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Bracovirus-derived genes in the genome of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) and their role in host susceptibility to pathogens.

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RESUMEN

SUMMARY

1

Resumen

La asociación entre los himenópteros parasitoides, polydnavirus (PDV) y lepidópteros representa un modelo interesante para estudiar la transferencia horizontal de genes. Está bien documentado que miles de himenópteros parasitoides pertenecientes a las familias Braconidae e Ichneumonidae han domesticado virus simbióticos denominados respectivamente Bracovirus o Ichnovirus. El virus se inyecta junto con los huevos del parásito en el hemocele del lepidóptero huésped, donde se expresan proteínas específicas. Estas proteínas inhiben el sistema inmune del lepidóptero y detienen su desarrollo, lo que beneficia el desarrollo de los huevos y luego las larvas del parasitoide. En este sistema único, tres genomas están continuamente en contacto. Como resultado, este contacto puede facilitar la transferencia de genes entre los tres genomas. En este contexto, el transcriptoma del insecto plaga *Spodoptera exigua* reveló la presencia de un número de unigenes que codifican para proteínas altamente homólogas a proteínas de bracovirus. Estas proteínas son en su mayoría miembros de la familia de lectinas tipo C, excepto uno que codifica una proteína perteneciente a la familia BV2 de *Cotesia congregata* bracovirus, BV2-5. El análisis de las secuencias genómicas de *BV2-5* y *BLL2* (un miembro de las lectinas homólogas a lectinas de bracovirus, BLLs) confirmó el origen vírico de estos genes. Análisis adicionales de las secuencias disponibles en varias bases de datos mostró la presencia de estos genes virales en otras especies de *Spodoptera*. Dicha presencia sugiere eventos de integración antiguos que se produjeron en una especie ancestral de *Spodoptera*.

Estos genes se expresan de forma constitutiva en diferentes tejidos, pero principalmente en los hemocitos, lo que sugiere que tienen un papel en la respuesta inmune del insecto. Sin embargo, uno puede preguntarse si la integración de los genes virales en la línea germinal del lepidóptero es el resultado de una estrategia del sistema virus/parasitoide para manipular el huésped o es el insecto que ha domesticado los genes virales con el fin de mejorar su inmunidad. Para responder a

tal dilema y como parte de esta tesis, nos decidimos ir más allá en la investigación del papel de estos genes en la interacción insecto-patógeno.

En primer lugar, hemos descifrado la función de BV2-5 (Gasmin). Para ello se construyeron varios baculovirus recombinantes expresando distintas formas de esta proteína. Observaciones mediante microscopia confocal demostraron que esta proteína es capaz de interactuar con la actina celular interrumpiendo su organización. Esta interrupción implica una drástica reducción de la multiplicación y la producción de partículas de baculovirus en condiciones de cultivo celular. Este resultado sugiere que el insecto ha domesticado este gen con el fin de hacer frente a uno de sus principales patógeno en la naturaleza: baculovirus. Por otro lado, la interrupción de la organización de la actina resultó en una disminución de la capacidad de los hemocitos para fagocitar las bacterias y, en consecuencia, resulta en un aumento en la susceptibilidad de las larvas a infecciones bacterianas. Por otra parte, se detectó una forma trucada no funcional de Gasmin en las poblaciones europeas de *S. exigua*. Este hecho, así como el papel divergente frente a diferentes patógenos, reflejan una posible adaptación al tipo de patógeno que es más abundante en cada localización geográfica.

A continuación nos propusimos investigar el papel inmunológico de las lectinas homólogas a bracovirus (Se-BLLs) en la respuesta inmune del insecto. De hecho, en el transcriptoma de *S. exigua* se detectó la presencia de ,al menos, treinta y dos unigenes que codifican proteínas de tipo lectinas, la mayoría perteneciente a la familia de lectinas de tipo C. Debido a la gran cantidad de estas proteínas, cuatro lectinas de lepidópteros (Se-LLs), además de la Se-BLLs han sido estudiadas en detalle. El estudio detallado de los dos grupos de lectinas reveló diferencias en distintos aspectos, como son la estructura de las proteínas, su distribución en los tejidos del insecto y su respuesta transcripcional a diferentes patógenos viricos y bacterianos así como endoparasitoides. Entre las lectinas estudiadas, Se-BLL2 respondió a todos los patógenos probados incluyendo la infección por baculovirus, lo que motivó su estudio en detalle a través de su estudio funcional. Para ello se

produjo de manera recombinante dicha proteína, y se comprobó que su administración conjunta con baculovirus disminuía la susceptibilidad de los insectos a baculovirus. Este resultado sugiere, nuevamente, que el insecto ha domesticado este gene de origen bracoviral con el fin de hacer frente a baculovirus.

En resumen, en este estudio hemos descrito la inserción de ADN bracoviral en un genoma de lepidóptero y su posterior domesticación con el objetivo probable de proporcionar protección frente a un patógeno importante como es baculovirus.

Summary

The association between parasitic hymenopteran, polydnavirus (PDVs) and lepidopteran host represent an interesting model to study the horizontal transfer of genes. It is well documented that thousands of hymenopteran parasitoids belonging to the families Braconidae and Ichneumonidae have domesticated symbiotic viruses called respectively Bracovirus or Ichnovirus. The virus is injected together with the parasitoid eggs into the hemocoel of the lepidopteran host, where it expresses specific proteins. These proteins immunocompromise the lepidopteran host and arrest its development making the development of the parasitoid eggs and then larvae successful. In this unique system, three genomes are continuously in contact which can facilitate gene transfer between them. In this context, the transcriptome of the beet armyworm *Spodoptera exigua* revealed the presence of a number of unigenes encoding proteins highly homologous to bracovirus proteins. All of them are members of the C-type family of lectins, except one unigene that codify for a protein belonging to the *Cotesia congregata* bracovirus protein family BV2, with high similarity to the viral protein BV2-5. Genomic sequence analysis of the *BV2-5* and the *BLL2* (a bracovirus-homolog lectin in *S. exigua*), confirmed the bracoviral origin of these genes. Further analysis of the available sequences showed the presence of these genes in other *Spodoptera* species suggesting ancient integration events in an ancestral *Spodoptera* species.

Those genes are sustainably expressed in different tissues, but mainly in hemocytes, which suggests an immune role of them. However, one can wonder if the germ line integration of such viral genes is a strategy of the virus/parasitoid system to manipulate the host or it is the insect who has domesticated the viral genes in order to improve its immunity. In order to answer such dilemma and as part of this thesis, we decided to go further in the investigation of functional role of these genes in the insect-pathogen interaction.

First, we have deciphered the function of the BV2-5 homolog, Gasmin. For that purpose, multiple recombinant baculoviruses, expressing different forms of

Gasmin, where prepared and analysed. Confocal microscopy observations showed that this protein is able to interact with the cellular actin, disrupting its arrangement. This disruption leads to a drastic reduction of the multiplication and the production of baculovirus particles in cell culture experiments. This result suggests gene domestication in order to face such type of pathogen. On another hand, the disruption of actin arrangement resulted in a decrease in the capacity of host hemocytes to phagocytise bacteria, a fact that was traduced with an increasing in larval susceptibility to bacterial infection. Moreover, a non-functional truncated form of Gasmin was detected in European populations. This fact as well as the divergent role in response to different pathogens, reflect possible adaptation to the type of pathogen that is most abundant in each geographic localization.

Next, we have also investigated the immune role of the *S. exigua* bracovirus-like lectins (Se-BLLs) on the insect immune response. Indeed, the insect transcriptome revealed the presence of at least thirty-two unigenes encoding lectin-like proteins, the majority belonging to the family of C-type lectins. Due to the large number of these proteins, four lepidopteran-like lectins (Se-LLs) in addition to the Se-BLLs have been studied in detail. Detailed study of the two groups of lectins revealed differences at different levels, such as protein structure, tissue distribution and the transcriptional response to different viral and bacterial pathogens and endoparasitoids. Among the studied lectins, Se-BLL2 responded to all the tested pathogens including baculovirus infection and its functional role was further investigated through its recombinant expression, purification and functional analysis. The administration of this protein together with the baculovirus to *S. exigua* larvae increased the resistance of the larvae to the baculovirus infection. Again, this result suggests an additional case of gene domestication in order to face baculovirus infection.

In summary, in this study we have described the insertion of bracoviral DNA into a lepidopteran genome and its further domestication probably to provide protection against an important pathogen such as baculovirus.

INTRODUCTION

1. The insect innate immunity, a well-developed immune system

Living creatures are continuously exposed to potential pathogenic microorganisms and eukaryotic parasites. Those pathogens continuously exert selection pressures that affect their hosts genome, physiology and development. Reciprocally, multicellular metazoans have evolved diverse strategies to defend themselves against the invading pathogens.

On the earth, more than 70% of the animal species are insects. The evolution of an effective innate immunity could be the main reason for the prosperity of insects (Tanaka and Yamakawa, 2011). Initial defences in insects include physical barriers that represent the first line of defence; those barriers include the insect cuticle, the integument, the peritrophic matrix of the midgut epithelium and the chitinous linings of the trachea (Lavine and strand, 2002; Wu *et al*., 2010). Although these barriers are effective, numerous invaders can pass them. In such case, the cuticle wounding activates proteolytic cascades leading to localized coagulation and melanisation (Royet, 2004). Once in the hemocoel, the pathogen faces a set of humoral and cellular responses. Humoral responses include the production of antimicrobial peptides, reactive oxygen and nitrogen species, and the activation of enzymatic cascades that regulate coagulation and melanisation of hemolymph (Castro *et al*., 2009; Lavine and Strand, 2002). Cellular response refers to hemocytes-mediated immune responses like phagocytosis, nodulation and encapsulation (Castro *et al*., 2009; Schmidt *et al*., 2010; Tamez-Guerra *et al.,* 2008). Humoral and cellular responses seem to be separated responses, but in fact, many humoral factors affect hemocytes functions and reciprocally hemocytes produce a multitude of humoral molecules (Fig. 1). In order to produce these responses, an efficient recognition system to differentiate between self and non-self is necessary.

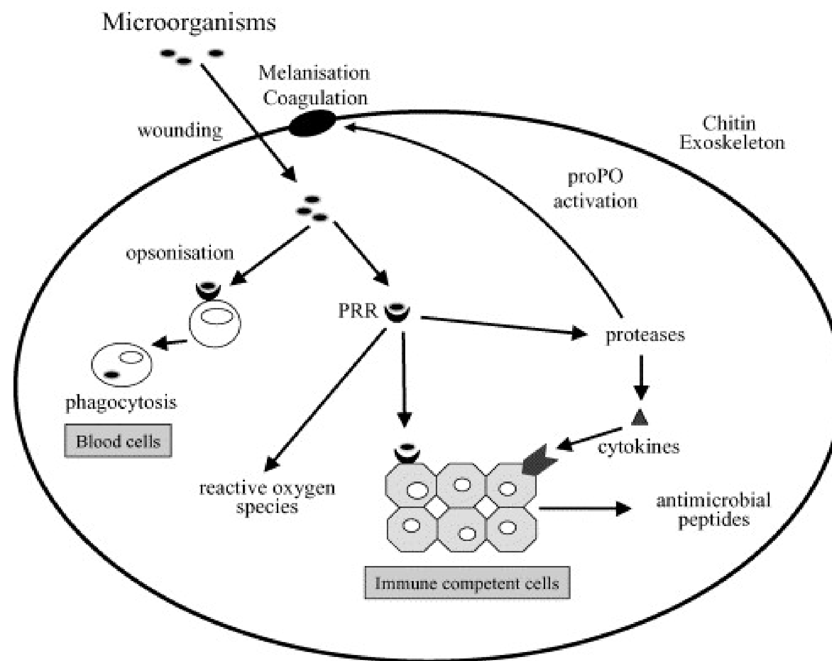


Figure 1. Schematic representation of insect immune response (Royet, 2004).

1.1. Identifying of non-self-organisms through pattern recognition receptors

A key step before the initiating of an immune response is the detection and recognition of the presence of non-self-molecules or organisms. Insects have developed efficient systems to recognize characteristic molecular patterns of microbial walls known as pathogen-associated molecular patterns (PAMPs) (Gillespie *et al*., 1997; Jiravanichpaisal *et al*., 2006). Such PAMPs have been well characterized for bacteria and fungi, but their existence for all other categories of parasites is conjectural (Schmid-Hampel, 2005). Those molecules are highly conserved in each class of pathogens and basically are lipopolysaccharides (LPS), lipoteichoic acid (LTA), peptidoglycans (PGN) and β-1,3-glucan (Wang *et al*., 2012). The recognition of these PAMPs are mediated by peptidoglycan recognition proteins (PRPs) that bind specifically to each class of PAMP molecules (Elftherianos *et al*.,

2007). The best characterized PRPs in insects are the C-type lectins, the peptidoglycan recognition proteins (PGRPs), the β-1,3-glucan recognition proteins (βGRPs), the hemolin and the integrin.

1.1.1.C-type lectins

Lectins are among the most diversified and studied family of PRPs. They are sugar binding proteins bearing one or more sugar binding site(s) and able to agglutinate cells and/or precipitate glucoconjugates (Malagoli *et al*., 2010). The increasing number of studies focusing on insects' lectins shows that these proteins are as abundant and diverse as it is in vertebrate genomes (Wang *et al*., 2012). As examples, *Drosophila melanonogaster*, *Aedes gambiae* and *Bombyx mori* genomes contain more than 30, 20 and 15 genes encoding C-type lectins, respectively. In lepidopteran species, lectins have been shown to be abundant in the different tissues and body fluids (Mosson *et al*., 2000).

In 1988, Drickamer suggested to organize animal lectins into several groups and classified Ca^{2+}-dependent lectins structurally similar to the asialoglycoprotein receptor as C-type lectin group. These lectins are the most diverse family of animal lectins and the best characterized lectin family in insects. C-type lectins are separated into 14 subgroups based on their domain architecture; such grouping correlated well with the results of phylogenetic analysis and functional similarities. In lepidopterans, C-type lectins are involved in various developmental events including regeneration, moulting, metamorphosis and embryogenesis. They have also been shown to be implicated in diverse immune responses such as phagocytosis, nodulation, encapsulation, hemolymph coagulation and phenoloxidase cascade (Liu *et al*., 2009).

To date, the best characterized and studied lepidopteran C-type lectins are those of *Manduca sexta*, *Helicoverpa armigera* and *Bombyx mori*. Four C-type lectins were purified from the plasma of *M. sexta* and named immunolectins (IML). All of them function as pattern recognition receptors involved in the prophenoloxidase

activation (Yu *et al*., 2002). Moreover, IML-2 has been shown to have a crucial role in the clearance of Gram-negative bacteria from the insect's hemolymph in the early phase of infection. It binds to both bacterial LPS and insect's hemocytes which suggest that IML-2 enhances phagocytosis and/or nodule formation (Yu and Kanost, 2003). Eight C-type lectins have been isolated from *H. armigera* hemolymph (Ha-CTL 1 to 8) (Wang *et al*., 2012). Chai et al. (2008) isolated the Ha-lectin which was induced in hemocytes in response to Gram-positive and Gram-negative bacteria, yeast and also in response to *H. armigera* nucleopolyhedrovirus HaSNPV, suggesting an antiviral role of this lectin. The immune role of C-type lectins in lepidopterans was also confirmed for *B. mori* lectins. A *B. mori* lipopolysaccharide-binding protein recognizes specifically the lipid A portion of LPS and play a key role in the clearance of bacteria from the hemolymph (Koizumi *et al*., 1997). Furthermore, the *B. mori* multibinding protein Bm-MBP binds with high affinity to bacteria that were easily trapped within nodules when they were injected to the hemolymph of larvae (Watanabae *et al*., 2006).

Lepidopteran C-type lectins seem to have key role in the recognition of different kinds of pathogens. They can also recognize the insects' hemocytes and enhance hemocyte-related responses. Some studies have shown that they respond rapidly to wounding (Chai *et al.,* 2008; Wang *et al*., 2012) which implicate that they respond in the early phase of infections. The diversity of these proteins in the lepidopteran genomes and their wide range of response make them an important subject for study in order to better understand the mechanisms of the innate immunity in insects, especially in the absence of antibody-mediated immunity.

