi-quantitative RT-PCR

cDNA samples corresponding to different tissues of both nonparasitized and parasitized *H. virescens* larvae at 6 hours after parasitization or those corresponding to hemocytes at 3, 6, 12, 24, and 48 hours after parasitization were used as templates in semi-quantitative RT-PCR reactions. Ribosomal SK4 RNA was used as internal control for all PCR reactions. All PCR reactions were performed under the same thermal cycles of 3 minutes of initial denaturation at 95°C and 24, 28, or 35 cycles for SK4, 102, and the viral antisense transcripts of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 58°C, 1 minute of at 72° C followed by 10 minutes of final extension at 72°C using Euroclone taq polymerase (Euroclone, UK).

7. Real Time PCR

Real-time quantitative RT-PCR was performed on applied biosystems 7900HT fast real time Quantitative Thermal Block using SYBR green chemistry and real-time fluorescence measurements. Gene specific primers of 102 gene were designed for real-time PCR to amplify the mid-open reading frame area of 102 based on the instructions provided by the thermal cycler producer with forward (5' CCAAGTTAATCTGCAAGGCAAGA 3') and reverse (5' TGCAGCAAATGGCCTTATTG 3'). Template cDNA samples were constructed as described before. Real-time RT-PCR was performed in SYBR green PCR master mix (Applied Biosystems, Life Technologies, CA, USA). The reaction mixture (20 μℓ) consisted of 1X Fast SYBR green Master mix, 200 nM each of RT primers, and 20 ng of cDNA. The reaction was performed under the following conditions; one cycle of 20 seconds at 95°C for activation of AmpliTaq Fast DNA Polymerase, 40 cycles under 94°C for 1 sec and 20 sec under 60°C to allow annealing of the primers and extension of PCR. The SK4 gene was also performed on each sample as an internal control for equivalence of template with forward (5’ TAGATGGCCTCATGATCCACTCT 3’) and reverse (5’

GGGCCGTTCTTGCCTTGT 3') primers. Fluorescence values were measured and amplification plots were generated in real time by the Exicycler program. Quantitative analysis of 102 transcript expression was done using the comparative CT (ΔCT) method (Livak *et al*., 2001).

8. Phylogenetic analysis

TnBV genome was compared to other three related species using Phylip package for phylogenetic analysis (http://evolution.genetics.washington.edu/phylip.html) for Neighbor Joining analysis based on multiple sequence alignments generated by ClustalX alignment tool (http://www.clustal.org). Nine divergent PTPs were removed by the Clustal program and the alignment file was edited by Geneious (http://www.geneious.com) for manual adjustment when necessary. Two gene families encoded by *TnBV* and the other 3 species (PTPs and IkB) were chosen for the analysis of Neighbor Joining followed by bootstrap analysis SEQBOOT software to find the most significant phylogenetic tree for both gene families (Felsenstein, 1985, 1989).

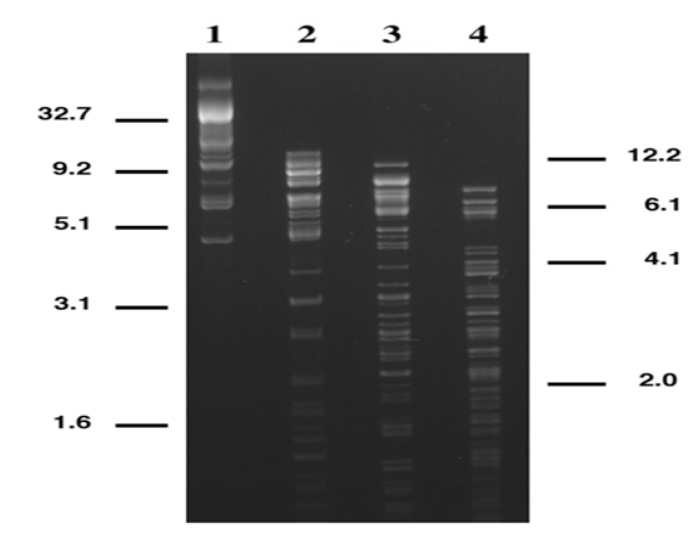
RESULTS

1. Genome sequencing of *Toxoneuron nigriceps* bracovirus.

1.1. *TnBV* genomic circles vary in size and abundance

The *TnBV* genome sequencing project required the construction of three different genomic libraries. The first two libraries contained viral DNA fragments, obtained by EcoRI or HindIII restriction enzyme digestion, ranging in size between 500bps and 7kb, cloned into the pGEM-3Z plasmid (Fig. 11a). Full sequencing of these genomic clones and assembly of the overlapping sequences allowed the identification of fourteen circles. However, many sequences remained unassembled. Therefore, a third library was constructed by mechanical fragmentation of the *TnBV* genome. Sequencing of this library followed by sequence assembly generated a collection of short supercontigs, some matching with sequences previously obtained and already assigned to defined circles and some representing novel sequences. The latter were used to design appropriate primers to be exploited in long PCR reactions. This strategy resulted in the amplification of the DNA sequences spanning the ends of the supercontings and led to the identification of additional circles. So far, 27 circles have been identified, varying in size from 3.9Kb to 13.9Kb. The aggregated genome size of *TnBV* determined in this work was 203,236 kilo bases. Based on ethidium bromide staining, the *TnBV* circles did not appear to have the same abundance, since some circles stained more intensely than other circles of similar size (Fig. 11a, lane 1). The overall GC content of the genome is 32.6% while the coding sequences are about 22%.

(A)



(B)

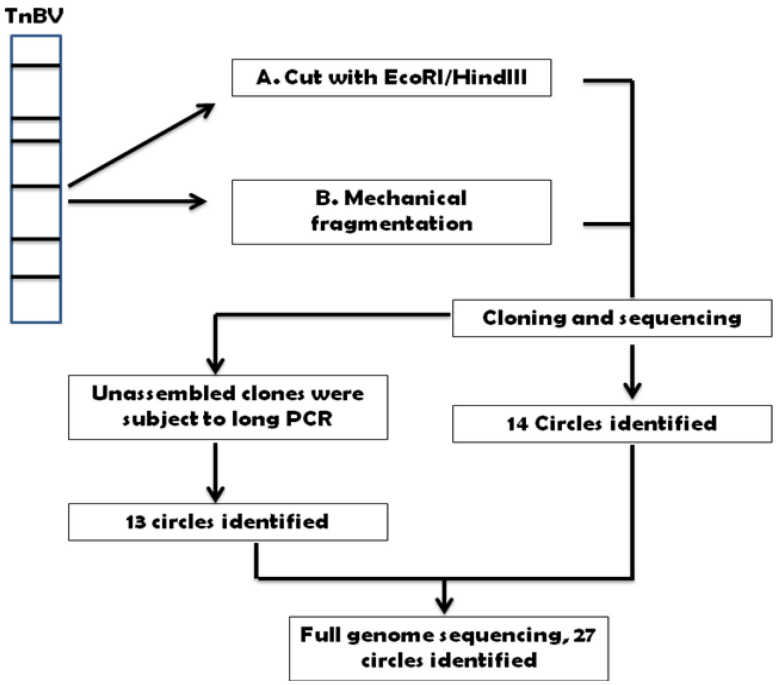


Fig. 11 (A) Polydnavirus genomic DNA undigested (1), digested with HindIII (2), EcoRI (3) Hind III/EcoRI (4). Numbers on either sides of the photo represent circular DNA sizes (left) and linear sizes (right). (B) Diagrammatic representation showing the sequencing strategy of *TnBV* genomic circles.

1.2 Genetic composition of *TnBV* circles

Forty two putative genes were identified in *TnBV* genome based on blast analysis combined with ORF prediction generated by several gene finder programs, as indicated in the methods section. The gene content of *TnBV*, like other bracoviruses, was mostly represented by multiple members of specific gene families and included genes coding for Protein Tyrosine Phosphatases (PTPs), Ankyrin repeat containing proteins (IkB), sugar transporters, BEN domain containing proteins, PHAO domain containing proteins and bracovirus hypothetical proteins. Beside gene families there were also single copy genes which included a gene coding for a putative aspartyl protease, a gene coding for a putative UDP glucose 6 phosphate dehydrogenase, a gene coding for a putative MFS transporter and a gene coding for a putative DNA helicase.

Gene	No. of genes	Predicted function
Protein Tyrosine Phosphatases (PTPs)	16	Signal transduction pathways
Ankyrin Repeats Containing proteins (IKB like)	4	Inhibitor NF-KB transcription factors
Hypothetical proteins	4	Unknown
Hypothetical proteins with BEN and PHAO domains	11	Unknown
NTP Sugar transporter	2	Bind and transport carbohydrates
UDP Glucose 6 phosphate dehydrogenase	1	Involved in phosphogluconate pathway
Aspartyl protease	1	Peptidase function using aspartyl residue
DNA Helicase	1	DNA unwind
Similar to CG2206-PA of *Nasonia vitripennis*	1	Unknown
Similar to CG25304 of *Drosophila erecta,* MFS transporter	1	Transport ions, sugar phosphates, peptides, amino acids and neurotransmitters across the cytoplasm

Table 2 Gene families and single copy genes in *TnBV* genome.

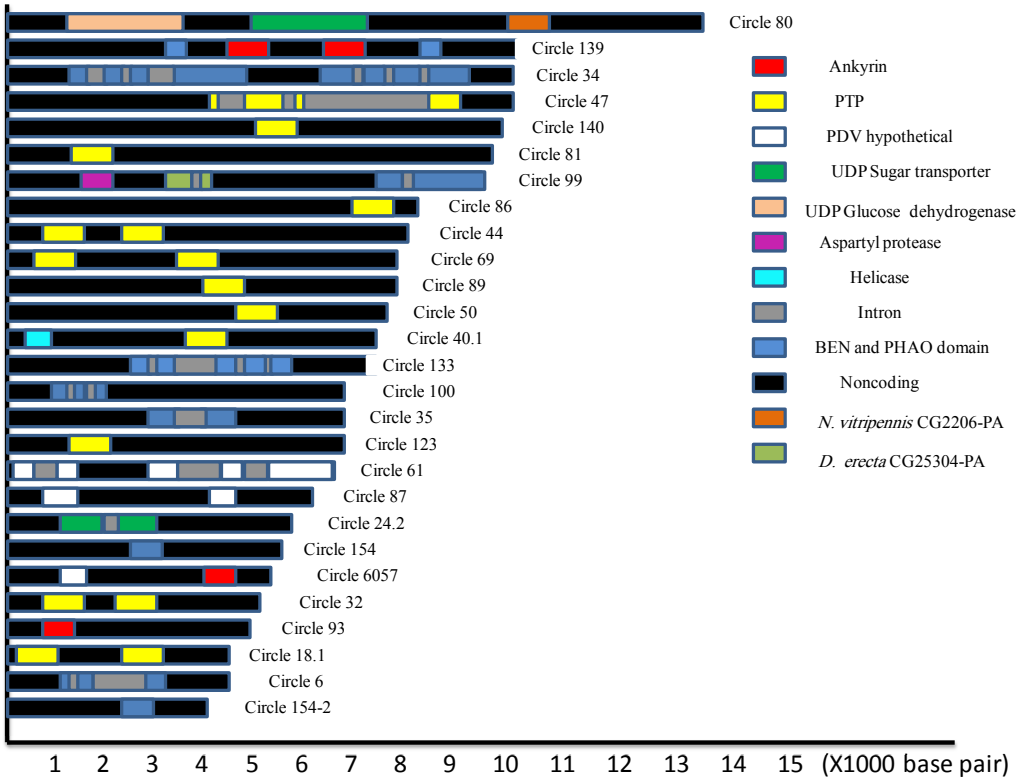


Fig. 12 Graphical representation of gene distribution in the *TnBV* genomic circles. Each circle is represented by a bar. Areas in black represent non-coding sequences. Areas in color represent coding sequences, with each gene and gene family indicated by a distinct color. Predicted introns are marked in grey color.

The largest gene family in *TnBV* was, like in other bracoviruses, the one coding for Protein Tyrosine Phosphatases (PTPs), which included 16 members. Like other PTPs encoded by different polydnaviruses, *TnBV*PTPs were classical cytosolic PTPs, characterized by a catalytic domain formed by 10 conserved motifs (Fig. 13a). *TnBV*-PTPs may contain mutations in their active site: this is the case for the cysteine catalytic residue that is mutated in 2 *TnBV*-PTPs out of 16 (Fig. 13a). Bracovirus PTP genes are known to be intronless, however one *TnBV*-PTP gene located in circle 47 was predicted to contain 3 introns. It should be mentioned that this putative PTP gene was only identified by the FGENESH gene finder program.

The second eukaryotic-like gene family found in the *TnBV* genome encoded for Ankyrin repeat containing proteins. This gene family comprised 4 members distributed in three circles (93, 139 and 6057). As described for other Bracovirus and Ichnovirus Ankyrin

proteins, *TnBV* ankyrin proteins were related to IkB proteins found in both vertebrates and invertebrates, but were shorter and contained a reduced number of the characteristic ankyrin repeats (Fig. 13b).

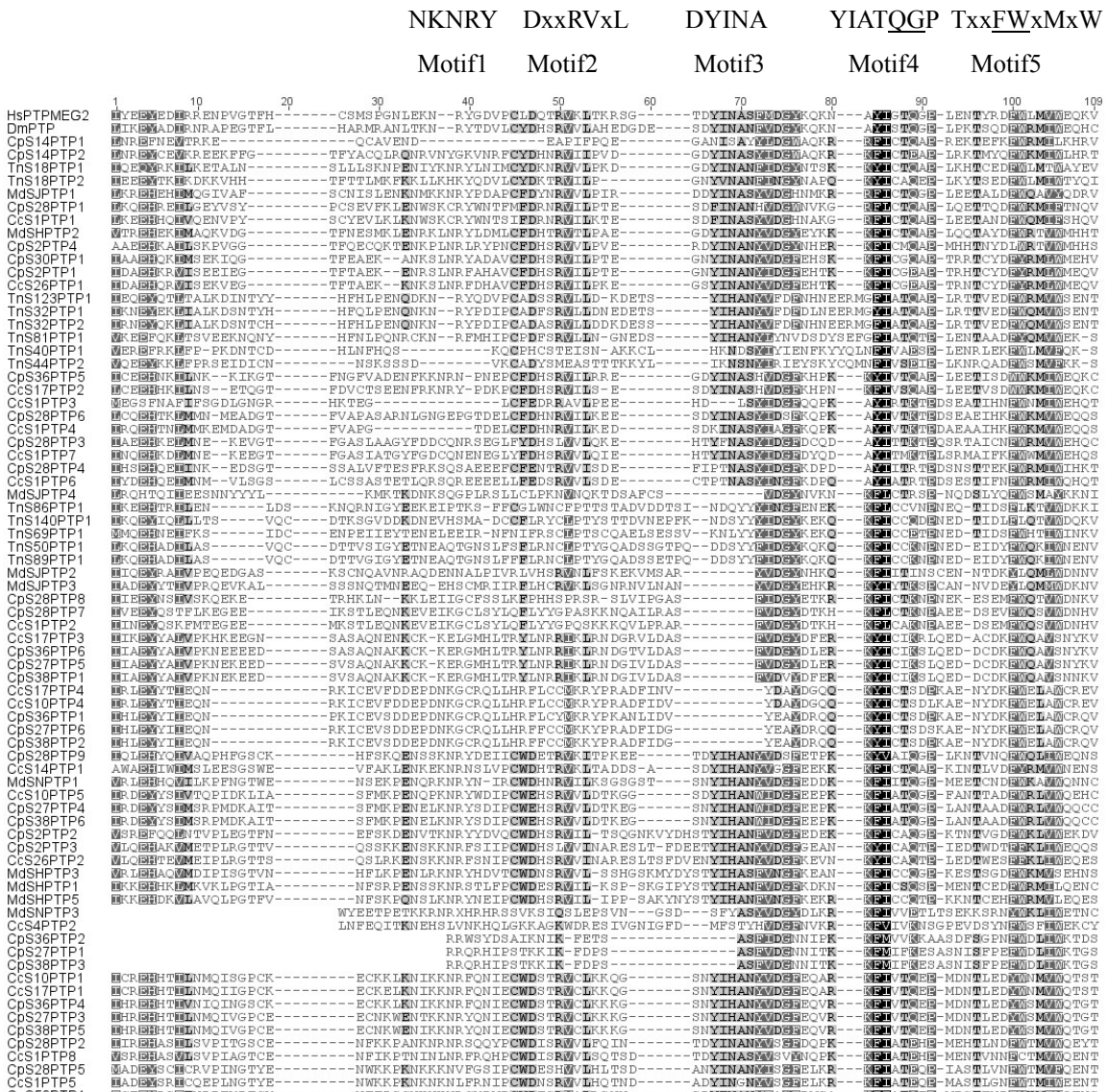
The third *TnBV* gene family comprised two predicted sugar transporter genes, located in two different circles (80 and 24.2).

Fifteen hypothetical genes found in the *TnBV* genome were also identified in other bracovirus and/or ichnovirus (Table 2). These genes comprised 38% of the total genes identified in *TnBV.* Four of them coded for hypothetical proteins ranging in amino acid length between 74 to 451 and were distributed in circles 61, 87 and 6057 (Table 3). Eleven putative genes encoded for hypothetical proteins containing two domains also found in other predicted polydnaviral proteins (BEN domain and PHAO domain) and ranging in length between 74 to 819 amino acids. The absence of obvious similarity with known proteins or protein domains with a clearly defined role did not allow any prediction about their function.

Four single copy genes with a predicted function were identified. They encoded for a putative aspartyl protease, a putative UDP glucose 6 phosphate dehydrogenase, a putative Major Facilitator Superfamily (MFS) secondary transporter and a putative DNA helicase and were located in three different circles (99, 80 and 40.1 respectively) (Fig. 12).

Finally, a gene identified in circle 80 showed significant similarity to a hymenopteran gene (CG2206-PA of *Nasonia vitripennis*). However, nothing is known about its function.

Concerning the gene structure, eleven genes were predicted to contain one or more introns, representing 26% of the total number of genes identified in the *TnBV* genome. Eight intron containing genes belonged to the group of genes coding for PDV hypothetical proteins, as well as BEN and PHAO domain containing proteins, one coded for a putative UDP sugar transporter, one coded for a MFS domain containing protein and the last one was the above mentioned PTP gene that differed from all the other bracoviral PTP genes because of its splitted structure.


NKNRY
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DYINA
YIATQGP
TxxFWxMxW
Motif1
Motif2
Motif3
Motif4
Motif5
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DmPTP
CpS14PTP1
CpS14PTP2
TnS18PTP1
TnS18PTP2
MdSJPTP1
CpS28PTP1
CcS1PTP1
MdSHPTP2
CpS2PTP4
CpS30PTP1
CpS2PTP1
CcS26PTP1
TnS123PTP1
TnS32PTP1
TnS32PTP2
TnS81PTP1
TnS40PTP1
TnS44PTP2
CpS36PTP5
CcS17PTP2
CcS1PTP3
CpS28PTP6
CcS1PTP4
CpS28PTP3
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TnS140PTP1
TnS69PTP1
TnS50PTP1
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CcS10PTP4
CpS36PTP1
CpS27PTP6
CpS38PTP2
CpS28PTP9
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MdSNPTP1
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CpS38PTP3
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CcS1PTP5
CpS50PTP1
CcS4PTP1
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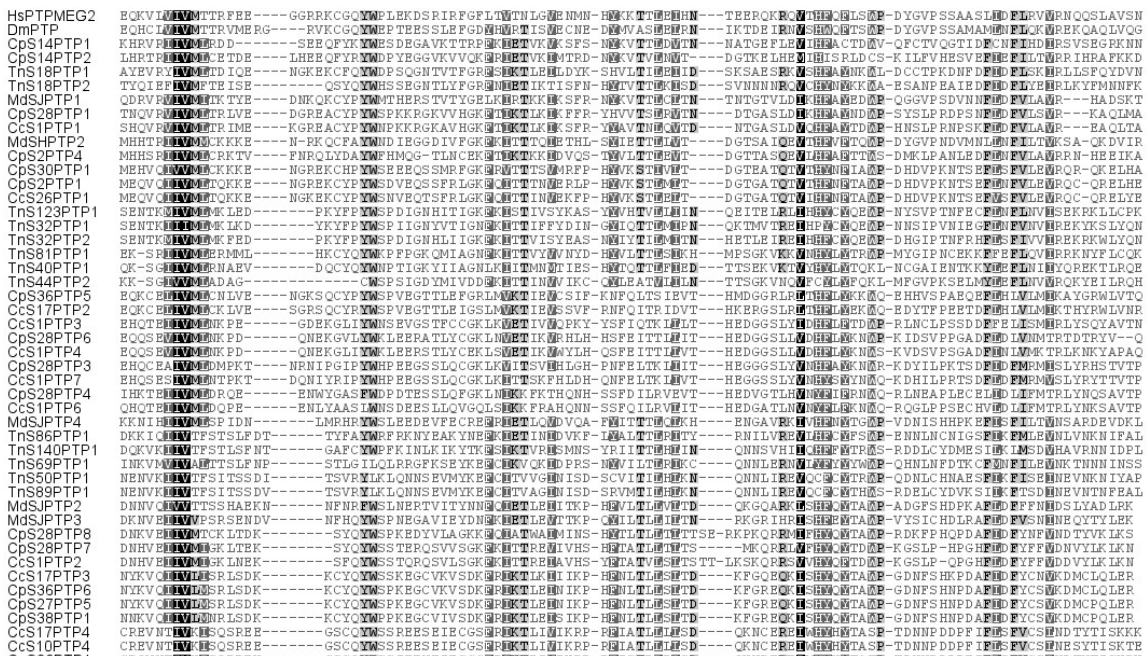
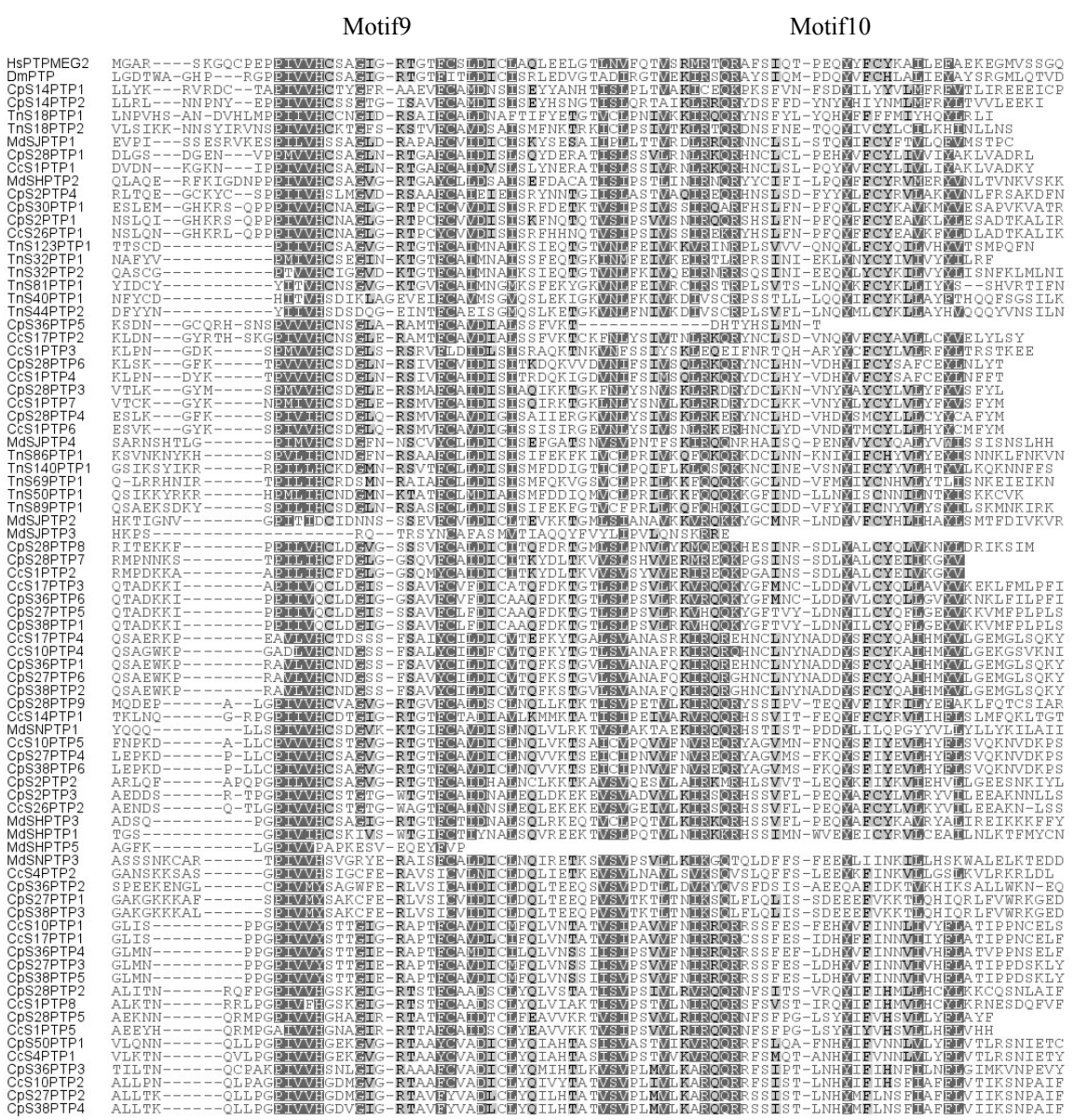
CpS27PTP2
CpS38PTP4