1.1.2.Peptidoglycan recognition proteins (PGRPs)

An essential component of the bacterial cell wall consists of a rigid layer of peptidoglycan (PGN), that is either exposed in association with lipoteichoic acid on the surface in the case of Gram-positive bacteria, or overlaid by another lipopolysaccharide layer in case of Gram-negative bacteria (Royet, 2004). Although

PGN's structure is variable from one bacterium to another, it is a big polymer of a repeating disaccharide of N-acetylglucosamine and N-acetylmuramic acid cross-linked by short peptide bridges (Lemaitre and Hoffmann, 2007; Yu *et al*., 2002). The peptidoglycan contain diamino acids necessary for the formation of the peptide bridges; in general Gram-positive bacteria contain diaminopimelic acid (DAP-type PGN) whereas Gram-negative bacteria contain lysine (Lys-type PGN) (Ferldhaar and Gross, 2008). Bacterial PGNs are elicitors of the insect's immune system. They were shown to induce the activation of different signal pathways. The Lys type PGN was found to be a potent inducer of the Toll pathway, whereas the DAP-type PGN induces the Imd pathway (Royet, 2004). These pathways are induced after the recognition of bacterial PGN mediated by small extracellular proteins named peptidoglycan recognition proteins (PGRPs). The first PGRP was detected in the hemolymph of the lepidopteran *B. mori* (Yochida *et al*., 1996). Orthologous genes were then characterized and cloned from other animals suggesting that PGRPs form a conserved family of proteins in animals (Royer, 2004). Insects PGRPs bind specifically to and hydrolyse bacterial PGN, activate the Toll and Imd signalling pathways or proteolytic cascades generating antibacterial effectors and stimulate phagocytes (Vogel *et al*., 2011). In lepidopteran insects, it has been shown that the recognition of PGNs by *M. sexta* and *B. mori* PGRPs induce the expression of antibacterial peptides and activate the protease cascade leading to phenoloxidase activation (Yu *et al*., 2002).

1.1.3. β-1,3-glucan and Gram-negative binding proteins (βGRP/GNBP)

βGRPs and GNRPs constitute an insect's protein family implicated basically in the recognition of fungus and Gram-negative bacteria. βGRPs recognize β-1,3-glucans, key structure components of fungal cell walls. GNBPs have two domains that bind gram-negative bacterial walls, and also some fungal and Gram-positive cell walls. The carboxyl-terminal domain of both βGRPs and GNBPs shares similarities with fungal β-1,3-glucanases, bacterial glucanases and/or LPS, recognizes them

through protein-protein interaction and initiates the prophenoloxidase activation system (Royet, 2004; Yu *et al*., 2002). The first GNBP was identified in the hemolymph of immunochallenged silkworm *B. mori* (Lee *et al*., 1996). In Lepidoptera, most GNBPs are produced in the fat body and secreted into the caterpillar's hemolymph and are up-regulated when challenged with gram-negative bacteria (Kim *et al*., 2000; Ma and Kanost, 2000). *B. mori* and *M. sexta* βGRPs have a restricted and a high affinity for β-1,3-glucans (Yu *et al*., 2002).

1.1.4. Hemolin and integrin

A hemolymph protein named hemolin is thought to have an important immune role in lepidopteran species. Hemolin is a 47-48 KDa protein composed of repeated immunoglobulin domains. This protein is commonly found in cell adhesion proteins of vertebrates and invertebrates including lepidopteran species but no orthologs have been found for *Drosophila* (Gillepsie and Kanost, 1997; Jiang *et al*., 2010; Yu *et al*., 2002). Several evidences suggest that hemolin have antibacterial and antiviral functions (Eleftherianos *et al.,* 2007; Terenius, 2008). In fact, hemolin can recognize glycolipids, lipopolysaccharides and lipoteichoic acid of bacterial cell walls. *M. sexta* hemolin has been shown to bind both *Escherichia coli* and the insect's hemocytes enhancing the phagocytosis of bacteria (Eleftherianos *et al*., 2007). It is also involved in the response to baculovirus infection (Terenius, 2008).

Another type of recognition proteins are the integrins which are surface proteins widely expressed in Metazoans (Giancotti, 2003). Those proteins recognise and bind a specific domain on the surface of foreign organisms and then initiate immune responses (Takada *et al.,* 2007).

1.2. Signalling pathways

The recognition of foreign invaders leads to the activation of different immune signalling pathways. They have been studied in detail in *Drosophila* species and are the Toll, Imd, JAK-STAT and RNA interference pathways. Even though each

pathway is activated basically by a class of pathogens, recent studies show that they collectively participate in the pathogens killing, phagocytosis, production of oxygen reactive species and the melanisation cascades (Merkling and van Rij, 2013).

1.2.1. Toll pathway

In insects, the Toll pathway was first discovered to be implicated in the specification of dorso-ventral polarity during embryonic development in *Drosophila* (Rajiv *et al*., 2002). Afterwards, it has been shown that this pathway is the major signalling pathway to induce immune reaction against Gram-positive bacteria, yeast and fungi (Feldhaar and Gross, 2008). The detection of Gram-positive bacterial peptidoglycans and fungal β-glucans by the corresponding specific recognition activate serine protease (SP) cascades in the insect's hemolymph. These receptors reactions initiate an extracellular signalling cascade leading to the activation of the Toll mature ligand, Spätzle. Spätzle is synthesized as an inactive precursor, pro-Spätzle. It is secreted in the hemolymph and its activation requires a proteolytic processing to form the active Toll ligand (Valanne *et al*., 2011). The Spätzle (Spz) activation is illustrated in detail in Figure 2.

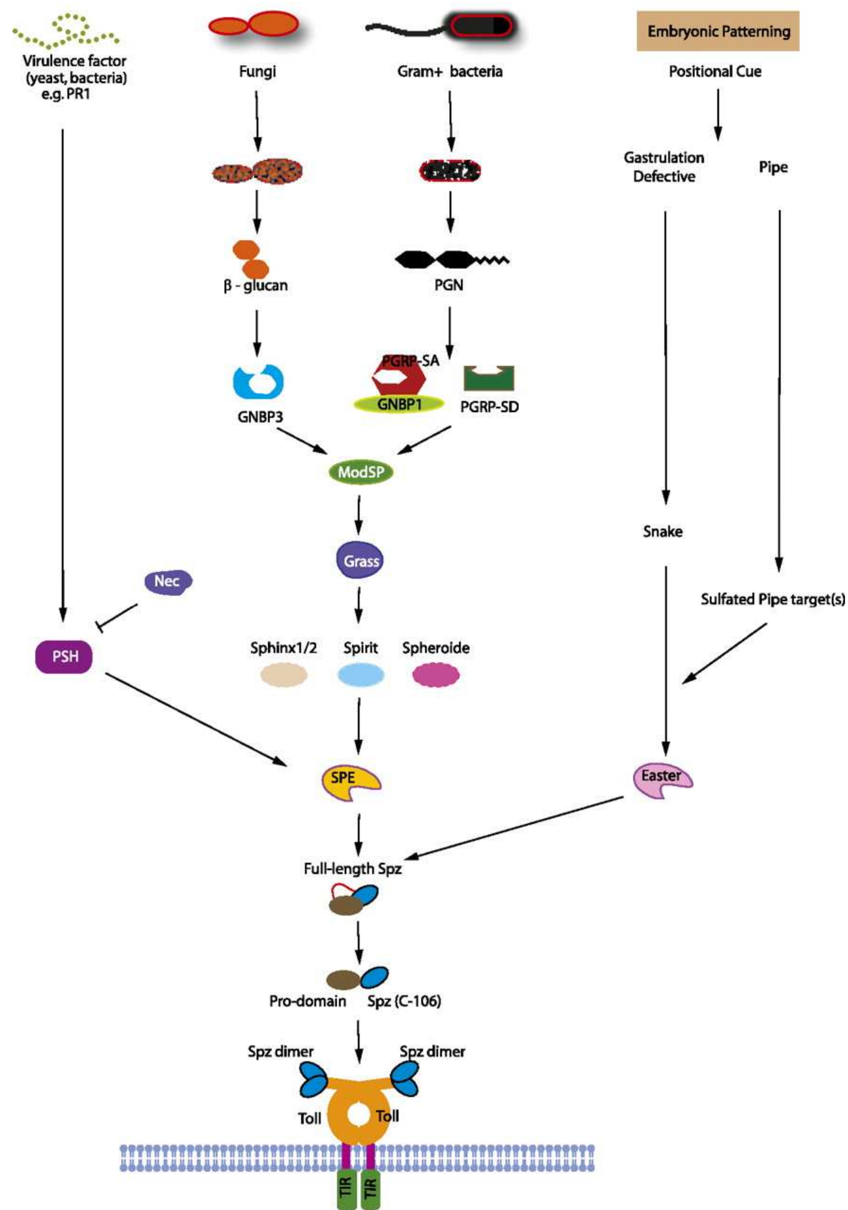


Figure 2. Activation of the Toll pathway mediating enzymatic cascades in the response to different stimuli (Vallane *et al*., 2011).

The mature form of Spätzle binds to the Toll receptor, which recruits the Tube/Myd88 complex followed by the activation of a protein kinase (Pelle). This

activation triggers an intracellular cascade involving different factors and resulting in the activation of the transactivator proteins belonging to the NF-kB family, Dorsal and Dif. The translocation of these proteins to the nucleus induce the transcription of the genes encoding antibacterial and/or antifungal peptides (Fig. 3) (Feldhaar and Gross, 2008; Welchman *et al.*, 2009; Jiang *et al.*, 2010).

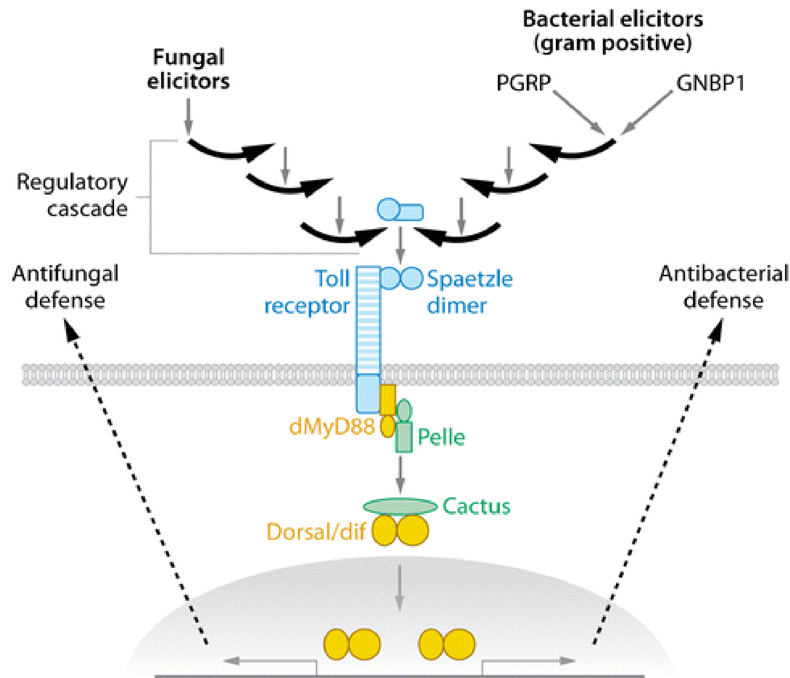


Figure 3. Schematic representation of microbial pathogens' recognition by the *Drosophila* Toll receptor (Scmidt *et al.*, 2010).

1.2.2. Immune deficiency (Imd) pathway

The Imd pathway was first described by the identification of a mutation named immune deficiency in *Drosophila* that impaired the expression of several antibacterial genes (Hoffman, 2003). Homologous of the *Drosophila imd* gene have been detected in the *S. exigua* transcriptome and in other lepidopteran species

(Pascual *et al.,* 2012). This pathway is primarily involved in the defence against Gram-negative bacteria and induces antibacterial genes via Relish, another NF-kB-like factor. Binding of the bacterial DAP-type peptidoglycan to the receptor PGRP-LC results on the recruitment of the intracellular Imd, and as a result, the activation of Relish after cleavage of its inhibitory IkB domain. Then, the Rel domain translocates into the nucleus and induces the transcription of antibacterial peptides and proteins (Merkling and van Rij, 2012) (Fig. 4).

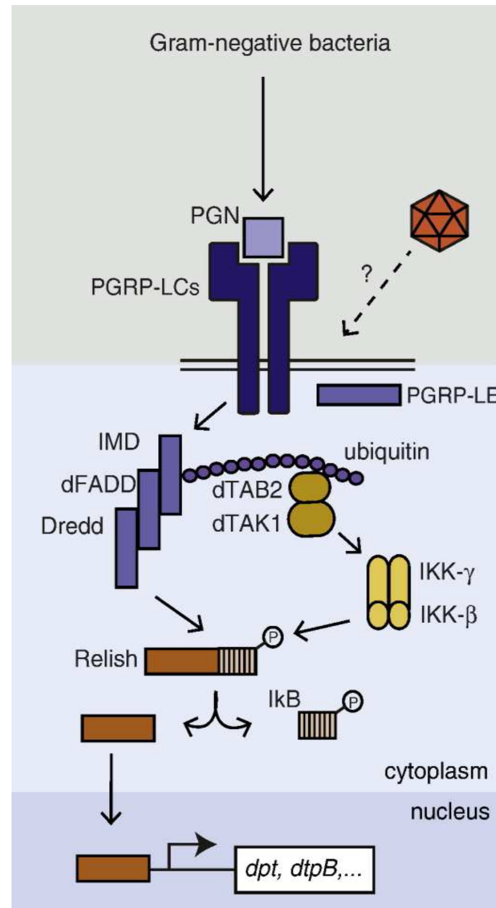


Figure 4. Imd signalling pathway activation in response to Gram-negative bacteria (Kingsolver *et al.,* 2013).

1.2.3. JAK-STAT pathway

A third major immune pathway named JAK-STAT was initially identified through its role in *Drosophila* development and hemocytes proliferation. Afterwards, it has been shown that it is implicated in antibacterial and antiviral responses (Merkling and van Rij, 2013; Kingsolver *et al.,* 2013). This pathway has three main cellular components: the receptor Domeless, the Janus kinase JAK and the STAT transcription factor (Lemaitre and Hoffmann, 2007).

Bacterial or viral infections induce the production of cytokine unpaired-3 (UPD3), a ligand of Domeless and Janus Kinase, by hemocytes. Upon binding of the UPD, the receptor Domeless dimerizes, allowing the transphosphorylation of the Hopscotch (hop) kinases. As a result, recruitment and transphosphorylation of the conserved tyrosine residue in the STAT transcription factor occurs. The phosphorylated STATs dimerize and are transported to the nucleus to regulate the expression of effector genes (Kingsolver *et al.,* 2013) (Fig. 5). In addition to this well described pathway, a link between the JAK-STAT and RNAi pathways through the Dicer-2 signalling pathway has been proposed (Kingsolver *et al.,* 2013).

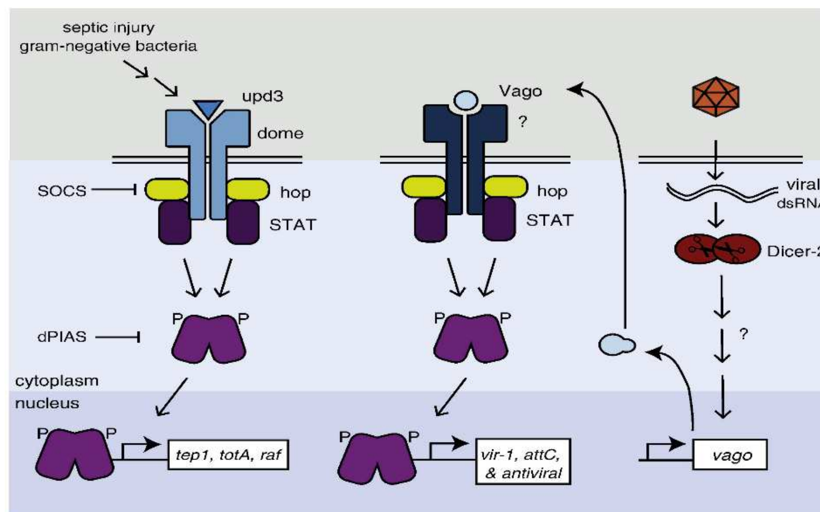


Figure 5. The JAK/STAT pathway (Kingsolver *et al.*, 2013).

1.2.4. RNA pathways: antiviral response

In response to viral infection, three different RNA pathways have been identified: the small interfering RNA (siRNA), the micro RNA (mi-RNA) and the piwi-interacting pathway (pi-RNA).

In the literature, the most documented pathway is the RNA interference (RNAi) pathway. This mechanism has been identified as a potent antiviral response in plants, and then was found to play a crucial role in the immune response of *Drosophila* as an essential antiviral response. Afterwards, numerous studies have defined the RNAi pathway as the most effective insect response when challenged with viruses. RNAi pathway in insects uses virus-generated ds-RNA to produce siRNAs targeting viral RNA for its degradation and then inhibit the virus replication (Kingsolver *et al.,* 2013). Recognition of this ds-RNA by dicer proteins, members of RNases III family of ribonucleases, results into the pre RNA-induced silencing complex (RISC). Unwinding of the complex occurs and defines target specificity based on complementarities and the targeted RNA will be degraded through the RNase activity of Argonaute (AGO) enzymes (Fig. 6) (Fullaondo and Lee, 2012; Kingsolver *et al*., 2013). Inhibition or suppression of the antiviral RNAi leads to effective propagation of the virus within the host.

The mi-RNA pathway is based on the use of non-coding RNAs of 22 nucleotides that are produced by all animals, plants and some viruses and their primary function consists on the regulation of gene expression at post-transcriptional level. Binding of mi-RNA to the target may inhibit the gene expression by either degradation of mRNA or suppression of the translation (Asgari, 2011). Recent studies have demonstrated that these mi-RNAs are involved in a variety of pathways and biological processes in animals such as development, immunity, viral infection and fat metabolism (Fig. 6) (Asgari, 2011; Fullaondo and Lee, 2012). In insects, an increasing number of mi-RNA are being identified. Insect mi-RNA may inhibit proliferation of microorganisms by targeting virulence genes (Asgari, 2011).