IVxMT KCxxYWT WPDxGxP

Motif6 Motif7 Motif8

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Motif10
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DmPTP
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CpS14PTP2
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TnS18PTP2
MdSJPTP1
CpS28PTP1
CcS1PTP1
MdSHPTP2
CpS2PTP4
CpS30PTP1
CpS2PTP1
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TnS50PTP1
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CcS26PTP2
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CcS1PTP8
CpS28PTP5
CcS1PTP5
CpS50PTP1
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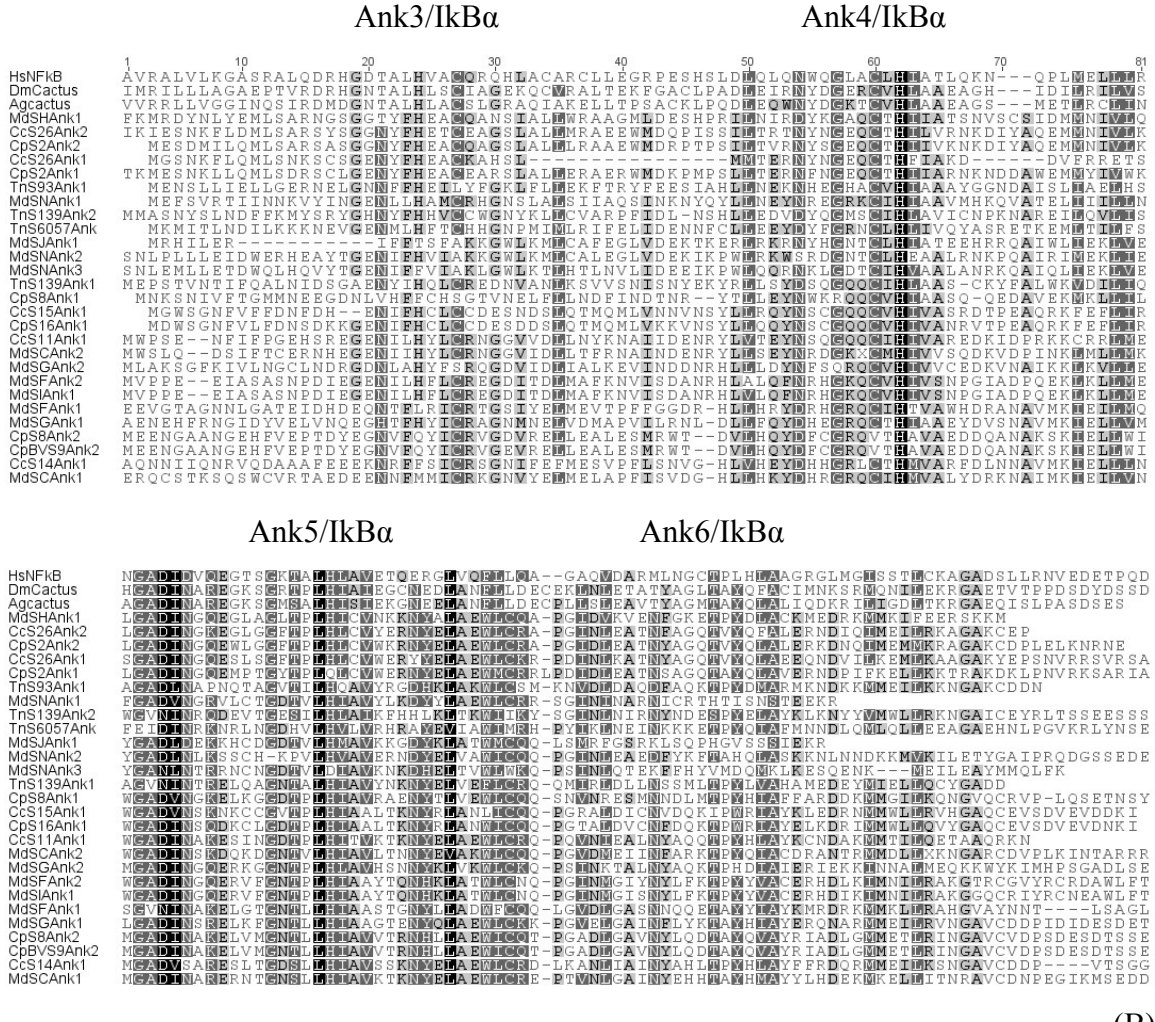
CcS10PTP2
CpS27PTP2
CpS38PTP4

(A)



(B)

Fig. 13 Multiple sequence alignment of Bracovirus PTP and ankyrin proteins. (A) Alignment of 79 PTP proteins encoded in four different Bracovirus genomes (*CpBV, CcBV, MdBV* and *TnBV*) with human and Drosophila PTP proteins of the MEG2 type (accession numbers M83738, L09247). 10 conserved motifs of classical PTPs are shown on the alignment (B) Alignment of 27 ankyrin repeat containing proteins from the same Bracovirus species as above with human IKappaBepsilon, *Drosophila* cactus, and *Anopheles gambiae* cactus proteins (accession numbers, AAM27002.1, Q03017, and EAA12805.2) Predicted ankyrin repeats are marked over the alignment. Ank3-Ank6 represent ankyrin repeats 3-6 of Drosophila cactus and human IkBα. These two alignments were used to generate phylogenetic trees of Bracovirus PTPs and ankyrin repeat containing proteins.

Circle number	Gene location (bps)	N. of exons	Amino acids	Database similarity (protein ID), % aa identity	Gene name (for PTPs and Ankyrin
123	1507-2400^{+}	1	297	*C. glomerata* bracovirus PTP *AAR99279.1, 39% (114/290)*	TnBV123PTP1
133	3020-6010^{+}	5	819	hypothetical protein ORF301 [*Cotesia plutellae* polydnavirus], BEN and PHAO2737 domain,, AAZ04269.1, 35%(219/689)	
139	3535-3771	1	78	hypothetical protein CcBV_20.2 [*Cotesia congregata* bracovirus], BEN and PHAO2737domain,YP184853, 37%(26/70)	
	5310-5831^{+}	1	173	viral ankyrin [*Glyptapanteles indiensis*], ACE75454.1, 38%(56/147)	TnBV139Ank1
	6428-6934^{+}	1	168	GfV-B29-ORF1 [*Glypta fumiferanae* ichnovirus, YP001029373.1, 33%(55/166)	TnBV139Ank2
	8962-9219^{+}	1	85	conserved hypothetical protein [*Glyptapanteles indiensis*], PHAO2744 domain, ACE75406.1, 32%(24/75)	
140	5107-5997^{+}	1	296	PTP 2 *Microplitis demolitor* bracovirus, YP239391.1, 28%(82/290)	TnBV140PTP1
154	2083-2442^{+}	1	119	hypothetical protein GFP_L2_0020 [*Glyptapanteles flavicoxis* bracovirus], ,PHAO 2747 super family, ACE75228.1, 25%(18/71)	
154-2	2083-2442^{+}	1	119	hypothetical protein GFP_L2_0020 [*Glyptapanteles flavicoxis* bracovirus], PHAO 2747 super family, ACE75228.1, 25%(18/71)	
34	2215-4927^{+}	4	678	conserved hypothetical protein [*Glyptapanteles flavicoxis* bracovirus], BEN and PHAO2737 domain, ACE75243.1, 31%(126/378|)	
	6690-8465^{+}	4	432	conserved hypothetical protein [*Glyptapanteles flavicoxis* bracovirus], BEN domain, ACE75153.1, 44%(92/216)	
35	3369-4678^{+}	3	378	conserved hypothetical protein [*Glyptapanteles flavicoxis* bracovirus], BEN and PHAO2737 domain , ACE75501, 36%(57/149)	
44	1309-1885^{+}	1	193	protein tyrosine phosphatase [*Cotesia plutellae* polydnavirus], AAZ04264.1, 26%(25/96)	TnBV44PTP1
	2710-3536^{+}	1	275	protein tyrosine phosphatase [*Cotesia plutellae* polydnavirus], AAZ04264.1, 37%(48/132)	TnBV44PTP2
47	4527-9117	4	419	PTP 2 [*Microplitis demolitor*	TnBV47PTP1

				bracovirus], YP239400.1, 40%(64/157)	
50	4777-5706^{+}	1	309	PTP 2 [*Microplitis demolitor* bracovirus], YP239391.1, 28%(88/304)	TnBV50PTP1
80	5367-7214^{+}	1	615	NTP-sugar transporter [*Apis mellifera*], XP395030.3, 46%(155/332)	
	1894-3245^{+}	1	450	UDP-glucose 6-dehydrogenase [*Apis mellifera*], XP396801.3, 69%(81/117)	
	11876-12682^{+}	1	271	similar to CG2206-PA [*Nasonia vitripennis*],XP001606517.1, 33%(45/135)	
61	3086-504^{+}	5	451	conserved hypothetical protein [*Glyptapanteles flavicoxis* bracovirus], ACE75505.1, 53%(119/225)	
69	660-1592^{+}	1	310	PTP 2 *[Microplitis demolitor* bracovirus], YP239393.1, 27% (80/287)	TnBV69PTP1
	3354-4182^{+}	1	276	PTP 2 [*Microplitis demolitor* bracovirus], YP239391.1, 27% (42/127)	TnBV69PTP2
81	1462-2379^{+}	1	305	similar to GA15974-PA [*Nasonia vitripennis*], XP001606932.1, 34%(103/300)	TnBV81PTP1
86	6484-7434	1	316	PTP 2 [*Microplitis demolitor* bracovirus], YP239393.1, 30% (91/302)	TnBV86PTP1
87	830-1330	1	166	hypothetical protein ORF904 [*Cotesia plutellae* polydnavirus], AAZ04286.1, 34%(29/85)	
	4097-4321	1	74	conserved hypothetical protein [*Glyptapanteles indiensis* bracovirus], ACE75434.1, 35%(25/71)	
89	3881-4819^{+}	1	312	PTP 2 [*Microplitis demolitor* bracovirus], YP239393.1, 28%(89/310)	TnBV89PTP1
93	601-1068^{+}	1	155	viral ankyrin [*Glyptapanteles indiensis* bracovirus], ace75303.1, 40%(63/154)	TnBV93Ank1
99	7851-9588^{+}	2	532	hypothetical protein [*Microplitis demolitor* bracovirus], BEN domain,YP239364.1, 42%(48/112)	
	3077-3595	1	172	CG25304 [*Drosophila erecta*], XP001979163.1, 31%(28/91)	
	1835-2257^{+}	1	140	family A2 unassigned peptidase (A02 family) [*Schistosoma mansoni*], XP002569446.1, 34%(33/97)	
100	1116-2618	3	219	DUF-like 1 [*Cotesia plutellae* polydnavirus], BEN and PHAO2737 domain, ABK63308.1, 28%(23/80)	
32	837-1718^{+}	1	293	protein tyrosine phosphatase	TnBV32PTP1

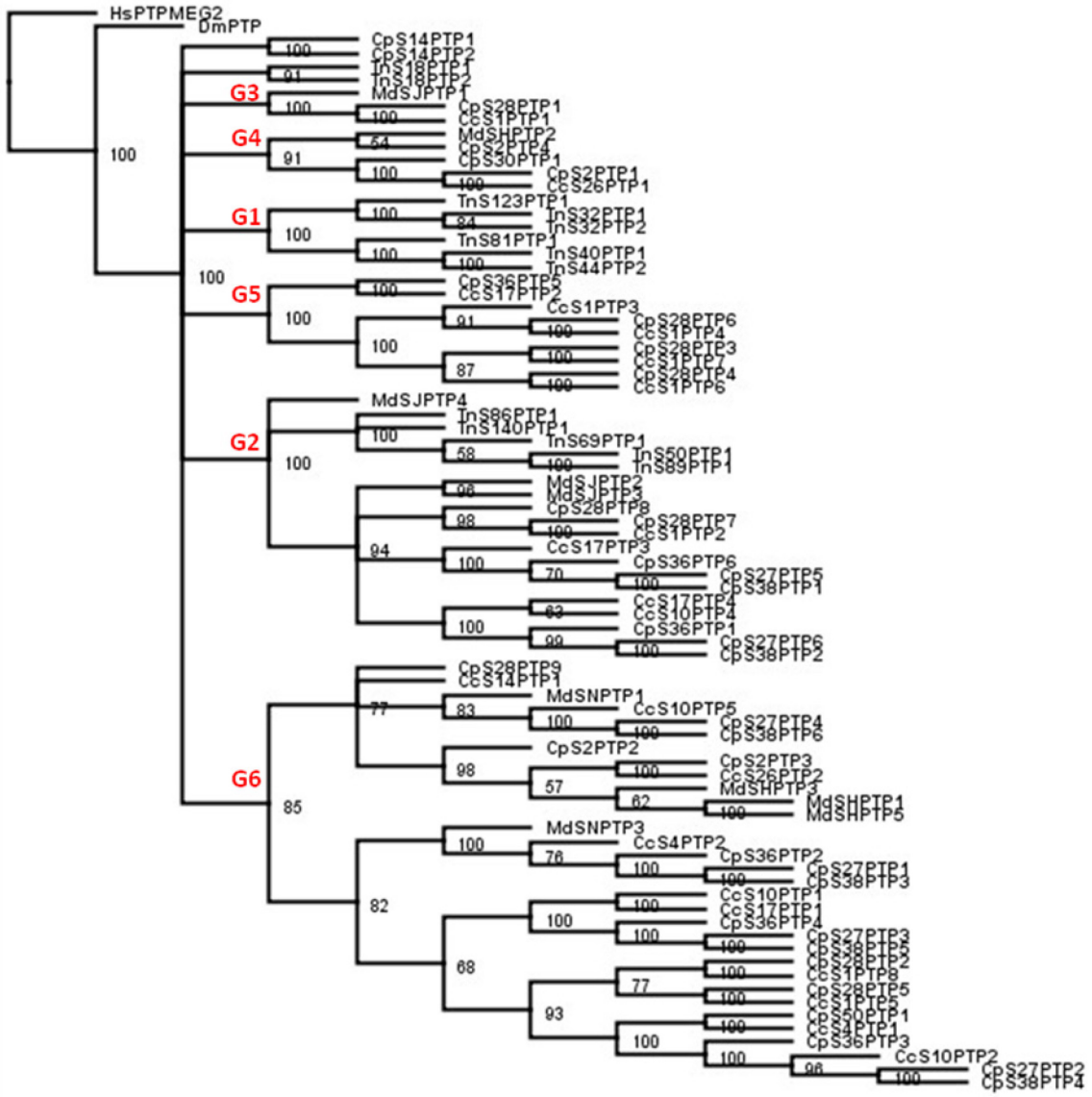
				[*Cotesia glomerata* bracovirus], AAR29979.1, 34%(103/300)	
	2245-3147^{+}	1	300	protein tyrosine phosphatase [*Cotesia glomerata* bracovirus], AAR29979.1, 36%(109/298)	TnBV32PTP2
6	1507-3379	3	318	hypothetical protein [*Microplitis demolitor* bracovirus], PHAO2744 domain, YP239392.1, 30%(42/140)	
18.1	325-1233^{+}	1	302	PTP1 [*Glyptapanteles indiensis* bracovirus], ACE30023.1, 35%(108/302)	TnBV18.1PTP1
	2475-3368^{+}	1	297	protein tyrosine phosphatase [*Glyptapanteles indiensis* bracovirus], ACE75313.1, 35%(108/303)	TnBV18.1PTP2
40.1	3967-4581^{+}	1	294	protein tyrosine phosphatase [*Glyptapanteles indiensis*], ACE75355.1, 29%(67/225)	TnBV40.1PTP1
	159-410	1	83	DNA helicase [*Adineta vaga*],AAZ67118.1, 64%(46/71)	
24.2	1121-2611^{+}	2	496	UDP-sugar transporter UST74c (fringe connection protein), putative [*Nasonia vitripennis*], XP001599500.1, 44%(116/260)	
6057	3922-4446	1	174	GfV-B29-ORF1 [*Glypta fumiferanae* ichnovirus], YP001029373, 34%(60/174)	TnBV6057Ank1
	1402-1644^{+}	1	80	hypothetical protein [*Cotesia plutellae* polydnavirus], ABK63307.1, 36%(27/65)	

Table 3 Predicted genes in *TnBV* genomic circles. (+) Refer to genes predicted on the positive strand.

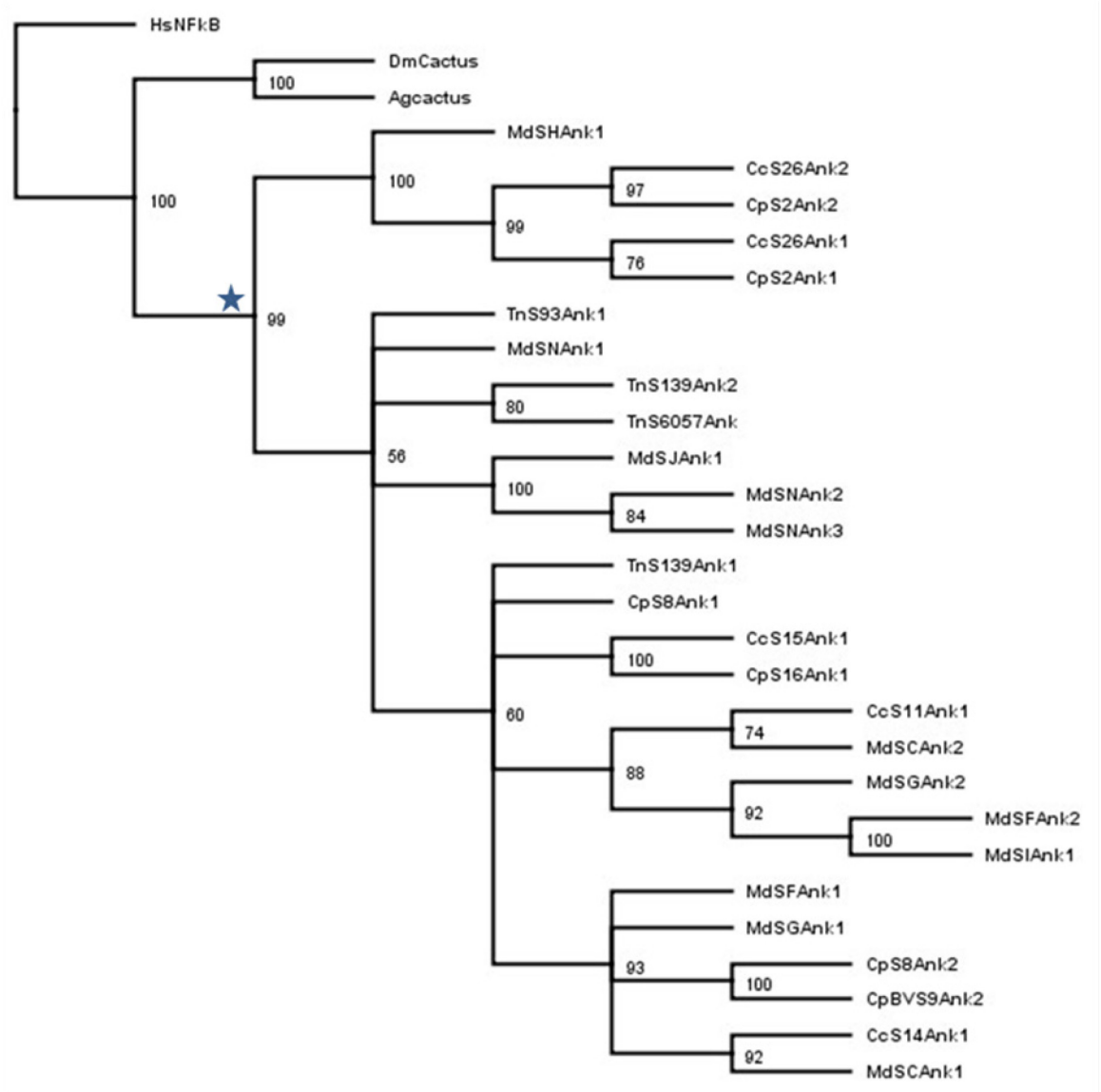
1.3 Phylogenetic analysis of *TnBV* encoded proteins

PTP and Ankyrin protein sequences from three different bracovirus were obtained from the NCBI Gene bank (http://www.ncbi.nlm.nih.gov/) and aligned with *TnBV*-PTP and *TnBV*ANK proteins, respectively, by using the Clustal X program (http://www.clustal.org/). Phylogenetic reconstructions were conducted separately on PTPs and Ankyrin repeat containing proteins. Three members of the four bracovirus species considered in this study belonged to the Microgastrinae subfamily (*CpBV, CcBV, MdBV*) while the fourth member belonged to the subfamily Cardiochiilinae *(TnBV)*. Construction of the phylogenetic tree was made by using the distance matrix method of analysis (Neighbor-Joining). Tree validation was performed by bootstrap analysis in which 100 trees were used to construct the consensus tree.