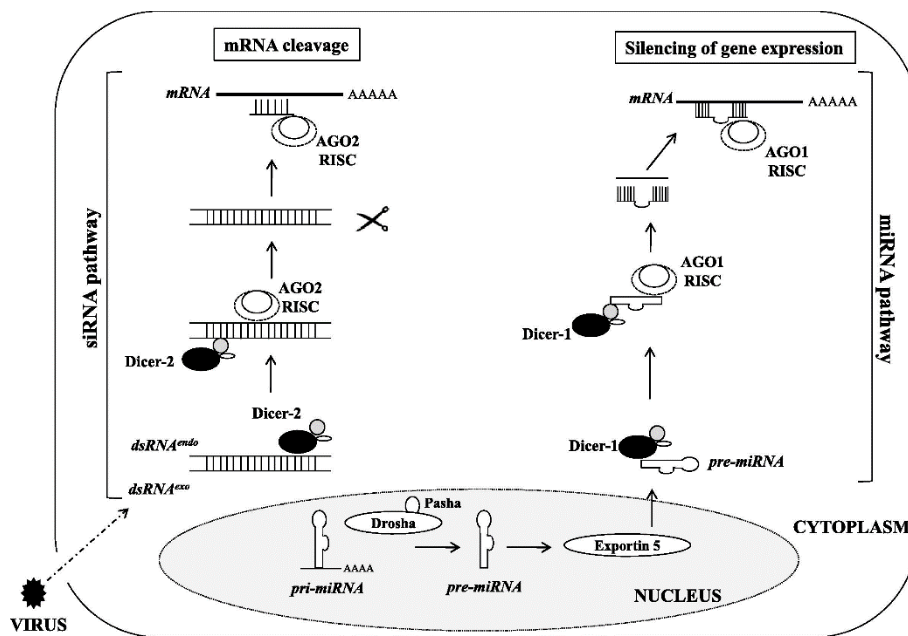


Figure 6. RNA interference pathway in *Drosophila*. (Fullaondo and Lee, 2012).

Even though the role of the pi-RNAs remains unsolved, it has been suggested that they may be involved in regulation of the antiviral response. pi-RNAs are transcribed from the cellular genome in the epigenetic control of genomic elements in the germline and their presence generally precede the si-RNA response (Kingsolver *et al.,* 2013).

1.3. Humoral defence

Insects contain open hemocoelic cavity in which the different organs are embedded. The fat body is the largest organ in insect's body cavity and is the major immune-responsive organ where the synthesis of different immune proteins takes place. Those proteins include the antimicrobial peptides and proteins, as well as proteins implicated in the different enzymatic cascades leading to melanisation and hemolymph coagulation.

1.3.1. Antimicrobial peptides (AMPs)

Microbial infections induce the expression of antimicrobial peptides essentially by the fat body and the hemolymph, which are secreted into the hemolymph. Some of these proteins are produced constitutively and others are synthesised only after microbial challenge. In general, AMPs can be divided into three groups according to their basic structures: α-helical peptides such as cecropins and moricins, cysteine containing proteins such as defensins, and linear peptides with abundance of particular amino acids such as prolin, glycine or tryptophan (Brown et al., 2009). They are usually small cationic peptides (< 10KDa) with an overall positive charge and exhibit action against a broad range of bacteria and/or fungi.

Insect cecropins are small cationic peptides composed of 35-39 amino acids that are almost exclusively restricted to the Lepidoptera and Diptera orders of insects. They have strongly basic N-terminal parts and long hydrophobic C-terminal stretches. These proteins act against Gram-negative and Gram-positive bacteria, but they are most effective against Gram-positive bacteria. They kill bacteria by forming ion channels in the bacterial membrane (Choi *et al*., 2000; Otvos, 2000; Tanaka and Yamakawa, 2011). Moricin is another highly basic α-helical peptide consisting of around 40 amino acid residues. It shows antibacterial activity against several Gram-positive and negative bacteria by attacking their membranes (Tanaka and Yamakawa, 2011).

Among cysteine rich peptides, defensins appear to be the most widely spread group of inducible AMPs in insects. They contain a cysteine-conserved motif that confers to these peptides high stability and resistance to proteases. Defensins act effectively on Gram-positive bacteria; they are able to destroy most Gram-positive bacterial cells by forming channels in the plasma membrane which leads to cell lysis (Choi *et al*., 2000; Lasz Otvos, 2000).

AMPs also act on Gram-negative bacteria. As an example, gloverins which are glycine-rich peptides, act actively against Gram-negative bacteria (Vogel *et al*.,

2011). Attacin, another glycine-rich peptide of about 20 KDa, acts against Gram-negative bacteria by inhibiting the production of the bacterial outer membrane protein (Vogel *et al*., 2011).

In lepidopteran species, transcriptome studies revealed the abundance of AMPs in response to microbial challenge. In *Bombyx mori*, six different families have been identified, among them cecropin, lebocin, gloverin, attacin and defensin (Tanaka and Yamakawa, 2011). Analyses of *Spodoptera exigua* transcriptome have shown the presence of a large number of AMPs belonging to the major families found in Lepidoptera such as cecropin, attacin, gloverin, diapausin, lobocin, moricin and cobatoxin (Pascual *et al*., 2012).

1.3.2. Reactive oxygen species

The production of antimicrobial peptides is known as the systemic immune response. This response involves also the production of oxygen and nitrogen reactive species. The production of reactive oxygen species (ROS) with potential cytotoxic properties is a key immune reaction in invertebrates. ROS are constitutively produced in the gut and induced in response to bacteria. The gut lumen is an environment hostile to bacteria and is continuously exposed to microbial infections. The induction of ROS in the gut is indispensable to keep the intestinal microbiota growth under control (Charroux and Royet, 2010). The production of ROS has been shown to be induced in association with encapsulation and nodule formation.

1.3.3. Phenoloxidase cascade

An efficient system of the insects immune response is the prophenoloxidase (proPO) system which consists of several proteins involved in the majority of the innate immune system reactions such as melanisation, cytotoxic reaction, cell adhesion, phagocytosis and encapsulation (Jiravanichpaisal *et al*., 2006). The proPO is an inactive precursor activated in response to microbial infection via proteolysis

by serine-protease activating enzymes. The serine cascades activate the proPO-activating proteinase (proPAP) to phenoloxidase-activating proteinase (PAP) which hydrolyses the proPO into PO. This activated form of the enzyme oxidises tyrosine to dihydrophenylalanine and then to quinones that are involved in different immune reactions (Fig. 7).

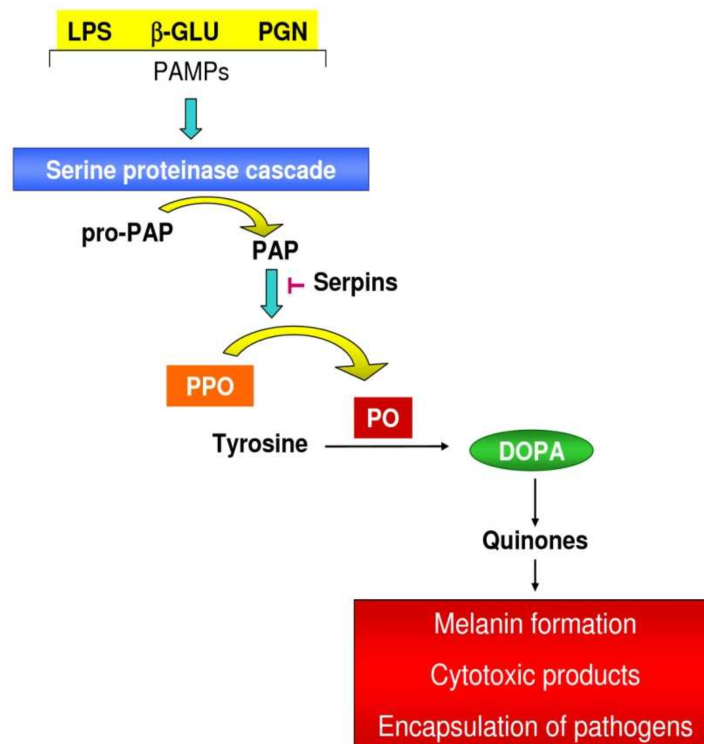


Figure 7. Prophenoloxidase activation occurs in response to the recognition of pathogens by pathogen-associated molecular pattern (PAMPs) (Garcia *et al.*, 2009).

1.3.4. Hemolymph clotting

Immediately after wounding and even before the activation of other immune reactions, hemolymph coagulation leading to clotting at the site of injury takes place. This serves to seal the wound, to prevent liquid losses and to immobilize the microbes at the wound site and contributes to the limitation of the infection. In

lepidopteran species, the clotting system consists of four steps. The primary step called soft clot consists on the degranulation of hemocytes which lead to the establishment of extracellular aggregates that seal the wound. In the second step, the activation of the proPO cascade and the transglutaminase activation take place cross linking the clot and forming the hard clot. Next, the plasmatocytes will be attracted and spread across the clot to seal it off from the hemocoel. Finally, epidermis regeneration take place cross the wound site (Jiravanichpaisal *et al*., 2006). Proteomic studies have identified several proteins involved in clotting. They include hemolectin, the humoral pro-coagulant lipophorin, hexamerin and its receptor fondue involved in initial clot formation and transaglutaminase providing the connection between bacterial surfaces and clot matrix (Fig. 8) (Feldhaar and Gross, 2008). The clotting process is very important to rapidly limit fluid loss in the soft-bodies larvae, but it may not occur in adults with hard cuticle (Royet, 2004).

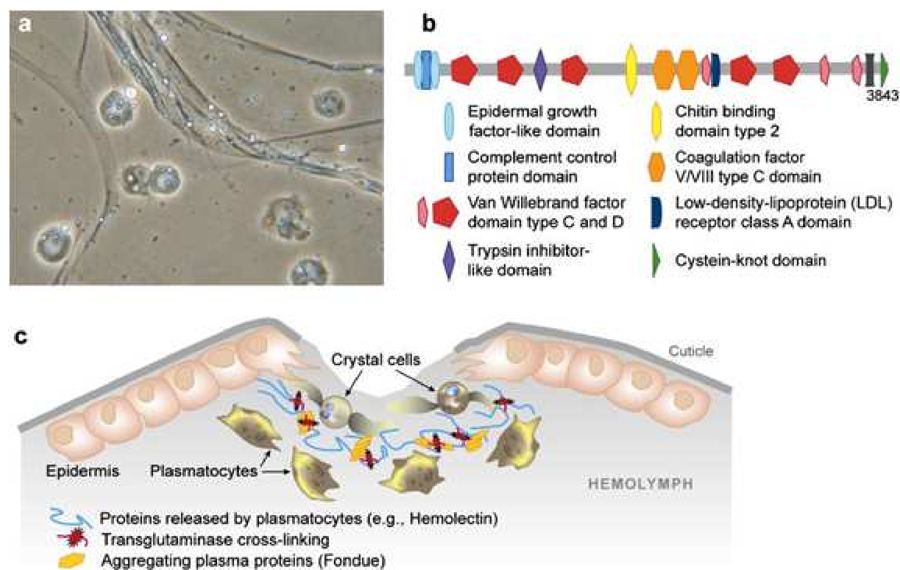


Figure 8. Clotting reaction in *Drosophila*. (a) A *Drosophila* clot with fibers and incorporated plasmatocytes. (b) Structure of Hemolectin. (c) A model for clot formation at an injury site (Lemaitre and Hoffmann, 2007).

1.3.5. Melanization

Melanzation is an immediate immune response in arthropods that leads to the physical encapsulation of pathogens in a dense melanin coat and generates toxic metabolites able to harm some pathogens (Yassine *et al.*, 2012). This response is triggered by PO catalyzing the oxidation of phenols (dopamine) to orthoquinones (dopaminoquinone) which then polymerize to melanin (Fig. 9). Melanization participate in wound healing and in encapsulation and nodulation in some lepidopteran and dipteran species.

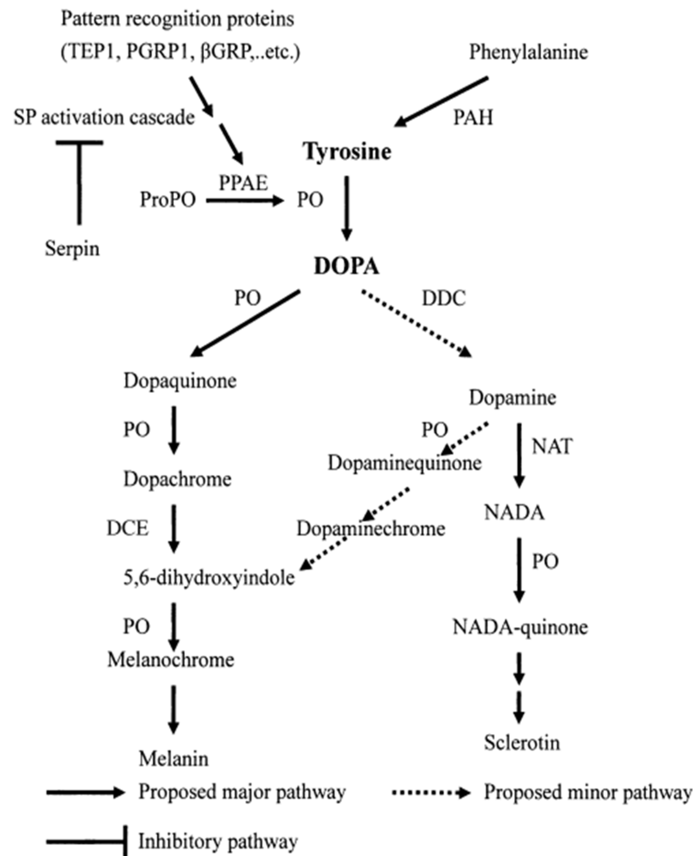


Figure 9. Proposed melanisation pathway in mosquitoes (Huang *et al.*, 2005).

1.4.Cellular responses

Cellular responses refer to hemocytes-mediated immune responses such as phagocytosis, nodulation and encapsulation. Hemocytes circulate in insect hemolymph and they derive from stem cells that differentiate into different lineages. In insects, at least seven types of hemocytes have been described: prohemocytes, granular cells (granulocytes), coagulocytes, crystal cells, spherules cells (spherulocytes), oenocytoids and thrombocytoids. The majority of insects do not dispose of all types of hemocytes. The most common types of hemocytes described in insects are granulocytes, plasmatocytes, spherulocytes and oenocytoids. Granulocytes have a small nucleus and their name is due to the abundance of granules in their cytoplasm. Plasmatocytes are usually larger than granulocytes with a leaf-like shape and their cytoplasm contain lysozymes. Spherulocytes have a variety of differing shapes and contain numerous small spherical inclusions. Finally, oenocytoids are large, bi-nucleate non-phagocytic cells (Fig. 10) (Browne *et al.*, 2013).

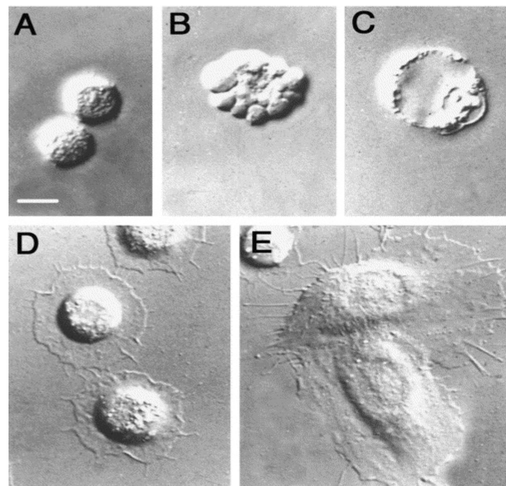


Figure 10. Nomarski images of the five types of hemocytes found in lepidopterans. (A) Prohemocytes, (B) spherule cell, (C) oenocytoid, (D) granular cells, and (E) plasmatocytes. Scale bar = 50 μm (Lavine and Strand, 2002).

The main function of hemocytes depends on the developmental stage of the insect. In embryos or pupae, they are involved in the removal of apoptotic degenerated tissues, while in larvae their major role is the uptake and destruction of invading microbes (Feldhaar and Gross, 2008). They were also described to participate in the clotting process and then the wound healing. At the larval stage of lepidopteran species, granulocytes and plasmatocytes represent more than 50% of the circulating hemocytes and they are the only hemocytes able to adhere to foreign bodies (Lavine and Strand, 2000; Levin *et al.,* 2005). The other hemocytes described in Lepidoptera are spherule cells and oenocytoids, both non phagocytic-cells. Spherule cells have been suggested to transport circulatory metabolites, while oenocytoids contain cytoplasmic phenoloxidase precursors (Jiravanichpaisal *et al.,* 2006; Lavine and Strand, 2002).