Phylogenetic analysis of 79 PTPs from the considered polydnavirus species indicated that they can be clustered in 6 monophyletic groups containing several members and two PTP protein pairs, one containing two *CpBV*-PTPs (CpS14PTP1 and CpS14PTP2) and the other one made of two *TnBV*-PTPs (TnS18PTP1 and TnS18PTP2) (Fig. 14a). Among the 6 main groups, the first group G1, contained only members of *TnBV*PTPs (TnS32PTP1, TnS32PTP2, TnS123PTP1, TnS81PTP1, TnS44PTP2, and TnS40PTP1) and was supported by high bootstrap value (100%). The corresponding genes might have been acquired, in the evolutionary history of bracoviruses, after the divergence of Cardiochiilinae from Microgastrinae in the Microgastroid lineage. The second group G2 was the only one with representation from all the species under investigation. It contained 20 members forming a clade that was supported by 100% bootstrap value. The corresponding genes have possibly derived, by gene duplication events, from a common ancestral gene, present in the bracovirus associated with the last common ancestor of Microgastrinae and Cardiochiilinae. All the remaining groups, G3-G6, only contained PTP members belonging to bracoviruses associated with the subfamily Microgastrinae (Fig. 14a). The phylogenetic analysis of 27 ankyrin repeat containing proteins from the above mentioned bracovirus species did not show a well defined clustering pattern.. Although few ankyrin proteins clustered together (Fig. 14b), a high divergence of sequences was observed. For this reason, it was not possible to reconstruct a complete phylogeny of bracovirus ankyrin genes.


HsPTPMEG2
DmPTP
CpS14PTP1
CpS14PTP2
TnS18PTP1
TnS18PTP2
MdSJPTP1
CpS28PTP1
CcS1PTP1
MdSHPTP2
CpS2PTP4
CpS30PTP1
CpS2PTP1
CcS26PTP1
TnS123PTP1
TnS32PTP1
TnS32PTP2
TnS81PTP1
TnS40PTP1
TnS44PTP2
CpS36PTP5
CcS17PTP2
CcS1PTP3
CpS28PTP6
CcS1PTP4
CpS28PTP3
CcS1PTP7
CpS28PTP4
CcS1PTP6
MdSJPTP4
TnS86PTP1
TnS140PTP1
TnS69PTP1
TnS50PTP1
TnS89PTP1
MdSJPTP2
MdSJPTP3
CpS28PTP8
CpS28PTP7
CcS1PTP2
CcS17PTP3
CpS36PTP6
CpS27PTP5
CpS38PTP1
CcS17PTP1
CcS10PTP4
CpS36PTP1
CpS27PTP6
CpS38PTP2
CpS28PTP9
CcS14PTP1
MdSNPTP1
CcS10PTP5
CpS27PTP4
CpS38PTP6
CpS2PTP2
CpS2PTP3
CcS26PTP2
MdSHPTP3
MdSHPTP1
MdSHPTP5
MdSNPTP3
CcS4PTP2
CpS36PTP2
CpS27PTP1
CpS38PTP3
CcS10PTP1
CcS17PTP1
CpS36PTP4
CpS27PTP3
CcS1PTP8
CpS28PTP5
CcS10PTP3
CpS36PTP3
CcS10PTP2
CpS27PTP2
CpS38PTP4
G1
G2
G3
G4
G5
G6

(A)



(B)

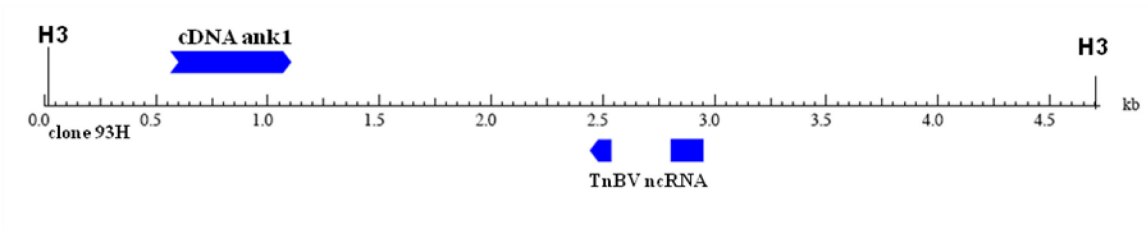
Fig. 14 Phylogenetic analysis of protein tyrosine phosphatases and ankyrin repeat containing proteins from different bracovirus species (Cc, *Cotesia congregata*, Cp, *Cotesia plutellae*, Md, *Microplitis demolitor*, Tn, *Toxoneuron nigriceps*). Phylogenetic trees, based on the Neighbor Joining method of analysis, were obtained by using the Phylip package, and the reliability of the groups were assessed by Bootstrapping using the program SEQBOOT to generate a total of 100 bootstrapped trees. Numbers before each node indicate bootstrap values. Clusters were supported by internal nodes with bootstrap values ≥85%. The six groups of Bracovirus PTPs are indicated as G1-G6. The tree shown in (A) was rooted by human and Drosophila protein tyrosine phosphatases of the MEG2 type (HsPTPMEG2 and DmPTP, accession numbers M83738and L09247) and the tree shown in (B) was rooted by human IKappaBepsilon, *Drosophila* cactus, and *Anopheles gambiae* cactus proteins (accession numbers, AAM27002.1, Q03017, and EAA12805.2),

2. Non-coding RNAs from *Toxoneuron nigriceps* polydnavirus target a host immune gene.

2.1 A *TnBV* gene gives rise to non-coding RNAs which display a complex splicing pattern

Previous work carried out in the lab identified a *TnBV* sequence which is transcribed into non-coding RNA molecules potentially implicated in the regulation of a *H. virescens* gene (unpublished data). This sequence was localized in the *TnBV* circle 93 which also includes a member of the ankyrin gene family (Fig. 15). Two partially overlapping cDNAs, differing in their 5' regions (cDNA 3: 550 base pairs and 5: 661 base pairs), were isolated from a haemocyte cDNA library. Sequence comparisons with the genomic circle 93 indicated that the corresponding transcripts were originated by alternative splicing and/or different usage of alternative transcription start sites (Fig 15b). Moreover, Northern blot analysis using a genomic probe resulted in a broad hybridization signal, ranging from about 400 to 600 bases (data not shown). To test the hypothesis that this size heterogeneity might derive from a complex splicing pattern, I performed several RT-PCR experiments using different primer pairs. The products generated were cloned into a suitable vector and sequenced. By this strategy, three different splicing patterns were identified in the 5' half of the ncRNA molecules generated from circle 93 (Fig. 15b).

(A)



(B)

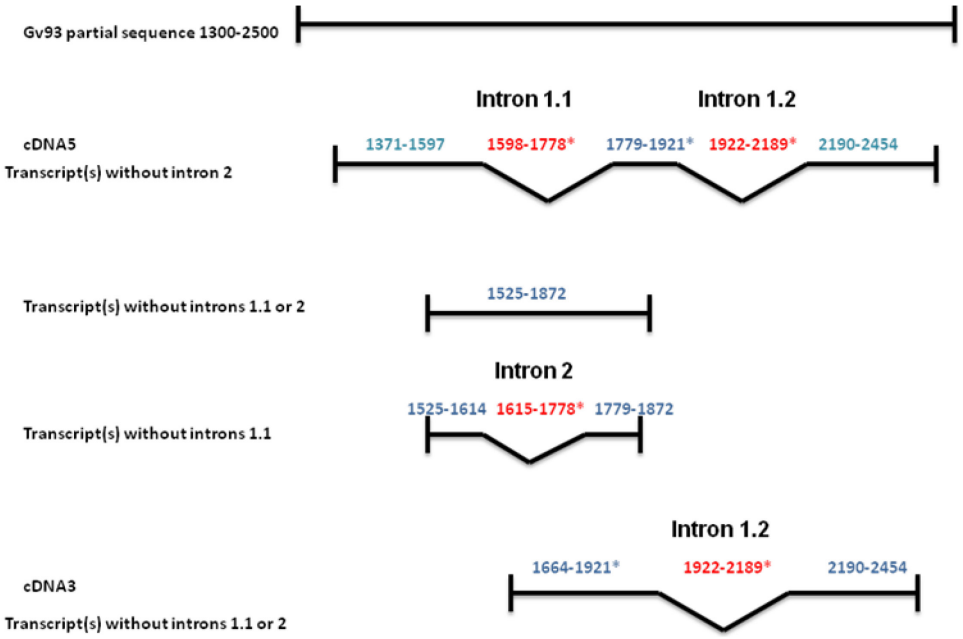


Fig. 15 Circle 93 contains a gene producing several ncRNAs. (A) Schematic representation of the circle 93in linear format. The circle contains a member of the *TnBV* ankyrin gene family along with a gene, transcribed into non coding RNA molecules, located on the opposite strand. (B) Diagrammatic representation of the detected splicing patterns of the ncRNA transcripts. Nucleotides are marked with reference to their positions in circle 93. Numbers in red represent intronic sequences while those in blue represent exons.

2.2 The putative target of the *TnBV* ncRNAs is activated by immune challenge

The identified ncRNAs shared a sequence which is the reverse complement of the 5'UTR of a *H. virescens gene*, named 102, expressed in haemocytes (Fig. 16). This peculiar structural feature suggested that the circle93ncRNAs might be able to silence the 102 gene, either by targeting its transcript for degradation or by blocking its translation. Several lines of evidence suggest that the 102 gene is implicated in the immune response (unpublished data). The circle93ncRNAs might therefore function in host immunosuppression that, as stated in the introduction, is one of the main physiological alterations induced by parasitization.