1.4.1. Phagocytosis

Phagocytosis is a highly conserved immune response in all Metazoan species and some Protozoa. It is a complex process allowing rapid engulfment of small particles like bacteria, yeast and apoptotic cells. Phagocytosis is a multiple step process beginning with attachment and recognition of non-self and implicating signal transduction, activation of pseudopodium formation, assembly of phagosomes and target ingestion via an actin-polymerization dependent mechanism (Gillepsie and Kanost, 1997; Lavine and Strand, 2002). In Lepidoptera, phagocytosis is achieved mainly by circulating granulocytes and plasmatocytes. Those hemocytes can recognize directly foreign objects by means of surface receptors such as calrecticulin and apolipophorin (Zhu and Zhang, 2013). In addition, some PRPs, such as lectins or hemolin have the ability to bind to the hemocytes after binding to the pathogen surface. Such binding induces the phagocytosis. As a final step of phagocytosis, the pathogen is destroyed by low pH, hydrolysis and radicals (Zhu and Zhang, 2013).

1.4.2. Nodulation

Phagocytosis is ideal to entrap small number of infecting microbes, whereas large number of bacteria leads to hemocytes aggregation and then nodule formation. Nodulation occurs when multiple hemocytes bind to clusters of bacteria allowing large numbers of bacteria to be cleared from the insect hemolymph (Browne *et al*., 2013). Those nodules are often melanized forming a melanin coat around the aggregation of hemocytes and bacteria. This response also generates quinones and ROS that may help to kill the entrapped bacteria (Jiang *et al*., 2010).

1.4.3. Encapsulation

When the target is too big to be phagocytized such as parasitoids or nematodes, it is cleared by encapsulation. During this immune response, the distribution of granulocytes and plasmatocytes in the hemolymph appear to be random in some lepidopteran species, while in others, granular cells are the first hemocytes to adhere to the pathogen and, in a second step, plasmatocytes attach (Lavine and Strand, 2002). Encapsulation requires a dramatic change in the adhesiveness of plasmatocytes. They transform from non-roughly spheroid cells freely circulating in the hemolymph to attached cells extending numerous filopodia (Levin *et al*., 2005). Such as nodulation, melanization through the PO polymerization and production of free radicals are associated with the formation of capsules.

1.5. Immune responses in the midgut

When the ingested pathogens arrive to the gut, they are in most cases detected and eliminated without harming the commensal and mutualistic microflora. The direct contact between pathogens and the gut epithelium results in the induction of AMP expression and ROS production (Fig. 11) (Wu *et al.,* 2010).

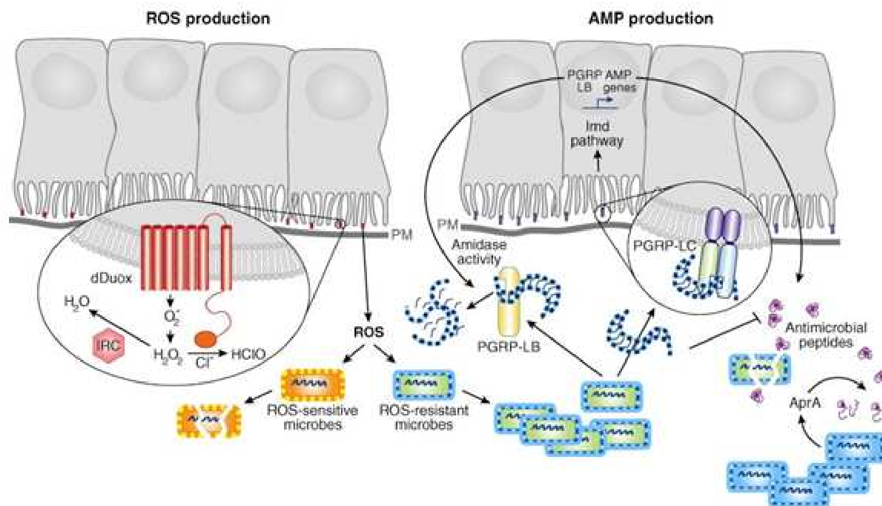


Figure 11. Gut immune response. Local production of ROS (left) and AMPs (right) provides two inducible defense mechanisms in the gut (Lemaitre and Hoffmann, 2007).

Recently, the systemic immune response in the epithelial tissues of *Drosophila* is being well studied and documented. It has been shown that in *Drosophila* gut two complementary effector mechanisms control bacterial infection, generation of reactive oxygen species (ROS) and local production of antimicrobial peptides (AMPs) (Buchon *et al*., 2009).

In general, AMPs expression is under the control of Toll and Imd signaling pathways, however in the midgut it depends only on the Imd pathway (Charroux and Royet, 2010). When an excesss of bacteria is introduced into the gut, AMP expression is induced upon the activation of Imd signaling cascades. This activation is afterwards regulated by the silencing of Imd pathway in order to maintain the equilibrium of the insect's commensal bacteria (Bae *et al*., 2010). In the silkworm *B. mori*, cecropin transcription is induced in the epithelial cells underlying the cuticle in the presence of bacteria. In addition, AMP levels in the silkworm gut changes dramatically with the different stages of development especially when the insect gut began to slough off; the same occurs in the gut of *M. sexta* (Lemaitre and Hoffman,

2007; Wu *et al.,* 2010).The production of reactive oxygen species is another element of the systemic immune response that is well described in insect's guts. ROS are continuously produced at a basal level in the gut by the membrane associated dual oxidase (DUOX), a member of the adenine dinucleotide phosphate (NADPH) oxidase family (Buchon *et al.*, 2009). Interaction between commensal microbiota and the epithelial cells triggers the modulation of DUOX enzymatic activity. When the level of present bacteria increases or new infectious bacteria came along with ingested food, the DUOX activity increases inducing ROS level (Charroux and Royet, 2010). Buchon and collaborators (2009) correlated the increasing of ROS with the renewal of gut epithelium cells in *Drosophila*. Naturally, the gut epithelium is constantly renewed by the division and differenciation of intestinal stem cells (Welchman *et al*., 2009). This renewal is accelerated in the case of bacterial infection in order to overcome the damage caused either by bacteria or by the protective ROS response (Buchon *et al*., 2009; Welchman *et al.,* 2009).

2. Bacteria, virus and parasitoid wasps in biological control of insect pests

The increasing human needs for food has generated great expansion of the area dedicated to agriculture. Thus, pest management has become necessary to protect the crops. During decades, application of chemical pesticides has been the best solution for pest control in agriculture. This intensive application has been shown to be harmful to other animals, plants and humans. Moreover, it has led to an increase resistance of insects to these pesticides. Therefore, the search for new alternatives has intensified leading to the discovery of a wide range of microorganisms and parasitoids that can be possible candidates for the development of biological insecticides replacing the chemical ones.

2.1. Entompathogenic bacteria: *Bacillus thuringiensis*

Entomopathogenic bacteria used as biopesticides are mainly based on the gram-positive bacteria *Bacillus thuringiensis*, even though commercial products from other bacterial species are also available.

Bacillus thuringiensis is a facultative anaerobic Gram-positive bacterium belonging to the *Bacillus cereus* group (Soberón *et al*., 2009). It is a spore-forming bacterium that can be isolated from different environments such as soil, the rhizosphere, the phylloplane, fresh water, grain dusts and from insects, crustaceans, annelids and insectivorous mammals (Raymond *et al*., 2010). The bacterium was first isolated in 1901 by the Japanese biologist Shegitane Ishiwatori and its insecticidal properties were discovered afterwards when dead flour moth caterpillars were found to be loaded with spores and crystals (Sanahuja *et al.,* 2011). *Bacillus thuringiensis* is well known for its ability to produce parasporal crystalline protein inclusions that are toxic to a wide range of insects and are named δ-endotoxins basically composed of one or more crystal proteins (Cry) and cytotoxic toxins (Cyt) (Bravo *et al*., 2007; Vachon *et al*., 2012). In addition to these proteins found in parasporal inclusions, during its vegetative growth the bacterium secrete other type of insecticidal proteins named vegetative insecticidal proteins (Vip) (Vachon *et al.*, 2012). *Bacillus thuringiensis* toxins are very effective and highly specific to their target insects, are innocuous for other animals, plants and humans and are completely biodegradable which make them an important alternative for pests control (Soberón *et al*., 2009). Indeed since the 1960s, a large number of successful formulations of *B. thuringiensis* toxins, such as Dipel, has been commercialized (Federici, 2005) (Fig. 12).

Figure 12. Field trials with Dipel, one of the first successful *B. thuringiensis* products used against forest pests, showing the difference between treated and non-treated trees (Federici, 2005).

In addition to the formulation of Cry proteins, these toxins have been used in the engineering of transgenic plants (Fig. 13). As a result, *B. thuringiensis* toxins applications have been successfully achieved in the control of defoliator pests, in the control of vector mosquitoes of human diseases and in the development of transgenic insect-resistant plants (Bravo *et al., 2007*).

Figure 13. *Bt* corn (right) that produces Cry1Ab to control the European corn borer, *O. nubilalis*, compared to conventional corn (Federici, 2005).

2.2. Entomopathogenic virus

Recent advances in molecular biology have greatly advanced the identification and characterization of insect viruses. These studies have revealed that insects, mostly in the orders Diptera, Hemynoptera, Coleoptera and Lepidoptera, are hosts of a wide range of viruses (Ibarra and del Rincon., 2011). Some cause extensive mortality and others are less harmful to their hosts. These viruses can be occluded or non-occluded, DNA or RNA viruses, and can replicate in the nucleus or in the cytoplasm of cells. They belong to different families that infect only invertebrates such as Ascoviridae, Baculoviridae and Polydnaviridae, or families that contain viruses infecting both invertebrates and vertebrates such as Poxviridae, Iridoviridae, Parvoviridae, and Reoviridae. These viruses can be good alternatives in the biological control of important pests, though the studies carried out so far are still limited. Among the insect viruses, baculovirus and polydnavirus are the best studied.

2.2.1. Baculoviridae

Baculoviruses are important insect pathogens used successfully in the microbial control of pests in agriculture and forestry (Cheng and Lynn, 2009). They usually have high degree of specificity and are restricted to the species from where they were isolated and closely related species (Ikeda *et al*., 2013; Szewczyk *et al.,* 2006). The majority of the members of Baculoviridae are isolated from the order Lepidoptera, but some were also isolated from the orders Diptera, Hymenoptera and Coleoptera (Harrison and Pophan, 2008, Szewczyk *et al*., 2006). In general, baculoviruses are large ds-DNA (90-240 Kb), with enveloped, rod-shaped viral particles that are occluded either within polyhedral or granular occlusion bodies (Cheng and Lynn, 2009, Ibarra and Del-Rincon Castro, 2011). These viruses are divided into two genera, Nucleopolyhedrovirus (NPVs) and Granulovirus (GVs), depending on their occlusion bodies. NPVs produce cubical polyhedra containing many occluded virions, while GVs produce occlusions containing a single virion (Szewczyk *et al.,* 2006). NPVs are also divided into single NPVs with only one virion per envelope (SNPVs) and multiple NPVs with several virions per envelope (MNPVs) (Fig. 14) (Ibarra and Del-Rincon Castro, 2011).

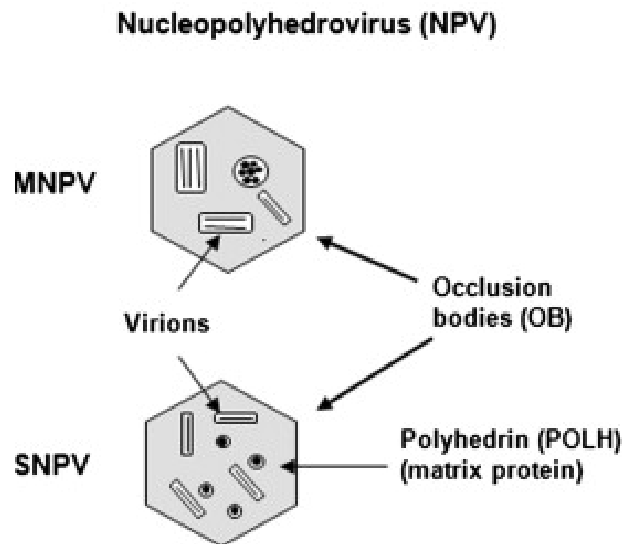


Figure 14. Representative scheme of baculovirus occlusion bodies (OBs) that consist of a polyhedrin matrix in which occlusion derived virions (ODV) are embedded. The ODVs contain single (SNPV) or multiple (MNPV) nucleocapsids (van Oers, 2011).

Baculoviruses have efficient replication in the insect host and are able to resist outside during long periods by forming two morphotypes, budded virus (BVs) and occluded virus or occlusion derived virus (ODVs) (Inceoglu *et al.*, 2001). BVs bud from the cell membrane and spread the infection from cell-to-cell in an infected host and ODVs are involved in virus transmission among insect larvae (Au *et al.*, 2013; van Oers, 2011). Viral infection of baculovirus starts when a larva ingests virion-containing polyhedra that are present on the vegetation resulting from a previous infection cycle (Cheng and Lynn, 2009). Following ingestion, the polyhedral matrixes rapidly degrade in the alkaline environment (pH 9-11) of the insect midgut and the virions are released. During the early stage of the infection and only within 15 h post infection, the cell releases hundreds of BVs. The progeny viruses bud then through the basal lamina to form BVs in other tissue cells, or are transmitted via the tracheal system to other parts of the fat body (Cheng and Lynn, 2009, van Oers, 2011). By 24

hours post infection, progeny production switches to the formation of occluded virions which accumulate within the infected cell nucleus. In the case of NPV-infected larvae, a critical accumulation of virions occurs generally by 5 days post infection, resulting in the insect death. In GV-infected larvae, this period is generally extended to 7-14 days (Fig. 15) (van Oers, 2011).

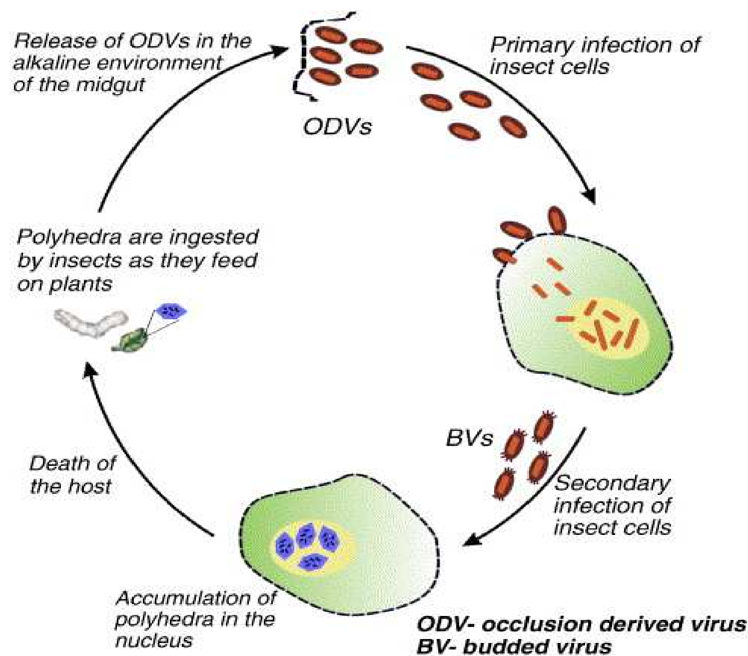


Figure 15. Life cycle of baculovirus (Szewczy *et al*., 2006).

The first baculovirus-based insecticide, Eclar™, was produced by Sandoz Inc. in 1975. It was a preparation of *Heliothis zea* baculovirus (HzSNPV) which is a relatively broad range baculovirus infecting many species belonging to the genera *Heliothis* and *Helicoverpa*. In 1982, the production of this pesticide was stopped, but the occurrence of resistance to many chemical insecticides revived the interest in HzSNPV and the same virus was registered under the name GemStar™ (Szewezy *et*

al., 2009). From then on, the generated baculovirus-based pesticides increases continuously and currently they exceed 50 formulations (Szewczyk *et al.,* 2006). Among these, *C. pomonella* granulovirus CpGV is the active ingredient of at least 5 products commercially used for protection of orchards in Europe against the codling moth, Carpovirusine™ in France, Madex™ and Granupon™ in Switzerland, Grawal™ in Germany and Virin-CyAP in Russia. In addition, at least 2 commercial products based on *Spodoptera* baculovirus are available to protect cotton, corn and tomates, SPOD-X based on *S. exigua* NPV and Spodopterin™ based on *S. littoralis* NPV (Szewezy *et al*., 2009).