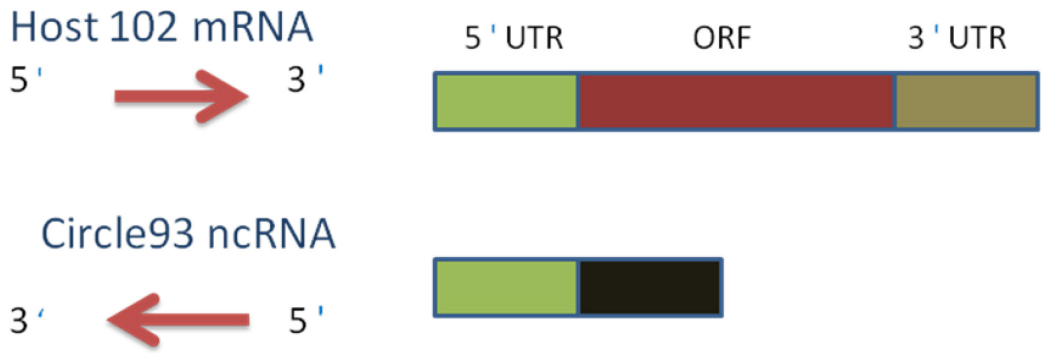


Fig. 16. Diagrammatic representation showing the structural relationship between *TnBV*ncRNAs transcribed from the *TnBV* genomic circle 93 and the 102 *H. virescens* transcript. Green bars represent the region of complementarity between the host and viral transcripts. In the host transcript, this region only includes the 5'UTR.

In the frame of a comprehensive study carried out in the lab to investigate the function of the 102 gene and its relationship to the circle93ncRNAs, my contribution was aimed at testing whether the 102 gene can be regulated by immune challenge. *H. virescens* larvae were immunochallenged by CM-Sepharose beads injection and the changes in the level of the 102 transcript were checked after 3, 6, 12, 24 and 48 h using Quantitative Real

Time RT-PCR. The relative quantification of the 102 transcript, referred to non-immunochallenged control larvae, showed an initial strong increase, reaching a peak 12 h after immune challenge, followed by a gradual decrease (Fig. 17). This result indicated that the 102 gene is activated by immune challenge, supporting the idea that the 102 protein is involved in immune response. A similar temporal profile of 102 transcript accumulation was found in parasitized larvae, analysed at 3, 6, 12, 24 and 48 h after parasitization (Fig. 17). This finding implies that also the injection of the parasitoid egg is able to trigger a molecular pathway leading to 102 gene activation. Moreover, the significant increase of the 102 transcript level observed after parasitization suggested that if circle93ncRNAs target this transcript they might inhibit protein translation rather than induce transcript degradation.

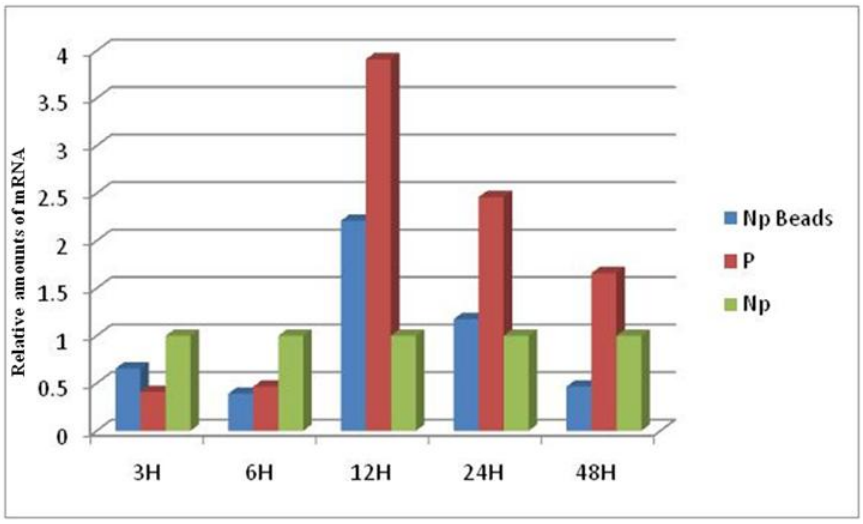


Fig. 17 Relative quantification of 102 mRNA using qRT-PCR. RNA samples from haemocytes of nonparasitized, parasitized or nonparasitized immunochallenged larvae were extracted at different time intervals as indicated above. Real Time PCR was performed using SYBR green technology. Data analysis was performed using ∧ cT method (Livak et al., 2001). All samples were calibrated against the nonparasitized control.

2.3 Temporal transcription pattern of *TnBV*ncRNAs in haemocytes of parasitized larvae

The expression pattern of *TnBV*ncRNAs in haemocytes, at different time-points after parasitoid egg injection, was analyzed by semi-quantitative RT-PCR using the SK4 ribosomal protein RNA as internal control. The appropriate number of cycles for both the control and the viral transcripts was determined separately to avoid saturation of PCR products. *TnBV*ncRNAs that spliced the intron situated in the 5' half of the gene displayed maximum accumulation level at 6 hours after parasitization, were reduced at 12 hours, and then remained constant up to 48 hours after parasitization. The *TnBV*ncRNAs deriving from the same genomic locus, but retaining the 5' end intronic sequences, displayed an accumulation pattern somehow complementary: their level was already high 3 hours after parasitization, decreased at 6 hours and again increased at 12 and 24 hours after parasitoid egg injection (Fig. 18).

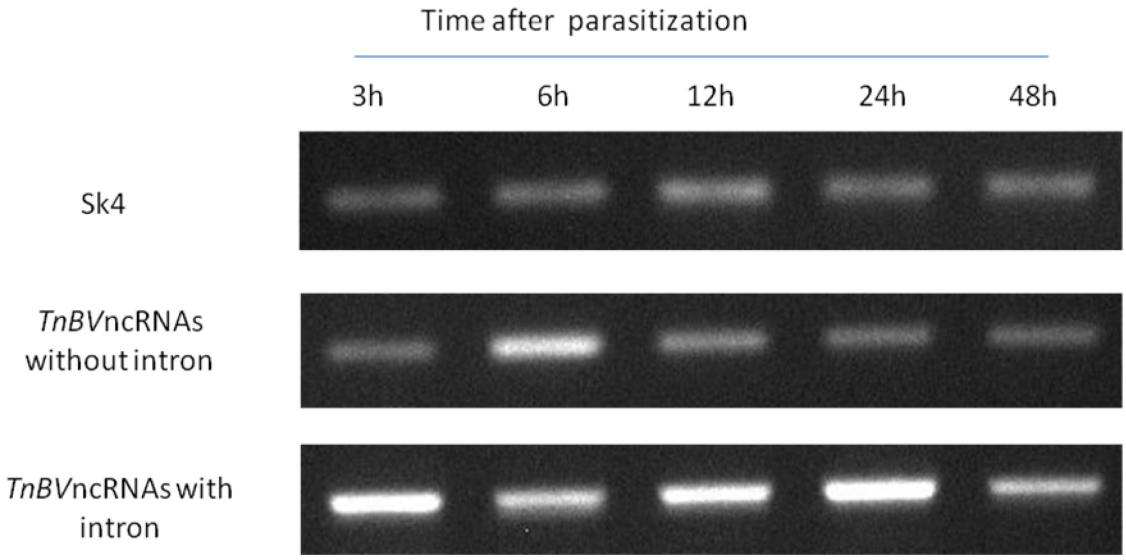


Fig. 18 Temporal expression pattern of differentially spliced *TnBV*ncRNA transcripts, detected by semi-quantitative RT-PCR. RNA samples were extracted from haemocytes of parasitized larvae at 3, 6, 12, 24, 48 h after parasitoid egg injection. Gene specific primers were used in PCR reactions for both *TnBV*ncRNAs and the internal control (Sk4).

2.4 Transcription pattern of 102 mRNA and *TnBV*ncRNAs in *H. virescens* tissues

The transcription levels of 102 mRNA and *TnBV*ncRNAs were compared in five *H. virescens* tissues, including haemocytes, fat body, gut, malpighian tubules and epidermis. The selected time-point was at 6 hours after parasitoid egg injection. In nonparasitized larvae, the 102 mRNA was detected in 3 out of the 5 tested tissues: hemocytes, fat body and gut, with no evident differences in levels (Fig. 19a). This pattern was reproduced in parasitized larvae (Fig. 19b). The alternatively spliced *TnBV*ncRNAs showed analogous profiles. They were found in the same tissues as the 102 transcript. Their level did not show significant differences in these tissues, but appeared to be higher than that of the 102 mRNA. In addition, *TnBV*ncRNAs were found in malpighian tubules, at a slightly reduced level compared to the other tested tissues (Fig. 19b).

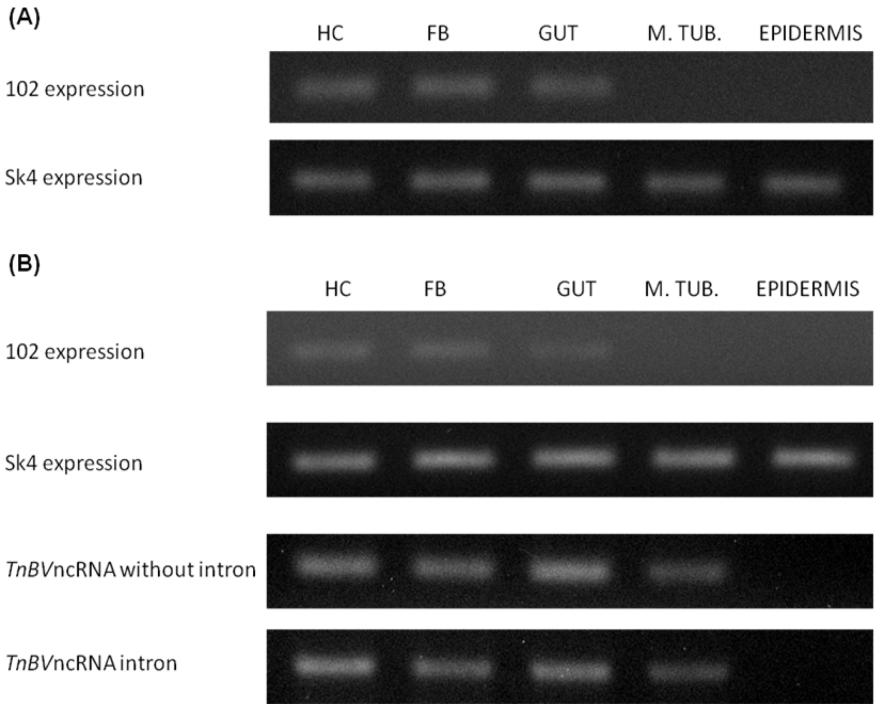


Fig. 19. Expression of 102 mRNA and *TnBV*ncRNAs in different tissues of *H. virescens* larvae, tested by semi-quantitative RT-PCR. A. Expression of 102 mRNA in hemocytes, fat body, gut, Malpighian tubules and epidermis of nonparasitized late fourth instar larvae. B. Expression of 102 mRNA and *TnBV*ncRNAs in the same tissues as above, dissected from parasitized larvae at 6 hours after parasitoid egg injection. Relative quantification was performed using the SK4 RNA as internal control.

2.5 Identification of *Drosophila melanogaster* homologues of the 102 gene

Blast analysis of the deduced amino acid sequence of the *H. virescens* 102 gene identified two putative *Drosophila melanogaster* proteins which display high similarity with it (e values: $3e^{-40}$ and $2e^{-48}$ respectively) (Fig. 20a). Then, as a first step to distinguish the presumptive orthologous protein, RT–PCR experiments were performed, looking for the presence of the corresponding transcripts in larval haemocytes. One of the two genes, CG3303, was only expressed at a very low level (Fig. 20b). On the contrary, the second gene, CG2145, was highly expressed in haemocytes (Fig. 20b) and a strong signal was also obtained from whole larvae (data not shown). Absence of genomic DNA contamination in the RNA preparation was confirmed by the two controls included in the experiment. Compared to the cDNA amplification product, the PCR fragment generated from genomic DNA had in fact a slightly larger size, while no amplification was obtained when using as template the RNA sample.

(A)

Sequences producing significant alignments:		Score (Bits)	E Value	
ref|NP_572668.1|	CG2145-PA [Drosophila melanogaster] >gb|AAF4...	190	2e-48	UG
ref|NP_650508.1|	CG3303-PA [Drosophila melanogaster] >gb|AAK9...	163	3e-40	UG
gb|AAL68194.1|	GH10845p [Drosophila melanogaster]	149	4e-36	U
ref|NP_732084.1|	CG31292-PA [Drosophila melanogaster] >gb|AAN...	34.3	0.24	UG
ref|NP_572175.2|	CG12179-PB, isoform B [Drosophila melanogast...	30.4	3.2	UG
emb|CAA20225.1|	EG:66A1.2 [Drosophila melanogaster]	30.4	3.3	
gb|AAX33541.1|	LD15043p [Drosophila melanogaster]	30.4	3.3	U
ref|NP_726937.2|	CG12179-PA, isoform A [Drosophila melanogast...	30.4	3.3	UG
ref|NP_610431.1|	CG8230-PA [Drosophila melanogaster] >gb|AAD3...	28.9	9.7	UG

(B)

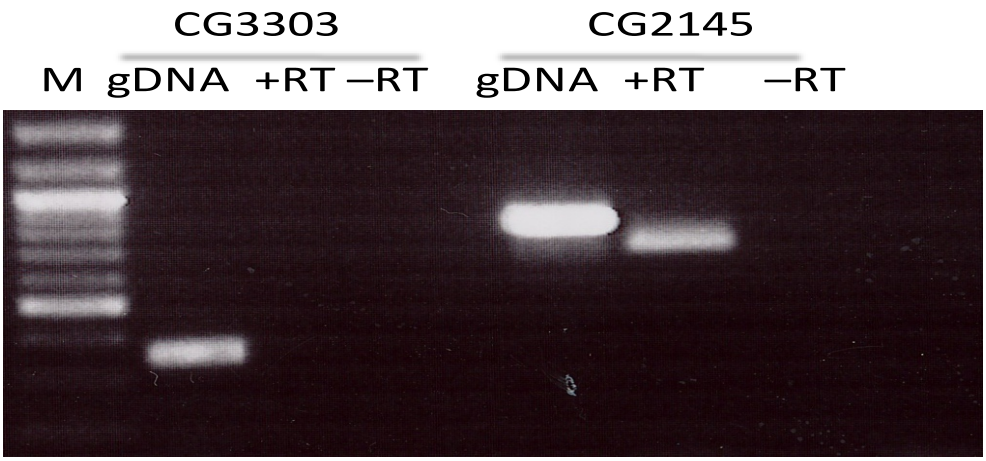


Fig. 20 *Drosophila melanogaster* genome contains two putative homologues of the 102 *H. virescens* gene. (A) Blast analysis identified two genes in Drosophila, CG2145 and CG3303. (B) Expression of CG2145 and CG3303 genes in Drosophila larval hemocytes, tested by RT-PCR. gDNA (positive control containing genomic DNA), +RT (reverse transcribed RNA), -RT (negative control, containing all components of the reverse transcriptase reaction except the enzyme).

DISCUSSION

The destructive effects produced by insect pests on crops represent a strong limiting factor to the increase of world food production. Effective strategies for pest management other than the indiscriminate usage of insecticides are therefore needed to cope with food demand, which is expected to rise more and more due to future population growth. In this frame, control strategies used by insect's natural enemies have attracted particular interest.

Parasitic insects are potent control agents as they developed very efficient tools to regulate the physiology of their hosts. Among these tools, polydnaviruses emerged as a really striking "machinery" used by parasitic braconid and ichneumonid wasps to transfer in their lepidopteran hosts a full set of key regulatory factors. Due to their pivotal role in the pathological syndromes associated with parasitization, polydnaviruses have received special attention in the last years. Therefore, along with functional analysis of individually isolated genes, full genome sequencing projects have been carried out on selected polydnavirus species (Espagne et al., 2004; Webb et al., 2006; Choi et al., 2009). The information retrieved from these studies will advance our knowledge on the molecular mechanisms underlying host-parasitoid interactions. In addition, it will allow the identification of a number of genes potentially useful for the development of novel bioinsecticides.

This PhD work focused on genome sequencing of the *Toxoneuron nigriceps* bracovirus. Following full genome assembly and annotation, evolutionary relationships among the members of two bracovirus gene families were studied. Finally, initial characterization of a non-coding region of the *TnBV* genome identified a number of alternatively spliced, non-coding transcripts, potentially involved in host immunosuppression.

The *TnBV* genome consists of 27 circles, varying in their size and abundance, with an aggregate genome size of about 203 Kb. The number of circles is similar to that found in other bracoviruses, i.e., *CcBV, CpBV*, *Glyptapanteles indiensis* bracovirus *(GiBV)* and *Glyptapanteles flavicoxis* bracovirus (*GfBV)*, while the total amount of the *TnBV* genome sequence is reduced. In fact, *CcBV*, *CpBV, GiBV* and *GfBV* comprise 30, 27, 29 and 29 circles, respectively, with aggregate genome size of 568 Kb, 471 Kb, 517 Kb and 594 Kb (Espagne et al., 2004; Webb et al., 2006; Desjardins et al., 2008; Choi et al., 2009). If we include in the comparison also the *MdBV* genome, that consists of only 15 circles, with a

total sequence of 187 Kb, it appears that *TnBV* circles have, in average, a smaller size than all other bracoviruses whose genome was sequenced to date.

The coding density in *TnBV* is about 22%. This is slightly less than that of *CcBV, CpBV, GiBV* and *GfBV,* which was reported to be 26.9%, 32.3%, 33%, 32%, respectively, while it is higher than that of *MdBV* which is 17% (Espagne et al., 2004; Webb et al., 2006; Choi et al., 2009).

Forty two genes were identified in the *TnBV* genome. This number is less than that predicted in other bracovirus genomes: 156, 125, 197, and 193 genes in *CcBV, CpBV, GiBV* and *GfBV,* respectively. This difference might be in part explained by the smaller size of the *TnBV* genome which is less than half compared to the other four species. However, *TnBV* genes are also less than the 67 genes of *MdBV*, which has a slightly smaller genome.

Eleven genes (24.2%) were predicted to have introns in *TnBV*, less than in *CcBV* (69%), *CpBV* (40.8%), *GiBV* (58%) and *GfBV* (63%), but more than in *MdBV*. (13.1%) (Webb et al., 2006; Lapointe et al., 2007). Sixteen genes were included in the largest *TnBV* gene family, coding for protein products that show high level similarity with protein tyrosine phosphatases (PTPs). PTP genes have been reported from various Bracoviruses, including *CcBV*, *Cotesia glomerata* bracovirus (*CgBV*), *Cotesia vestalis* bracovirus (*CvBV*), *GiBV*, (*GfBV*), *MdBV* and *CpBV* (Kroemer and Webb, 2004; Provost et al., 2004; Webb et al., 2006; Ibrahim et al., 2007; Lapointe et al., 2007; Shi et al., 2008a). Interestingly, one PTP gene present in the *TnBV* genome contains an intron, which is not the case for all other bracovirus PTPs.

All the 16 *TnBV*-PTPs are classical non-receptor PTPs because, unlike receptor PTPs, they lack a transmembrane domain and possess only a single phosphatase domain (Paul and Lomborso, 2003).

PTPs have a catalytic domain of about 250 amino acids containing a highly conserved 11-residue sequence called the HC motif, (I/V)HCXAGXXR(S/T)G (Andersen et al., 2001). In this motif, there is a cysteine residue that is critical for PTP activity (Guan and Dixon, 1991, Barford et al., 1994, Bliska and Black, 1995). Two *TnBV*-PTPs lack this cysteine residue in the catalytic site (Fig 13a). This is also true for 12 out of 27 *CpBV*PTP and 3 out of the 14 PTPs characterized in the *CpBV* genome (Provost et al., 2004;

Ibrahim et al., 2007). In *C. congregata* bracovirus, it was demonstrated that one PTP family member lacking the critical cysteine residue did not show catalytic activity while another member containing this key residue was fully functional (Provost et al., 2004). Four PTP genes were organized in the *TnBV* genome in two pairs. The two genes located in circle 32 (*TnBV*S32PTP1, *TnBV*S32PTP2) coded for PTP proteins which displayed high level of similarity with a single *Cotesia glomerata* bracovirus PTP. The two *TnBV* PTP genes located in circle 44 (*TnBV*S44PTP1, *TnBV*S44PTP2) coded for PTPs that shared high level of similarity with a PTP from *Cotesia plutellae* bracovirus. (Table 2). These findings support the hypothesis that each pair of PTP genes located on the same circle originated as a consequence of a gene duplication event.

Four genes coding for ankyrin repeat containing proteins were predicted in the *TnBV* genome, in comparison with 12 found in *MdBV*, 8 in *CpBV*, 5 in *CcBV*.. Ankyrin genes have been also reported for other bracoviruses including *CvBV, GiBV* and *GfBV* (Espagne et al., 2004; Kroemer and Webb, 2004; Kroemer and Webb, 2005; Webb et al., 2006; Lapointe et al., 2007; Tian et al., 2007; Shi et al., 2008b). In addition, ankyrin genes were predicted in ichnoviruses (Kroemer and Webb, 2005; Tian et al., 2007).

The smallest gene family found in the *TnBV* genome includes 2 members coding for putative UDP sugar transporters. Similar genes were found also in *GiBV* and *GfBV* genomes, in number of 3 and 5, respectively (Desjardins et al., 2008). It can be hypothesized that bracoviral UDP sugar transporters may affect the glycosilation profile of host cells, by altering the transport balance of the UDP-sugars across the Golgi membrane. Interestingly, the *TnBV* genome also contains a gene coding for a UDP glucose 6 phosphate dehydrogenase, an enzyme that is also involved in protein glycosylation (see below).

Previous analysis of bracoviral genomes identified several genes that are present only in one or few species. Sequence comparisons with the *TnBV* genome extended these observations, by clearly recognizing the absence of particular bracoviral genes, as well as by identifying *TnBV* specific genes. *TnBV* lacks EP1 genes, found in Bracoviruses associated with *Cotesia plutellae* (Chen et al., 2009) *Cotesia kariyai*, *Cotesia congregata* and *Glyptapanteles indiensis* (Tanaka et al., 2002, 2003; Harwood et al., 1998; Desjardins

et al., 2008). One member of this protein family was suggested to be involved in the disruption of hemocyte activity in parasitized hosts, since it was highly expressed in hemocytes at early times after parasitoid egg injection (Tanaka et al., 2002).