2.2.2. Polydnaviridae

During the 1960s, George Salt determined that some endoparasitoids eggs gained protection as they passed through the calyx region of the female wasp reproductive tract. From then on, further studies have proved that the calyx region contains DNA-containing particles responsible for the eggs protection (Vinson, 1972; Vinson and Scott, 1975). Later on, it has been confirmed that these particles are viruses that were classified into a new family named Polydnaviridae. The Polydnaviridae family was formally recognized as a virus family in 1995 and to date approximately 40000 wasp species belonging to two families named Braconidae and Ichneumonidae are known to carry polydnavirus (PDVs) (Strand and Bruke, 2013). PDVs are then divided respectively into two genera, Bracovirus (BVs) and Ichnovirus (IVs). The association between these parasitic wasps and their hosts is unique since it is the only case where an eukaryotic organism use a virus to manipulate the physiology of another eukaryote (Drezen *et al*., 2003). PDVs are also unique because of their segmented genome composed of multiple double stranded DNA circles (Drezen *et al.*, 2003). PDVs genome segments are integrated as proviral DNA in the germ line and somatic cells of their associated hymenopteran hosts and are vertically transmitted from parents to their offspring (Strand and Bruke, 2012). Even though PDVs DNA are found in all individual wasps including males, replication only occurs

in pupal and adult stage female in the nuclei of specialized calyx cells located in the ovary (Drezen *et al*., 2003; Strand and Bruke, 2013; Webb et al., 2006). IVs and BVs particle replication occurs within the nuclei, but both types of particles differ in many ways (Drezen *et al*., 2003) (Fig. 16). In the case of BVs, the nuclei enlarge greatly to occupy most of the cell volume at the end of the virus replication. Calyx cells lyse then to release single enveloped virions that accumulate in the lumen of the reproductive tract where eggs are stored (Drezen et al., 2003, Webb *et al*., 2006). In contrast, IVs particles acquire a second membrane and are released continuously through a budding process that does not damage the cell (Drezen *et al*., 2003, Kroemer and Webb, 2004).

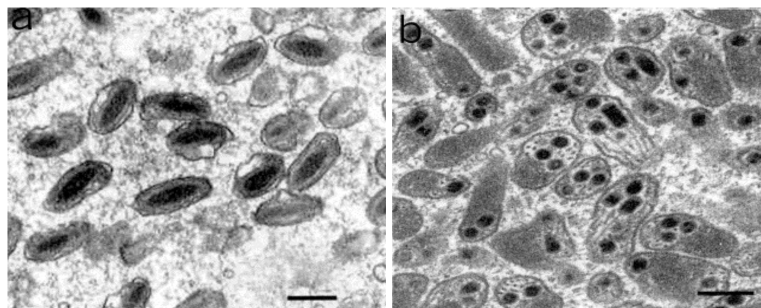


Figure 16. Transmission electron micrographs of polydnavirus virions. a) Ichnovirus virions; b) Bracovirus virions (Federici and Bigot, 2003).

Virions accumulate in high densities in the lumen of the reproductive tract along with the wasp eggs until they are injected into the lepidopteran host (Strand and Bruke, 2013). PDVs do not replicate into the lepidopteran host but virions rapidly infect and discharge their DNAs into the nuclei of host cells which is followed by the early expression of virus-encoded genes (Webb *et al*., 2006). PDVs expressed genes immunocompromise the parasitized host favoring the survival of the parasitoid egg and larvae. They also alter the host metabolism and growth which

promotes the wasp larva development and ultimately cause the host to die (Strand and Bruke, 2012). The PDV life cycle is summerized in Figure 17.

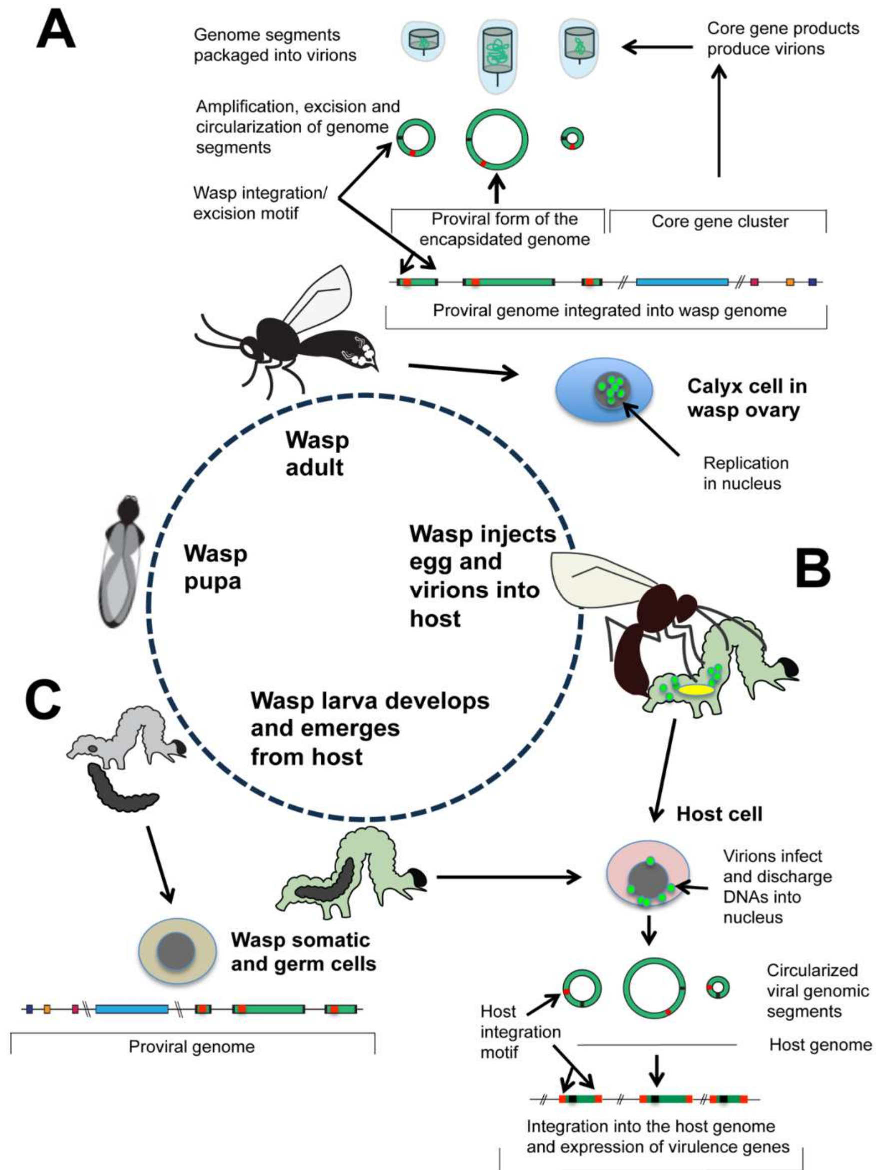


Figure 17. Life cycle and genome organization of BVs (Strand and Bruke, 2012).

2.4. Parasitic wasps

Parasitoids occur in several orders of insects (Diptera, Coleoptera, Lepidoptera, Trichoptera, Neuroptera, Strepsitera), but they are especially common in the Hymenoptera order. Recent estimates suggest that 10 to 20% of all insects might be parasitic wasps (Pennacchio and Strand, 2006). Parasitoids are generally divided into idiobionts and koinobionts. Idiobionts are either endoparasitoids that develop in non-growing host stages such as eggs or pupae or ectoparasitoids that inject permanently paralyzing venom into the host. In contrast, koinobionts attack hosts that continue to feed and grow after parasitism (Harvey, 2005; Pennacchio and Strand, 2006).

The successful parasitism usually depends on gene products that the adult wasp injects at oviposition or that offspring produce during the course of development (Beckage and Gelman, 2004). Among these factors virus and virus-like particles described in many parasitoid wasps have a crucial role in the disruption of the lepidopteran host immunity and development, Polydnavirus associated with some families of endoparasitoid wasps are a good example. In several host-wasp systems, the viral transcripts are observed in the host hemolymph just few hours after oviposition, while in others this transcription can be delayed for several days or occur throughout the course of development of the parasitoid (Beckage and Gelman, 2004). In all these cases, the expression of the viral proteins interferes with the host immunity and development. Venom is another product injected into the host at the time of the oviposition. Venom has been defined as a toxic fluid that produces sudden death or paralysis in the host or prey. It can be also defined as secretions delivered through a wound that interferes with normal physiological processes to facilitate feeding or defense by the animal that produces the venom (Asgari and Rivers, 2011). In this context, parasitoid venoms consist on complex mixtures of components involved in host manipulation (Asgari and Rivers, 2011). The venom of ectoparasitoids contains paralytic factors and endocrine disrupters that immobilize the host and program its development to favor that of the parasitoid

(Asgari and Rivers, 2011; Pennacchio and Strand, 2006). Most of the components of endoparasitoids venoms affect the host immune functions and delay or arrest its development (Asgari and Rivers, 2011). In addition to their venom, some endoparasitoids inject into their hosts hemocytes specialized cells named tetratocytes originated from the cells that surround the parasitoid embryo during its development. They are believed to absorb nutrients from the host hemolymph in favor of the parasitoid feeding. They also interfere with the host immunity and development (Beckage and Gelman, 2004).

In conclusion, the parasitoid can inject one or different products in order to disrupt the host immunity and development. Such disruption ensure the protection of the wasp eggs and then the development of the larvae either inside or on the surface of the host. Those associated viral particles, cells, chemical compounds and proteins present important subjects of study in the aim to develop new pesticides or to improve the existing ones.

OBJETIVOS

OBJECTIVES

OBJETIVOS

En la presente tesis, nos hemos planteado determinar y estudiar una serie de genes del lepidóptero *Spodotera exigua* con elevada homología a genes de bracovirus. Nuestro objetivo principal fue investigar el origen de estos genes así como su implicación en la respuesta inmune del insecto. Este trabajo se ha realizado a través del abordaje de los siguientes objetivos.

1. **Caracterización genética de los genes de *Spodoptera* derivados de bracovirus**
    - 1.1. Identificación y análisis filogenética de los genes relacionados con bracovirus y expresados en *Spodoptera spp*.
    - 1.2. Determinación de las secuencias genómicas de algunos de estos genes con el fin de obtener evidencias de la integración de los genes viricos en el genoma del insecto.
2. **Estudio del papel de BV2-5 en el sistema inmune del insecto**
    - 2.1. Localización celular de BV2-5 y estudio de su posible interacción con los componentes celulares.
    - 2.2. Análisis del efecto de BV2-5 en la susceptibilidad a patógenos de insectos, tanto viricos como bacterianos.
3. **Estudio del papel de las lectinas derivadas de bracovirus en el sistema inmune del insecto**
    - 3.1. Identificación, análisis filogenético y determinación del patrón de expresión de lectinas de tipo C en *S. exigua*.
    - 3.2. Análisis de la función de una lectina de origen virico en la susceptibilidad de *S. exigua* a patógenos viricos.

OBJECTIVES

In the present thesis, we have planned to determine and study bracovirus (BV)-derived genes found in the beet armyworm *Spodoptera exigua* genome. We aimed to investigate the origin of these genes and their implication in the insect's immune response. This work has been performed through the accomplishment of the following objectives.

1. **Genetic characterization of *Spodoptera* BV-derived genes**

   1.1. Identification and phylogenetic analysis of BV-related genes expressed in *Spodoptera spp.*

   1.2. To determine the genomic sequences of some BV-derived genes in order to obtain evidences of the horizontal gene transfer and genomic integration of these viral genes in the genome of Lepidoptera.

2. **Study of the role of BV2-5 on the insect's immune system**

   2.1. Cellular localization of BV2-5 and analysis of its possible interaction with cellular components.

   2.2. Analyses of the role of BV2-5 on the insect susceptibility to viral and bacterial pathogens.

3. **Study of the role of BV-derived lectins on the insect's immune system**

   3.1. Identification and phylogenetic analysis and expression pattern of C-type lectins from *S. exigua*.

   3.2. Analyses of the role of a BV-homolog lectin in the susceptibility of *S. exigua* to viral pathogens.

CHAPTER 1

Gene transfer of bracoviral DNA into the germ line of the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) genome

Results from this chapter are included in:

Gasmi, L., Boulain, H., Gautier, J., Hua-Van, A., Musset, K., Jakubowska, A. K., Huguet, E., Herrero, S. and Drezen, J.M. Recurrent domestication by Lepidoptera of genes from their parasites mediated by bracoviruses. Submitted to "Plos Genetics".

1.1.Introduction

Several thousands of parasitic wasps take advantage of the virus family *Polydnaviridae* to successfully colonize their lepidopteran hosts. This association is a unique example of virus domestication by an eukaryotic organism in order to manipulate the physiology of another organism. Indeed, polydnaviruses (PDVs) are the only symbiotic viruses associated to insects (Drezen *et al*., 2003). They get their name from their polydisperse genomes organized as series of different circular DNAs (Glatz *et al*., 2004). These viruses replicate only in specific calyx cells within the wasp female reproductive tract (Gundersen-Rindal and Daugherty, 2000). PDVs are divided into two groups, Ichnovirus (IVs) and Bracovirus (BVs). The IVs, associated with species belonging to Ichneumonidae, are characterized by lenticular nucleocapsids surrounded by two membranes and are released in the ovary lumen by budding from the female calyx cells. Whereas, the nucleocapsids of BVs, found in Braconidae, are localized within a protein matrix surrounded by a single unit envelope and are released during the lysis of the calyx cells (Burke and Strand., 2012; Cui and Webb., 1997; Gruber *et al*., 1996).

PDV DNA is integrated into the parasitoid wasp genome and uses the wasp machinery to replicate (Cui and Webb, 1997). Once injected together to the wasp eggs into the host, polydnavirus infect the host tissues and express specific proteins involved in the suppression of the insect immunity, but no virus replication occurs in lepidopteran tissues (Strand and Burke, 2012; Strand and Burke, 2013). Therefore, PDVs were described as gene transfer agents used by parasitoid wasp to facilitate the development of their progeny in the body cavity of the lepidopteran host (Dupuy *et al*., 2011).

Since PDVs do not replicate inside the lepidopteran hosts, the viral genome or at least, a part of this genome, has to persist long enough in the host infected cells. The persistence of sufficient viral particles is required to express enough protein able to induce developmental and immune alterations. In this regard, there was recently suggested that the viral segments may undergo integration into the

lepidopteran genome DNA. *In vitro* and *in vivo* experiments aiming to confirm such hypothesis were the subject of various studies. It was shown that DNA from the braconid *Glyptapanteles indiensis (GiPDV)* was permanently integrated and maintained for generations into the genome of the lepidopteran *Lymantaria dispar* cells that survived from the viral infection (Gundersen-Rindal and Dougherty, 2000; Gundersen-Rindal and Lynn, 2003). Integration of viral DNA into genomic DNA of lepidopteran cells was also shown in the case of the lepidopteran host *Choristoneura fumiferana* derived cells infected with *Tranosema rostrale* ichnovirus (TrIV) (Doucet *et al*., 2007). Another study showed that 15 genomic segments of the *Microplitis demoliter* bracovirus (*MdBV*) persisted for a long-term in infected CiE1 cells derived from the lepidopteran host *Pseudoplusia includens*. The same study demonstrated for the first time that polydnavirus DNA is able to integrate into the genome of parasitized larvae (Beck *et al*., 2011).

Here we report evidence of recurrent horizontal transfers taking place in a unique system: symbiotic virus, endoparasitoid wasp and Lepidoptera. To prove such gene transfer, we have investigated seven genes highly homologous to *Cotesia congregata* bracovirus and *Cotesia vestalis* bracovirus found in the transcriptome of the lepidopteran *S. exigua*. After showing germ line integration of some of these viral sequences into the insect's genome we have analysed the influence of those genes in the insect susceptibility to the viral pathogen, baculovirus.

1.2.Materials and methods

1.2.1.Insect rearing

Four different colonies of *Spodoptera exigua*, derived from different geographic locations, were continuously reared on artificial diet at 25± 3°C with 70±5% relative humidity and a photoperiod of 16h light: 8h dark. The FRA strain was kindly supplied by M. López-Ferber, INRA (St.Christol les Alés, France) (Hernández-Martínez *et al.*, 2008). The ALM strain was established from successive collections from southern Spain (Hernández-Martínez *et al.*, 2010). The XEN-R strain

was established from insects collected from cotton fields in Pattville, AL. (USA) and was later selected for resistance to *Bacillus thuringiensis* (Hernández-Martínez *et al.*, 2010; Park *et al.*, 2014). The SUI population was kindly provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland). DNA and insects from a Mexican population were kindly provided by P. Caballero (Universidad Publica de Navarra), than the population was maintained in the laboratory conditions. Finally, DNA representing *S. exigua* from Japan was obtained from the *S. exigua* cell line Se301 that was originally derived from insects from Japan (Hara *et al.*, 1995).

1.2.2.Phylogenetic analysis

The putative bracovirus-like proteins were determined with the EditSeq program from the DNASTAR and their homologs in other insect species were obtained using BLASTp at NCBI site (http: //www.ncbi.nlm.nhi.gov/). The predicted amino acid sequences were aligned using the ClustalX software (Thompson *et al.*, 1997) and visualized in GenDoc program (Nicholas *et al.*, 1997). The evolutionary distance was calculated for the aligned sequences by the Maximum-likelihood method and phylogenetic trees were performed with the MEGA5 program (Tamura *et al.*, 2011). Reliability of an inferred tree was determined using the bootstrap test (1000 replicates).

1.2.3.*BV2-5* and *Se-BLL2* genomic sequence

A universal genome walking kit (Clontech, Maintain View, CA) was used to obtain the genomic sequences of *BV2-5* and *Se-BLL2* and their flanking regions. For this purpose, three DNA libraries from *S.exigua* genomic DNA (Hernandez-Rodríguez *et al.*, 2009) were subjected to primary and secondary PCRs using the general primers provided by the kit and specific primers designed to amplify 5' and 3' flanking regions of the *BV2-5* and *Se-BLL2* open reading frames (ORFs) (Table 1.1). The amplified fragments were purified, cloned into the pGEM-T easy vector (Promega) and sequenced. Sequences were assembled using the Seqman program.

Table 1.1. Primers designed in order to determine *BV2-5* and *Se-BLL2* sequences.

Primer name	Sequence (5´-3´)	Application
5p-BV2-5	TTCATGCTCCTCTGCTTGTATCCTCG	Genome walking (*BV2-5*)
5s-BV2-5	GCAACGTTAGTATGGTAATAGGCAACA	Genome walking (*BV2-5*)
3p-BV2-5	CTTATTTGTCCGTTAGGTTTGGTGTGCTTG	Genome walking (*BV2-5*)
3s- BV2-5	GGTGATGGTGATGGTGATACTGGAATTGG	Genome walking (*BV2-5*)
5p-Se-BLL2	CTGGGCTTCATCAAATGTTGCAGCAT	Genome walking (*BLL2*)
5s-Se-BLL2	GTTCTGAGGTCCTGGATTCGGGTAGCTT	Genome walking (*BLL2*)
3p-Se-BLL2	CAAATCAGTCTTGCCGTTTTTGGCTTGA	Genome walking (*BLL2*)
3s-Se-BLL2	GATTTTCAGAGTAGCCACAGAGTGTTAAG	Genome walking (*BLL2*)
BV2-5ORF-F	CGTGCGAGTTGAAAGCATAG	*BV2-5* amplification in different populations
BV2-5ORF-R	TCCACTGAGTTCCGCTTTTAG	*BV2-5* amplification in different populations

1.2.4.DNA sequencing of *BV2-5* alleles from different *S. exigua* populations

Initial *BV2-5* sequences were obtained from the transcriptome of *S. exigua* larvae exposed to different types of pathogens (Pascual *et al.*, 2012). Primers flanking the coding domain sequence were designed (Table 1.1) and used to amplify cDNA from different populations. Total RNA was extracted from the samples using the RNAzol reagent (Molecular Research Centre, INC) according to the manufacturer's protocol. Purified RNA was subjected to DNAse (Invitrogen) treatment in order to remove any contaminant DNA and then used for cDNA synthesis using PrimeScript RT reagent kit from Takara Bio Inc (Otsu Shiga, Japan) following the manufacturer´s protocol. PCR-amplified alleles of *BV2-5* were directly sequenced or cloned into the pGEM-T Easy vector (Promega) and sequenced using standard primers. At least two independent clones were sequenced for each insect population. Resulting sequences were assembled using the Seqman program from the DNASTAR package (DNA STAR, Madison, WI).

1.2.5.RNA extraction and quantitative PCR

The presence and abundance of mRNA for the *BV2-5* and the *lectin* genes in different larval tissues were analyzed by quantitative reverse transcription PCR (RT-qPCR). Total RNA from fat body, midgut and hemocytes were isolated from 5th instar larvae using RNAzol reagent (Molecular research centre, INC) as described in the manufacturer's protocol. One µg of total RNA was reverse-transcribed into cDNA with oligo (dT) primer using Super ScriptTM II reverse transcriptase (Invitrogen). Resulting cDNAs were used to determine the level of transcripts for each gene by quantitative PCR (qPCR). qPCR was carried out using an ABI Prism 7700 thermocycler from Applied Biosystems. SYBR green Ex Taq™ master mix (Clontech) was used in the qPCR reactions in a total volume of 20 µL. Specific primers for each gene were designed by Primer Express Software (Applied Biosystems) (Table 1.2). Expression enrichment in the analyzed tissues was observed by estimation of the expression level in relation to the average expression level from the three studied tissues. Data are normalized to the internal control gene, ATP synthase and presented as fold change using the $2^{-\Delta\Delta Ct}$ method and using the GraphPad Prism program (GraphPad software Inc., San Diego, CA, USA).

In order to check the effect of PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 27 mM KCl; pH 7.0) injection in gene expression, *S. exigua* FRA fourth instar larvae were intrahaemocoelical injected with PBS. Larvae without any treatment has been considered as control larvae. Height hours post injection (hpi), hemocytes, midgut and fat body from treated and non-treated larvae were collected. Finally, and in order to study the effect of parasitism in genes expression, early fourth instar larvae has been stung individually by the ichnomonid *Hyposoter didymator*. Larvae were then incubated in the conditions described above for 24 hours or 72 hours. At these point times, hemocytes, midgut and fat body were extracted from parasitized and non-parasitized larvae. RNA and cDNA was extracted and produced such as described previously. Differences in the response compared to the control samples were detected by quantitative PCR. Data are presented as described previously.

Three independent replicates were performed and statistical analyses were performed by Dunnet test using the GraphPad Prism program (GraphPad software Inc., San Diego, CA, USA).

Table 1.2. Primes used to realize the qPCR.

Gene name	Forward primer	Reverse primer
Se_BV2-5	CGTGCGAGTTGAAAGCATAG	TCCACTGAGTTCCGCTTTTAG
Se-BLL1	ACAACCCAGCAACCCAACA	CCATGGCTCCTTGTAGCAACA
Se-BLL2	TCCAGGACCTCAGAACACAGAA	GGGCTTCATCAAATGTTGCA
Se-BLL3	GTGGAAATCTTGCCGTCGTT	TCCACAGAGCCAGCATTTCA
Se-BLL4	GGAAGTGGGCTATGGCAAAC	GGTTGTACCCCCATCCAG
Se-BLL5	AATGCTGGCCCTGTGGAA	GCTTGAGCATTCAATCCATGTG
Se-BLL6	ACAGCCAAAGTGGGCAATG	CGTTGCGACTCCAGTTAAGGT

1.2.6.Recombinant baculovirus expressing *BV2-5*

The full ORF of the two main allelic forms of *BV2-5* (complete and truncated) were amplified by PCR from cDNA obtained from *S. exigua* FRA and XEN-R larvae, respectively. They were cloned into pFBD-pH downstream the p10 promoter to generate pFBD-pH-BV2-5 (for the complete form) and pFBD-pH-BV2-5t (for the truncated form). pFBD-pH refers to the dual vector pFBD (Clontech) containing AcMNPV polyhedrin gene downstream the ph promoter. In order to generate recombinant baculoviruses, *Escherichia coli* strain DH10Bac, which contains the AcMNPV ΔCC bacmid (Kaba *et al.*, 2004) and the pMON7124 helper plasmid (Luckow *et al.*, 1993), was transformed with pFBD-pH-BV2-5, pFBD-pH-BV2-5t, or pFBD-pH plasmids according to the standard procedure described for the Bac-to-Bac system (Invitrogen, Carlsbad, CA). Recombinant bacmids were selected based on white-blue screening of DH10Bac colonies and the positive clones were confirmed by PCR. Bacmid DNAs were isolated from bacterial cells according to the standard procedure and used to transfect *Spodoptera frugiperda* ovary-derived cell line Sf21 using Insect

Gene Juice Transfection Reagent (Novagen, Madison, WI, USA). Four to six days post transfection, the recombinant ΔCC-pH, ΔCC-pH-BV2-5 and ΔCC-pH-BV2-5t bacmid-derived viruses were collected and multiplied to produce high-titer stocks for further experiments.

1.2.7.Effect of Se-BLL2 on susceptibility to baculovirus

Recombinant Se-BLL2 was expressed and produced in *Escherichia coli* expression system and purified with affinity chromatography using the HiTrap™ Chelating HP column (GE Healthcare). *S. exigua* (SUI) third instar larvae were infected with SeMNPV by the drop-feeding method. OBs ($5x10^5$) were added to a solution containing sucrose and phenol red colorant (10% and 0.05%, respectively) in presence or absence of purified Se-BLL2 (0. 15mg/mL). The larvae were allowed to drink from the virus and control solution in Petri dishes and then transferred individually to the assay plates. Mortality was then recorded every 12 h until death or pupation of all the larvae. Sixteen larvae were used for each treatment and three independent replicates were performed. Mortality was expressed as the percentage of dead larvae. The time to death was assessed by comparing the mortality curves using the Kaplan Meier method (GraphPad Prism 5). The statistical significance was determined using the log-rank analysis (Mantel-cox test).

1.2.8. Effect of BV2-5 expression on baculovirus multiplication

Effect of BV2-5 on baculovirus multiplication in cell culture was determined by one-step growth curve assay. Sf21 cells were infected with the different recombinant baculoviruses at a multiplicity of infection (MOI) of 2. After infection, cells were washed and incubated in fresh medium. At different time points, an aliquot of the medium was harvested and the viral titer (amount of budded viruses) in each sample was determined by qPCR. For that purpose, viral DNAs were extracted using Prepman reagent (Applied Biosystems) following the manufacturer

protocol and were quantified by comparing the obtained Ct values against a standard curve of known viral concentration. Three independent replicates were performed for each sample.

In a second experiment, about 10^6 Sf21 cells were infected with each type of virus in T75 flasks at MOI 2. Four days post infection, infected cells were collected from each flask, pelleted and treated with 1% SDS for 5 min to liberate the occlusion bodies (OBs). OBs were pelleted by centrifugation and washed twice with water and counted using a Neubauer chamber. Three independent replicates were performed and values for each sample were compared by Dunnett's multiple comparison test.

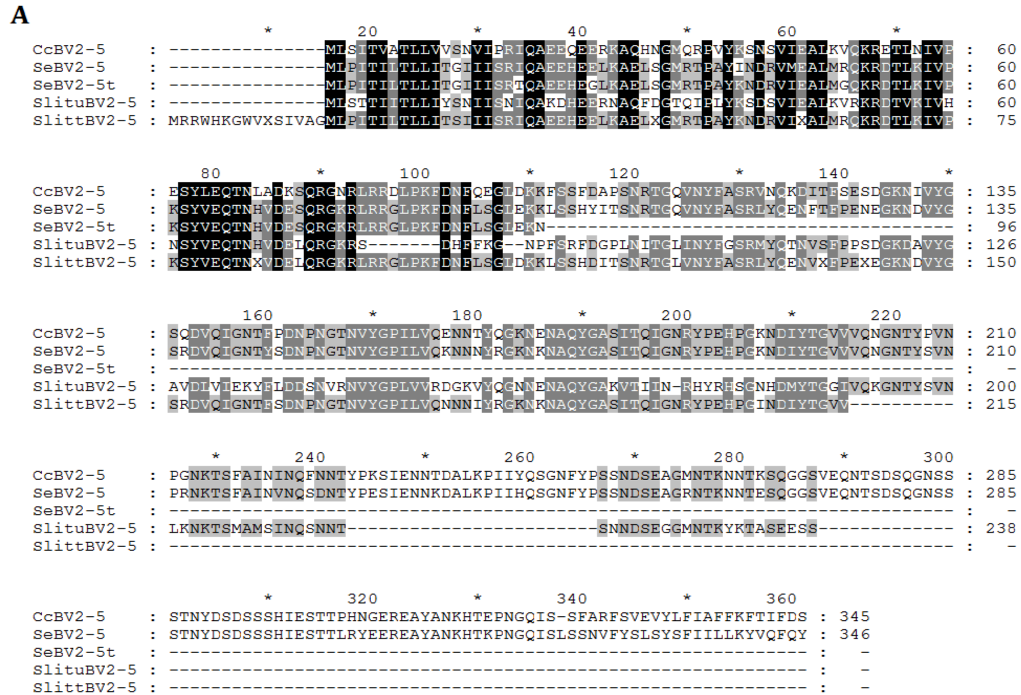
1.2.9. Susceptibility to *H. didymator* of different populations of *S. exigua*

In order to avoid the interference of genetic backgrounds of the different insect populations on the susceptibility to parasitism, a hybrid population containing individuals expressing both the active and the truncated form were generated. Genetic crosses between insects from the SUI population and insects from the MEX population were performed. The offspring (F1) was allowed to mate again to generate the F2 that, according to the standard genetic segregation should contain homozygotes for the functional and for the truncated alleles as well as heterozygotes. The resulting population was named MESUI and maintained in laboratory conditions such as described above. Early third instar larvae from the three different populations SUI, MEX and MESUI were introduced separately into glass vials containing 2-days-old adult female wasps (Dorémus *et al.*, 2013). Stung *S. exigua* larvae were isolated and maintained at 26°C until the emersion of all parasitoid pupae. Each caterpillar was then classified according to the following categories: larvae that supported the wasp development and emergence between 8 and 10 days post parasitism (dpp); larvae that supported the wasp development and emergence in a period of time longer than 10 dpp; larvae that died during the process of parasitism; larvae that escaped the parasitism and reached the final development stage (L5); and finally larvae that escaped parasitism but did not develop (L3/L4).

1.3. Results

1.3.1. Bracovirus homologs in *Spodoptera exigua* genome

Seven sequences similar to those of bracovirus were previously identified from the analysis of the *S. exigua* larvae transcriptome (Pascual *et al*., 2012). One sequence contained an insertion (1548 bp long) highly similar to CcBV C25 (>90% sequence identity at the nucleotide level) that contains an ORF encoding a protein that does not share any homology with lepidopteran proteins (Fig. 1.1). This ORF encodes BV2-5 a member of the bracovirus specific gene family BV2 comprising 8 genes in *Cotesia congregata* bracovirus. Based on that we named this new gene in *S. exigua* as *Se_BV2-5.* Different alleles from *Se_BV2-5* were found in *S. exigua* DNA obtained from different geographic sources. Asian and North American samples encoded a complete protein while European populations contained a frame shift mutation that generates a truncated form of the protein (Fig. 1.2). The intron present in the CcBV gene is also present in *S. exigua BV2-5,* indicating that the *BV2-5* gene organization has been conserved.



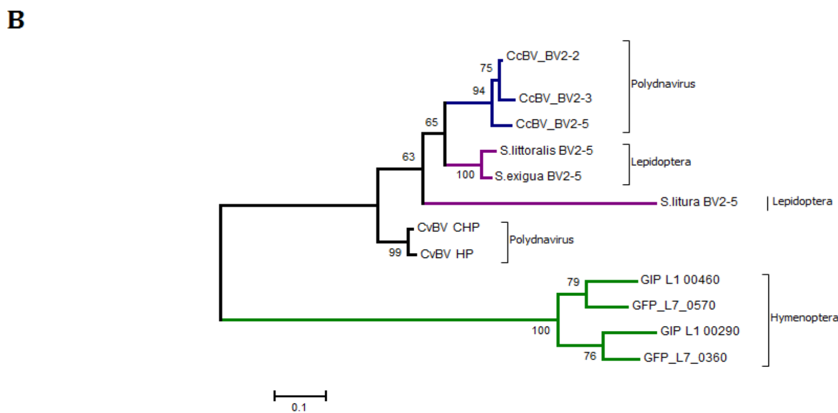


Figure 1.1. BV2-5 alignment and phylogenetic reconstruction. **A)** ClustalX alignment of BV2-5 members in different *Spodoptera spp* and CcBV-BV2-5. **B)** Phylogenetic tree obtained by Maximum likelihood of BV2-5 from the different *Spodoptera* species and their homologs from polydnavirus and parasitic wasps. Names and access numbers are in the Annex 1.1.

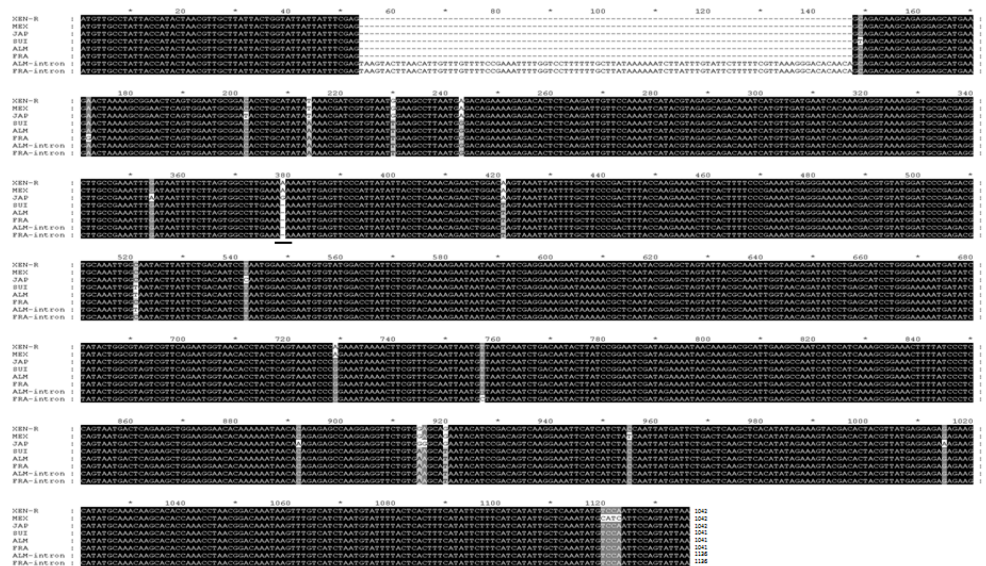


Figure 1.2. Sequence comparison of *Se_BV2-5* gene derived from different populations of *S. exigua* showing the presence of two forms of the expressed protein. XEN-R: Xentari resistant population originated from America, MEX: a population originated from Mexico, JAP: Japanese-derived cells, SUI: Switzerland population, FRA: French population, ALM: population originated from Almeria (Spain). The bold line represents the mutation site (see clearer Figure in Annex 1.2).