TnBV does not contain E94–related genes. E94 is a gene originally identified in *Autographa Californica* nucleopoly-hedrosis virus as an apoptosis inducer (Freisen et al., 1987; Clem et al., 1994). A gene similar to E94 was found in *CpBV.* It was proposed that this gene might impair the immune system of *Plutella xylostella* larval hosts, by causing apoptosis of host hemocytes (Ibrahim et al., 2005).

H4 histone-related genes were found in *CpBV*, *CcBV* and *CgBV* genomes. Functional analysis performed on the *CpBV* gene suggested that it may regulate host gene expression (Turner, 1991; Gad and Kim, 2009). H4-related genes were not found in the *TnBV* genome.

The list of *TnBV* specific genes, not found in any other bracoviral genome, contains four members. One codes for a putative aspartyl protease that is expressed abundantly in several tissues of parasitized larvae, including fat body, haemocytes and prothoracic glands (Falabella et al., 2003). The other *TnBV*-specific genes code for a putative DNA helicase, a protein with MFS secondary transporter domain, and a UDP glucose 6 phosphate dehydrogenase. The functional analyses of the last gene have recently started in our lab. UDP-glucose dehydrogenases play multiple roles in different biological systems. These enzymes catalyze the oxidation of UDP-glucose, to generate UDP-glucuronic acid, which, in turn, originates important polymers. For example, in plants, a significant amount of matrix polysaccharides may derive from monosaccharides donated from UDP-glucuronic acid derivatives (Zablakis et al., 1995). In animals, the synthesis of glycoproteins and proteoglycans is dependent from pathways originating from UDP-glucuronic acid (Roden, 1980). Moreover, UDP-glucose dehydrogenase is involved in the production of an antiphagocytic capsule, which contains UDP-glucuronic acid derivatives (Griffith et al., 2004). Therefore, the expression of a viral encoded UDP-glucose dehydrogenase, combined with the expression of the *TnBV* genes encoding UDP-sugar transporters (see above), may have a significant impact on carbohydrate metabolism. A likely consequence would be the alteration of the cell surface characteristics, which are important in cell-cell interactions, including those involved in capsule formation during

the immune response against foreign intruders.

Several hypothetical proteins with BEN and PHAO domains were found in *TnBV* as well as other bracoviruses. The BEN domain is a 90-100 amino acid long domain that derives its name from the three proteins in which it was originally found: human BANP, vaccinia virus E5R and vertebrate NAC1. Its function is currently unknown, but it was predicted to mediate protein–DNA and protein–protein interactions during chromatin organization and transcription (Abhiman et al., 2008).

There is considerable diversity in the number of copies of the BEN domain coded by different polydnaviruses. For instance, the *Cotesia congregata* bracovirus has 11 BEN domain containing proteins, while *Microplitis demolitor* bracovirus codes a single BEN domain. In this respect, *TnBV* genome displays a somehow intermediate situation since it contains 7 sequences coding for BEN domains. The presence of genes coding for hypothetical proteins with BEN domains in the bracovirus particles suggests a possible role in transcriptional regulation of viral and/or host genes. It might be also possible that these viral proteins are used to modify host cell functions by mimicking molecular interactions of endogenous proteins containing BEN domains. The only report concerning the impact of BEN domain proteins on host physiology indicated a role for a *CpBV* member of this class of proteins in host immunosuppression (Park and Kim, 2010).

The PHAO domain, which is also present in several *TnBV* hypothetical proteins, has not been characterized and its biological functions are still fully unknown.

On the basis of detailed phylogenetic studies, it was shown that the bracovirus associated wasps form a monophyletic group known as microgastroid complex and it was hypothesized that a single integration event of a viral genome, as a provirus, occurred in the microgastroid lineage (Whitfield, 2002, Murphy et al., 2008). This hypothesis was corroborated by the finding that bracovirus-associated wasp species share genes coding for structural components of polydnavirus particles and that these genes are related to genes of nudivirus, a sister group of baculovirus. These genes are not integrated in the packaged bracoviral genomes, that do not contain any nudivirus-related gene, but display structural and functional characteristics that are typical of eukaryotic genomes. It has been therefore suggested that, shortly after initial integration of the nudivirus ancestor, viral DNA might have been replaced by wasp DNA in the viral particles and that most

genes promoting parasitism were acquired later in bracovirus-associated wasps (Bezier et al., 2009).

Sequence aligment of 88 PTPs from four BV genomes (*CpBV, CcBV, TnBV*, and *MdBV*) indicated that 9 are very divergent and were therefore discarded from further analyses. The remaining 79 PTPs were used for phylogenetic studies aimed at establishing their evolutionary relationships. These studies indicated a clear clustering pattern of PTP proteins from viruses of the subfamily Microgastrinae (*CpBV, CcBV, and MdBV*). *TnBV* PTPs, on the other hand, mostly clustered together, but few of them were included in a clade that contained also members from the other bracovirus species, suggesting that they might have all derived from a common ancestral gene. This hypothesis would imply that gene duplication events, which occurred before and/or after the divergence of the Microgastrinae from the Cardiochiilinae, as well as during the evolution of the two subfamilies, generated all members of the PTP clade shared by the four bracovirus species. Unlike PTPs, bracoviral ANK proteins did not indicate a clear evolutionary relationship. The non-coding portion of the *TnBV* genome, as well as other polydnaviral genomes, is unusually high when compared to typical viral genomes, while it is similar to that found in insect genomes. In *Drosophila melanogaster*, for example, the total amount of non-coding sequences is about 80% (Halligan and Keightley, 2006). Unlike polydnaviral protein coding regions, which are actively studied at molecular and functional level, up to now the huge amount of polydnaviral non-coding sequences did not receive much attention. The only report dealing with the role of non coding RNA species in host-parasitoid interactions concerned the identification of 27 miRNAs that were up regulated in *Lymantria dispar* parasitized by *Glyptapanteles flavicoxis* in response to parasitization (Gundersen-Rindal and Pedroni, 2010). However, these non coding RNA species were produced by the host genome and the study did not give any hint about the possible presence, in the small RNA population isolated from parasitized individuals, of non coding RNAs deriving from the *GfBV* bracovirus.

Therefore, the *TnBV* non coding RNA species described in this PhD thesis work are the first identified in a polydnavirus. These ncRNA molecules were expressed in several tissues, including haemocytes, fat body, gut and malpighian tubules. Interestingly, they all derived from a single locus located in the *TnBV* genomic circle 93. The definition of their

full structure and number is still in progress, but the identification of at least three different splicing patterns anticipate a complex picture consisting of a network of partially overlapping transcripts. It is at the moment unclear whether this complexity has a functional meaning. It should be anyway noticed that the different non coding RNA isoforms seem to be differentially regulated during parasitization, at least in hemocytes.

The two non-coding cDNAs, isolated from a haemocyte cDNA library, share a common sequence that is complementary to the 5'UTR of a host gene. This gene codes for a protein product that is highly conserved throughout evolution. In particular, it displays 85% identity to a protein present in the venom of the bristles of *Lonomia obliqua* (Lepidoptera, Saturnidae) larvae, which is a member of a novel protein class, the "XendoU family" (Snjider et al., 2003; Renzi et al., 2006). The members of this protein family were thought to be serine protease-like enzymes, based on a study on Human Placental Protein 11 (PP11) (Grundmann et al., 1990), which has been recently reconsidered, assigning to PP11 RNA binding and hydrolytic activities (Laneve et al., 2008).

The sequence complementarity between the identified *TnBV* ncRNA species and the 5'UTR of the 102 host gene suggests a possible role of the ncRNAs in the silencing of this gene.

Antisense transcript pairs have been described in different organisms (Werner et al., 2005). They can be transcribed from opposite strands at the same genomic locus (*cis*-sense/antisense pair) and thus potentially lead to overlapping, perfectly matching RNA-RNA hybrids, or can derive from different genomic loci and in this case may have imperfect sequence complementarity (trans-sense/antisense pair). While cis-antisense transcripts have been extensively studied both computationally and experimentally, studies of trans-antisense RNAs have mainly focused on small RNAs, which play important regulatory roles (Korneev et al., 1999). There is evidence suggesting that long trans-antisense RNAs may also perform key regulatory functions. In eukaryotes, to date, the activity of long trans-antisense has been experimentally characterized in only three cases. Translation of the nitric oxide synthase (NOS) protein in the central nervous system of the snail *Lymnaea stagnalis* was prevented by the an antisense transcript produced by a NOS pseudo gene that formed a stable hybrid *in vivo* with NOS mRNA

(Korneev et al., 1999). Variant δ of the meiotic recombination gene Msh4 contains antisense RNA for the endoplasmic reticulum chaperon gene Hspa5. This variant forms a double stranded RNA structure with Hspa5, possibly inducing Hspa5 mRNA degradation (Hirano and Noda, 2004). MBP antisense RNA produced by gene duplication in the *mld* mouse mutant formed a RNA duplex with the MBP gene transcript, resulting in MBP protein decrease, either by reduced nuclear export or degradation of the RNA duplex (Okano et al., 1991).

Real time PCR relative quantification experiments demonstrated that the 102 gene is activated in haemocytes by immune challenge, supporting the hypothesis that this gene is involved in immune response. Interestingly, 102 transcript level strongly increased not only after chromatographic bead injection, but also after parasitoid egg injection. Since two types of stimuli that have a very different nature induced a similar effect on the 102 gene, the molecular pathways leading to this effect might be rather unspecific or might be simply triggered by injection.

It should be stressed that, in the haemocytes of parasitized larvae, the amount of the 102 transcripts reached its maximal level at a time-point when also the *TnBV* ncRNA species that are complementary to their 5'UTR were actively transcribed in the same cell type. Based on this finding, it is very unlikely that these *TnBV* ncRNAs exert a negative control on the stability of the 102 transcripts. Therefore, if these *TnBV* ncRNAs play any regulatory role on the 102 transcripts, this must occur at the translation level. In this respect, their mechanism of action would be similar to that reported above for the NOS/anti-NOS transcript pair in the nervous system of *Lymnaea stagnalis*.

The widespread conservation of the 102 protein sequence all along the evolutionary tree, makes the study of this protein particularly interestingly. The finding that a putative *Drosophila melanogaster* homologue is highly expressed in larval hemocytes raises the possibility of a conserved function, at least in insects. The investigation of this issue in this model organism can benefit from the huge amount of molecular and genetic tools it can offer, the availability of the full genome sequence and the possibility to use transgenesis for functional analyses.

In conclusion, this study identified the putative genes present in *TnBV* genome and provided preliminary support to the hypothesis that also its non-coding regions may have

important functional roles.

As already stated in the introduction, polydnaviral genes are of potential interest for the development of safe bioinsecticides useful for crop protection against insect pests. Genetically modified plants expressing immunosuppressive genes of viral origin, making pests more susceptible to other natural pathogens, might be produced, or the genomes of insect pathogens like baculoviruses and *Bacillus thurinogenesis* might be modified by the introduction of polydnaviral genes, to increase their ability to induce mortality in phytophagous insects. This scenario raises a question about the criteria to be used in the choice of polydnavirus genes suitable for either control strategy. Since an ideal bioinsecticide should be harmful only to pest target species and should not affect other living organisms, polydnavirus hypothetical proteins might turn to be good candidates. These proteins might be in fact rather specific; as they were not described in any other biological organism. Non-coding RNAs from polydnavirus are also a very promising tool. In this case, specificity would be guaranteed by the specificity of base pairing with the target transcript.

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