A second group of *S. exigua* ESTs with similarities to bracovirus sequences is composed of 6 sequences (about 500 bp long) with significant similarity to C-type lectin genes of bracovirus (close to 70% nucleotide similarity). Accordingly, these genes were named *S. exigua* bracovirus-like lectins (Se-*BLLs*). Phylogenetic analysis of the *Se-BLLs* clearly showed that they share a common evolutionary history with bracovirus lectins and are more distantly related with C-type lectins from other Lepidopteran species (Fig. 1.3, Annex 1.3).

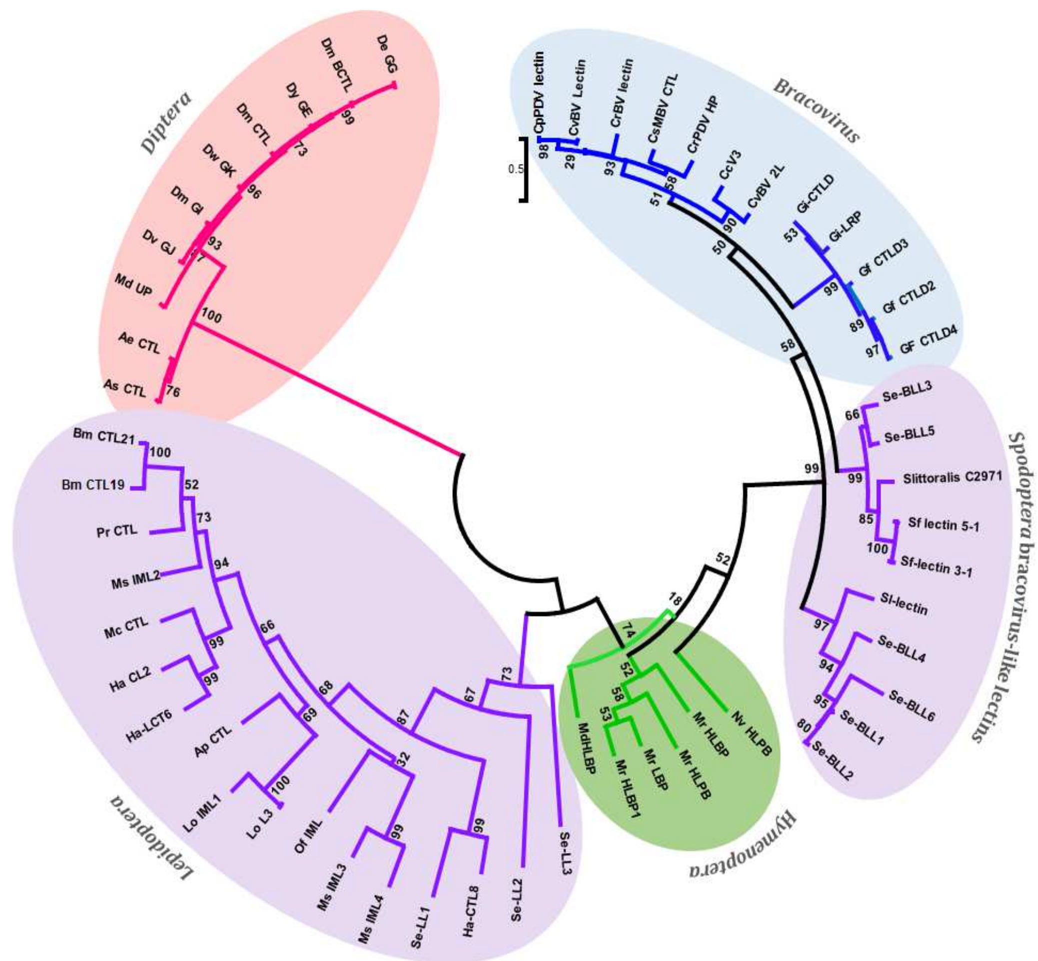


Figure 1.3. Phylogenetic tree of the deduced amino acid sequences of bracovirus-lectin like proteins from different *Spodoptera* species and their homologs from polydnavirus, hymenopteran, lepidoptera and diptera species. Evolutionary distance was calculated for aligned sequences by Maximum likelihood analysis. 1000 replicates were analyzed by the bootstrap analysis. The alignment and the names and access numbers of the sequences are present in the annexes 1.3 and 1.1.

Additional searches of ESTs databases from other *Spodoptera* species revealed the presence of BV2-5 homologous in *S. litura* and *S. littoralis* but not in *S.*

frugiperda. In addition, a bracovirus-like lectin ortholog in *S. littoralis*, another in *S. littura* and thwo in *S. frugiperda* available databases were detected. *S. littoralis* and *S. frugiperda* bracovirus-like lectins are grouped with Se-BLL3 and Se-BLL5. This group of proteins has the highest similarity to polydnaviral proteins. *S. litura* lectins is more similar to Se-BLL4, Se-BLL2 and Se-BLL1 (Fig. 1.3).

To determine the genomic organisation of bracovirus-derived genes in *S. exigua*, genomic regions of the *BV2-5* and *Se-BLL2* genes were obtained by genome walking. Fragments of 3687 and 2529 bp were obtained for *BV2-5* and *Se-BLL2*, respectively (Fig. 4). Analysis of the *BV2-5* fragment revealed the presence of a sequence (2246 bp long) highly similar to CcBV C25 containing the *BV2-5* gene and its bracovirus upstream and downstream sequences flanked by lepidopteran sequences. Remarkably the analysis of *BV2-5* insertion revealed the presence of a single direct repeat junction (DRJ) highly similar to those of CcBV circles (Table 1.3), which is a signature of the viral origin of this sequence, clearly indicating that the direction of the gene horizontal transfer was from bracovirus to lepidoptera (Fig. 1.4 A). The *Se-BLL2* insertion contains a short bracovirus upstream sequence, but no downstream sequences nor the intron present in bracovirus genes (Fig. 1.4 B). Both genomic sequences are characterized by high A+T percentages (63.71% and 53.49% for *BV2-5* and *Se-BLL2* respectively) especially in the sequences ends.

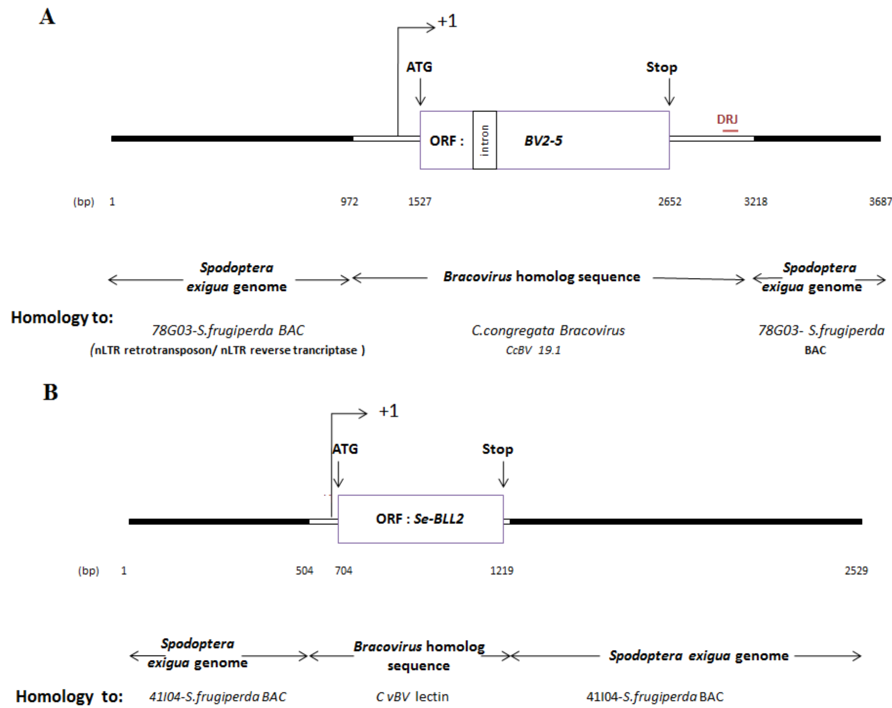


Figure 1.4. Schematic representation of the genomic integration of two different *S.exigua* bracovirus-like genes. (A)*BV2-5* gene and (B) *Se-BLL2* gene. DRJ indicates the integration site into the lepidopteran genome.

Table 1.3. Sequence comparison of Se_BV2-5 direct repeat junction with the DRJ of some *Cotesia congregata* bracovirus circles. The red and blue characters represent the conserved region.

Gene name	DRJ
Se_BV2-5	CACTTTTATAGCTTTTTGAATGATGAACAAAAATAGTGAACAACTCTCCACAACATGTC
CcBV C25	TAATCAAGTAGCTTTCTGAGTGATGAACAAAAATAGTGAACAACTCTCCACAACATGTC
CcBV C23	TAAATGAATAGCTTTCTAAATAATGAACAAAAAGTGTGATTAACTACCTAGAACCCGTC
CcBV C16	TATTCTTATAGCTTTTCTAATACTGAATAAAAATATACCACAAATGCACATACGGATGT
CcBV C15	TATTTGTATAGCTTATTAAATGTTGAACAAAAATACTGAACAAATCCAGTAAATAAGC
CcBV C5	GTTTCTTATAGCTTTTTGAATAATGAATTGAAATAACGATCAGATATTCTA-AAATAGA
CcBV C3	AAATTAAGAAGCTTTTTAAATGACGAACAAAAATACTGAATAAAAT—CTCAGGATATT

1.3.2. *S. exigua* bracovirus-like genes are highly expressed in hemocytes and respond to injection and parasitism

In order to accumulate information about the possible origin and function of the bracovirus-like genes, we determined their tissue distribution in the larval stage. When compared with the rest of the tested tissues, expression of all the bracovirus-derived genes was enriched in the hemocytes (Fig. 1.5 A).

When we determined the effect of PBS injection (simulating parasitoid wasp stinging), we noted a general repression of these genes. This general down-regulation was detected in hemocytes and midgut for all the studied genes and in the fat body only for *BV2-5* (Fig. 1.5 B).

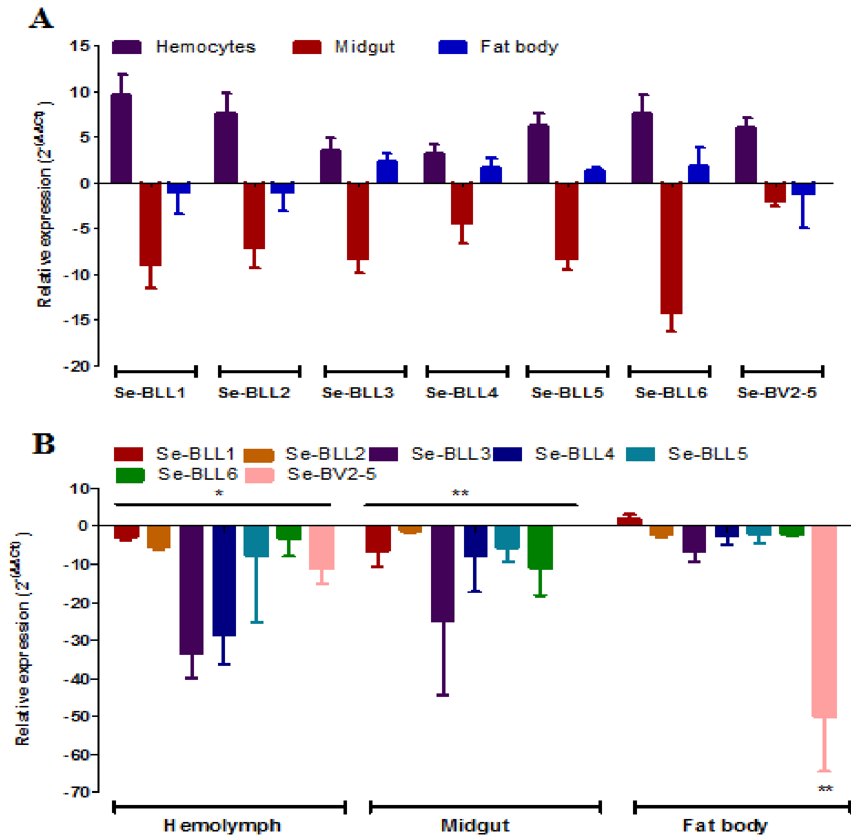


Figure 1.5. A) Relative expression pattern of the different *S. exigua* bracovirus-like proteins in three different tissues, hemocytes, fat body and midgut. The results are presented as the mean ± standard deviation of three independent replicates. **B)** Relative expression pattern of the different *S. exigua* bracovirus-like proteins in response to PBS injection in different tissues measured 8 hours post injection. The results are presented as the mean ± standard deviation of three independent replicates. The * represent $p<0.05$ and ** represent $p<0.001$.

Moreover, some *S. exigua* bracovirus-like genes responded to parasitism by the endoparasitoid *Hyposoter didymator*. Twenty-four hours post parasitism, *Se-BLL6* was induced in hemocytes and *Se-BLL4* in midgut; while, *Se-BLL2* was

repressed in midgut. Seventy-two hours post parasitism, another pattern of response was observed. In hemocytes, *BLL2* was induced; in midgut, *BLL1*, *BLL4*, *BLL6* and *BV2-5* were induced (Fig. 1.6).

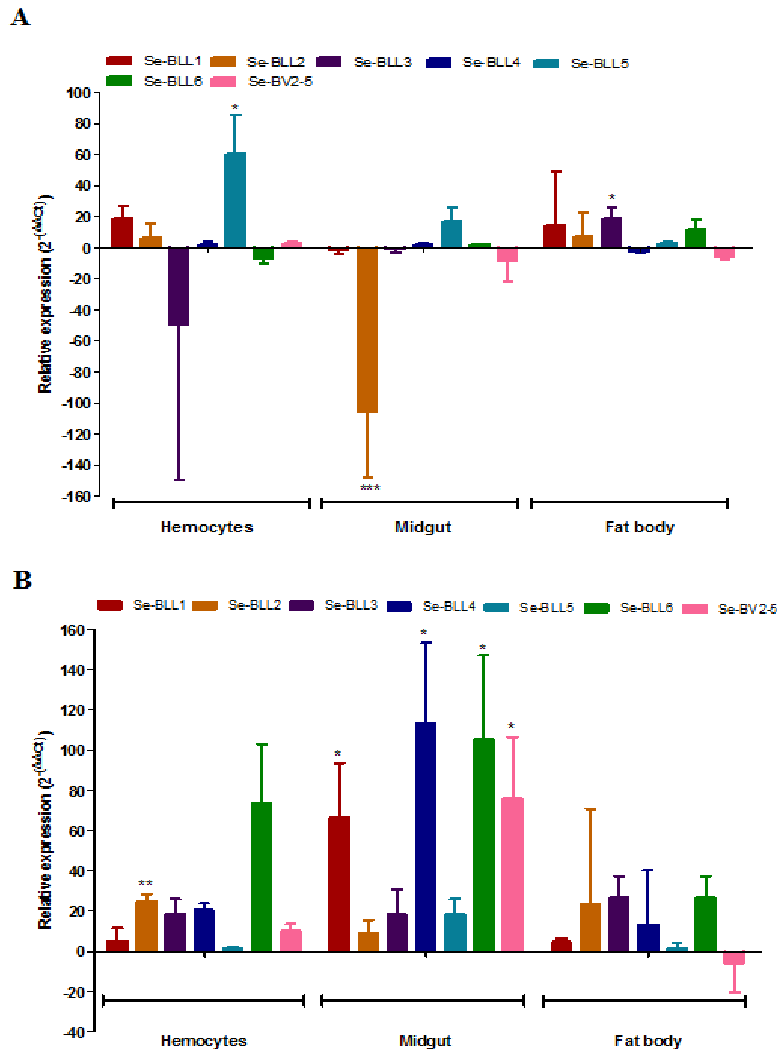


Figure 1.6. Response to *S. exigua* lectins in response to parasitism at 24h (A) and 72h (B) post injection. The results are represented as the mean ± standard deviation; the * represents p<0.05, ** represents p<0.001 and *** represents p<0.0001.

1.3.3. Different populations of *S. exigua* show different susceptibility after parasitation by an ichneumonid

Early third instar *S. exigua* larvae were parasitized with *Hyposoter didymator*. The larvae were parasitized individually in order to be sure that all the larvae included in the assay were parasitized. In the case of SUI population, homozygote for the truncated form of BV2-5 protein, parasitoid wasps emerged from 70% of stung larvae 8 to 10 days post parasitism. In contrast, the parasitism in larvae from the MEX population (homozygote for the full form of the protein) was successful for only 20%. In an important number of larvae (about 11%), a delay in the parasitoid wasp emergence was observed (more than 10 days and up to 15 days). Remarkably, more than 30% of parasitized larvae escaped the parasitism and reached the last instar. The rest of parasitized larvae (about 30%) died. These results suggest that MEX population is more resistant to parasitism by *H. didymator*. Finally, the MESUI population (the hybrid population) showed similar susceptibility to the MEX population, with low percentage of successful parasitism (about 33%). In addition, and unexpectedly half of parasitized larvae died within 2 to 4 days post stinging (Fig. 1.7).

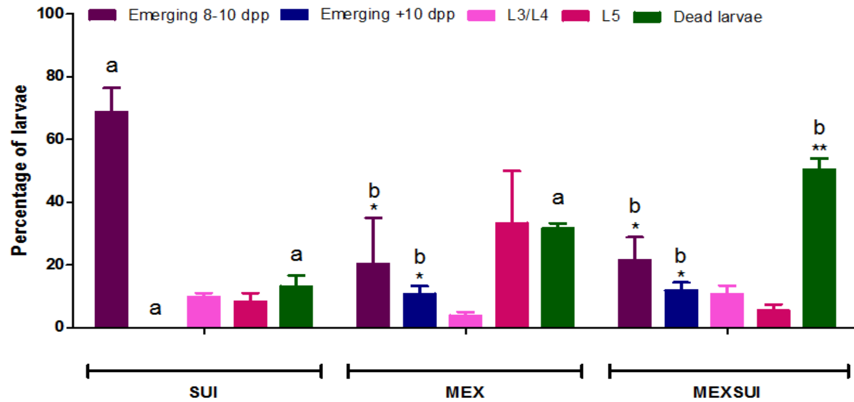


Figure 1.7. Comparison of the response to parasitism studied in three different populations of *S. exigua*.

1.3.4. BV2-5 and BLL2 affect baculovirus infection

One can wonder what could be the functional role of the bracoviral proteins in Lepidoptera that could confer a selective advantage promoting its selection. As many bracovirus virulence proteins are interfering with host immunity we hypothesized that they could modify some features of the lepidopteran immune response resulting in an impact on other pathogens.

To evaluate the possible role of Se-BLL2 in the insect interaction with the viral pathogen, baculovirus, Se-BLL2 was expressed in *E. coli* and purified using affinity chromatography. Then we determined the effect of Se-BLL2 on *S. exigua* susceptibility to baculovirus, a deadly and common lepidopteran pathogen encountered by larvae in the field. Third instar larvae were infected with its native baculovirus (SeMNPV) in the presence or absence of the recombinant Se-BLL2 protein and larval mortality was registered at different time points. When purified Se-BLL2 was added to the viral inoculum, larval mortality produced by baculovirus was reduced by half (Fig. 1.8 A). These results suggest that Se-BLL2 expression in *S. exigua* seem to play an important role in increasing host resistance to baculovirus infection.

A similar approach was planned with BV2-5 protein, however given the difficulties to express the recombinant protein in *E. coli*, an alternative methodology was used. We generated recombinant baculovirus expressing the BV2-5 protein or its truncated form (BV2-5t). The effect of BV2-5 expression on baculovirus multiplication was determined by comparing the replication and production of the different viral constructs in Sf21 cells. The progression of the baculovirus multiplication was reduced for the BV2-5-expressing virus compared to the control virus. At 96 hours post infection, the concentration of viral particles allowing cell-to-cell spreading of infection (budded virus or BV) in the cell culture medium was reduced by more than two order of magnitude in the baculovirus producing BV2-5 (Fig 1.8 C). In contrast, the recombinant virus expressing the truncated form (BV2-5t) did not show any significant difference with control virus (Fig. 1.8 B). Moreover, BV2-5-expressing virus had also a lower production (Fig. 1.8 C, D) of viral occlusion bodies (OBs), a second type of viral particle that allows persistence and horizontal transmission of the virus in the field. As previously observed for the Se-BLL2, bracoviral-derived proteins seem to contribute to increase insect fitness in the field by increasing its natural resistant to baculovirus.

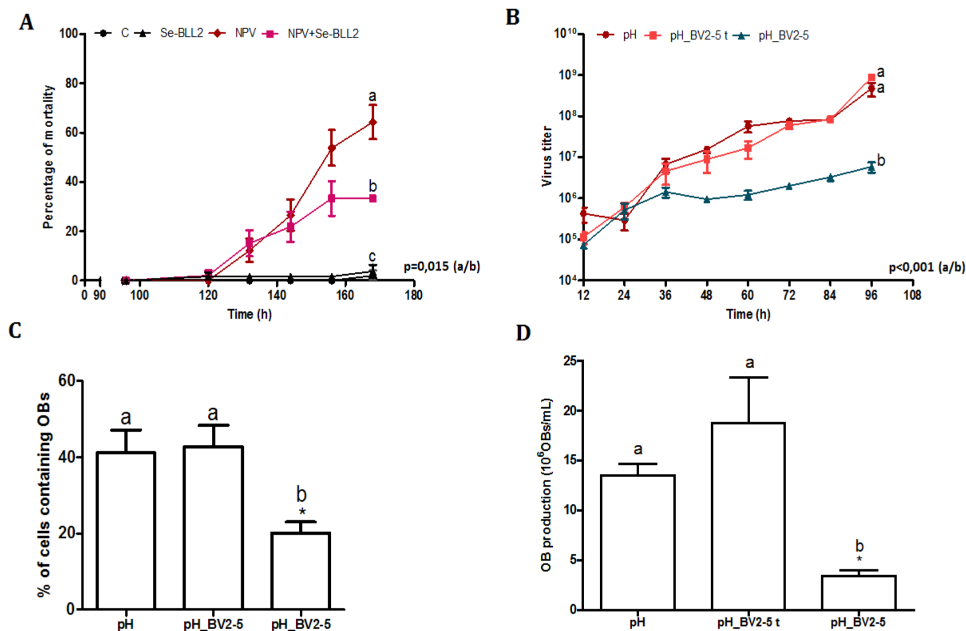


Figure 1.8. *Spodoptera exigua* bracovirus-like genes affect baculovirus infection. **A)** The effect of Se-BLL2 on baculovirus infection. The time to death was assessed by comparing the mortality curves using the Kaplan Meier method (GraphPad Prism 5). The statistical significance was determined using the log-rank analysis (Mantel-cox test), p=0,015. **B)** Baculovirus titres of the different constructs. **C)** Percentage of the cells containing OBs in the presence or absence of BV2-5; **D)** Number of viral occlusion bodies produced by each recombinant baculovirus. In B, C and D, pH represent the wild baculovirus, ph_BV2-5 represent the recombinant baculovirus expressing *S. exigua* BV2-5 and ph_BV2-5t represent the recombinant baculovirus expressing a truncated form of *S. exigua* BV2-5. In both cases the bars represents the mean ± SEM. Means were analysed by Dunnett's test and different letters denote significant differences and the * represents $p<0.05$

1.4. Discussion

The association parasitic wasp/PDV/lepidopteran host can represent an interesting model to study the horizontal transfer of genes (HGT) since three different genomes are continuously in contact with each other. Although, HGTs mainly occur in prokaryotes (Hotopp *et al*., 2007), it has been described that many insects such as pea aphids, mosquitoes and fruit flies have been recipients of HGT

from bacteria and in rare cases fungi (Dunning Hotopp *et al.,* 2007; Moran and Jarvi, 2010; Zhu *et al.,* 2011). In Lepidoptera, 14 putative HGT cases from bacteria to *Bombyx mori* have been reported (Zhu *et al*., 2011). Therefore, horizontal gene transfer from PDVs to the lepidopteran genomes can occur. Interestingly, and in this context, we report here the presence of at least 7 bracovirus-related sequences in the *S. exigua* genome. All those genes are mainly expressed in the hemolymph, where the parasitoid is supposed to inject its eggs during parasitism. Analysis of the genome sequences of two of the *S. exigua* bracovirus-like genes, *BV2-5* and *Se-BLL2,* revealed that they are characterized by a high A+T percentage. In fact, it has been shown that polydnavirus genomes have a strong A/T bias compared to other viruses (Webb *et al*., 2006) with a high AT content of the segments or sequences extremities (Beck *et al*, 2011; Wyder *et al*., 2002). Those extremities are highly conserved among the segments within a species or among related species (Beck *et al.*, 2011; Bézier *et al*, 2013; Desjardins *et al*., 2008) and correspond to the direct repeat junction (DRJ). They are specific DNA circularization motifs flanking each segment and common to all bracoviruses. Individual circles are produced by a recombintion between the DRJs from large precursor molecules, which are amplified during viral DNA replication, and a single DRJ resulting from the recombination is present on the circle (Bézier *et al*., 2013; Herniou *et al*., 2013; Strand and Burke, 2013). In the case of *BV2-5*, the 3' extremity juxtaposed to the lepidopteran genome corresponds to the DRJ conserved in *Cotesia* bracovirus segments. The presence of such a regulatory sequence involved in the bracovirus life cycle indicate clearly that the direction of the transfer was from the virus to the lepidopteran host. This hypothesis holds true just in the case where the host received a very low amount of viral particles that would be insufficient to provoque a developmental effect, or if the parasitoid wasp carrying the bracovirus parasitizes a less sensitive host that is able to survive and pass the integrated genes to the progenety. In fact, even though braconids parasite a wide range of lepidopteran species, noctuids are not natural hosts of these parasitoids (Beckage and Tann, 2002; Harwood, 1998). However, the noctuid *Trichoplusia ni* was found to

support *Cotesia congregata* development to maturity (Beckage and Tann, 2002). It seems, therefore, that noctuids can be semi-permissive hosts for *Cotesia sp.*. Moreover, there has been suggested that since the polydnavirus is unable to replicate inside the lepidopteran host, it may integrate a part of its DNA into the insect genome (Drezen *et al*., 2014). During parasitism, the particles do not replicate in the host tissues and the role of the integration into host cells DNA is probably to allow the persistence of the bracovirus DNA in lepidopteran larvae that continue to develop. Integration would permit continuous expression of viral genes in newly divided cells and, thus, persistence of host manipulation throughout wasp larva development (Herniou *et al*., 2013). In our case, an explanation of the presence of PDV genes in *S. exigua* transcriptome can be that *Cotesia sp.* was able to parasitize *Spodoptera sp*. which allowed a part of the bracovirus to integrate into the lepidopteran genome. A significant number of larvae might succeed to escape the parasitism and the viral sequences have been transmitted vertically to the descendants. It is likely that after circle integration, bracovirus sequences get lost, unless they provide a selective advantage to the insect. Therefore, complete circles would correspond to sequences recently inserted and not fixed in the species, such as those normally detected in the wasp genome. We hypothesize that *BV2-5* insertion corresponds to a remain part of the circle integrated into the lepidopteran genome that have undertaken arrangements since its integration. The *BV2-5* insertion corresponds mostly to a single gene with its regulatory sequences, which could represent an ultimate stage of domestication, where most of the circle sequence have been lost.

Even though, the *Se-BLL2* sequence does not contain the conserved DRJ, we suspect that this sequence, as well as the other bracovirus-like lectins, are viral genes integrated into the genome of *S. exigua*. They can be the result of a more ancient integration event or events that have been the subject of selective phenomen which resulted in the deletion of the viral DRJ. Another explanation would be that the lectins acquisition by the lepidopteran germline has been mediated by a broader

mechanism, such as DNA repair. Actually, it has been reported that viral infections can activate transposable elements and enhance the frequency of DNA recombination, which increases the possibility of viral DNA or RNA integration into the host genome (Dong, 2004; Kovalchuk *et al*., 2003). Another hypothesis is that the acquisition of the *BLLs* and particularly *Se-BLL2* might be the result of the integration of a cDNA after retrotranscription of a viral transcript. This process is thought to be mediated by reverse transcriptases of endogenous retrotransposons that are abundant in most genomes including those of Lepidoptera.

The acquisition of these genes appears to be ancient since homologs are present in *S. litura* which is an ancient species in the *Spodoptera* lineage (Kergoat *et al*., 2012). However, no homolog for *BV2-5* has ever been detected in *S. frugiperda*. We suppose that the bracovirus sequence containing *BV2-5* has either been acquired by the three species in different integration events or more likely the integration has occurred in an ancestor of the three species and then the sequence has been lost in the case of *S. frugiperda* but conserved in the other species. As for the lectins, homologs were detected in *S. frugiperda*, *S. littoralis* and *S. litura*. A Phylogenetic analysis has shown that these lectins are clustred in two different groups. The integration of the bracovirus-like lectins into an ancestral *Spodoptera* species germ line might has occurred in a single or multiple events. After speciation of the different species studied, gene duplications might have been occurred in order to increase the ability of the insect to face pathogens. Indeed, selection pressure may act on virulence genes causing radiation toward various targets increasing the possibility of evolution and adaptation of the host (Huguet *et al*., 2012).

HGT can confer advantages to the host such as diet changes, colonizing new habitats or survive in previously lethal conditions. Therefore, functional analyses in lepidoptera indicate that HGT may increase their survival and fecundity (Sun *et al.,* 2013). Thus, conservation of bracovirus genes in the *S. exigua* genome is likely associated to an increase in fitness due to the expression of the viral genes. This hypothesis is sustained by functional studies with the Se-BLL2 and BV2-5 proteins

from *S. exigua* showing that they have an impact on baculovirus infection. This suggests that host domestication of these genes might increase insect tolerance to this natural pathogen playing an important role in regulating population dynamics in the field. The alteration of lepidopteran immunity parameters may also have a cost by increasing the susceptibility to other pathogens. It is noteworthy that two different alleles have been found for the *BV2-5* and one of them is no longer functional. It is tempting to speculate that the frequency of one or the other form might depend on the abundance and local selective pressure exerted by pathogens and/or parasite. *S. exigua* is a palearctic species whose introduction in America was first recorded in 1876 (Wilson, 1932). The fact that Lepidoptera collected in Europe encode a truncated form suggests that a recent mutation has spread in the populations. This suggests that there might be a cost to maintain baculovirus resistance conferred by *BV2-5* in the European populations. This cost might also explain why *BV2-5* is not present in *S. frugiperda* while it was detected in *S. litura* a more basal species in *Spodoptera* lineage.

We have described, in this report, several insertions of bracovirus DNA sequences in the lepidopteran *S. exigua* genome. Moreover, we showed that domestication of different bracovirus genes confer a protection against baculovirus, a common pathogen in the field. A specific bracovirus circle integration mechanism into lepidopteran host DNA, operating during parasitism and resulting occasionally in circle reintegration, has been characterized. However, what remains of bracovirus-like lectin sequences in the lepidopteran genome does not allow to determine whether their acquisition involved this bracovirus-specific mechanism or a broader one such as DNA repair. Once integrated, bracovirus genes might be readily domesticated by Lepidoptera since they are already adapted for expression in lepidoptera tissues during parasitism. Altogether the ability of bracovirus particles to mediate integration, along with the structure of their genes adapted to the expression in Lepidoptera, suggests that in the future numerous evidences of

integration and domestication of bracovirus sequences will be identified with the exponential rise of genomic data provided by next generation sequencing.

CHAPTER 2

Gasmin (BV2-5), a polydnaviral-acquired gene plays a role in response to bacterial and viral infections in *Spodoptera exigua* by interacting with the cytoskeleton

Results from this chapter are included in:
Gasmi, L., Jakubowska, A.K. and Herrero, S. Gasmin (BV2-5), a polydnaviral-acquired gene plays a role in response to bacterial and viral infections in *Spodoptera exigua* by interacting with the cytoskeleton. Submittes to “Cellular microbiology”.

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2.1. Introduction

Insects represent a large and vast group of animals distributed worldwide and adapted to different types of environments. Although they lack adaptive immune systems, they have developed a strong innate immunity that allowed them to face a world surrounded by natural enemies (Beckage, 2008). In response to pathogens, insects activate a large repertoire of cellular and humoral components aimed to block or reduce the progress of the pathogen as well as to minimize the detrimental effects produced by the pathogen (Feldhaar and Gross, 2008).

Before activation of the immune response by the host, pathogens need to overcome the primary line of defense which consists of the cuticle barrier, gut and trachea. If the invader succeeds to penetrate these barriers, the non-self recognition is crucial to stimulate the insect immune system. Although insects lack antibody based immunity, it has been shown that they produce a set of proteins that recognize and bind conserved domains on the pathogen surface, named pattern recognition proteins (Gillespie *et al*., 1997, Jiravanichpaisal *et al*., 2006). Once pattern recognition proteins bind to the target, a number of signaling pathways leading to humoral and cellular responses take place. In general, humoral immune response refers to the expression of peptides that are produced mainly by fat body, but also by some other tissues such as hemocytes and gut. These peptides have the ability to kill the foreign intruders. Such responses also include the activation of enzymatic cascades regulating hemolymph coagulation and melanisation (Cerenius and Soderhall, 2004). The cellular defense is mainly mediated by hemocytes through the phagocytosis and encapsulation of the pathogen (Lavine and Strand, 2002). Phagocytosis consists of the recognition and uptake of microorganisms followed by their destruction (Jiravanichpaisal *et al*, 2006), whereas encapsulation refers to the binding of hemocytes to larger targets such as parasitoids (Browne *et al*., 2013).

Parasitic insects rely on the parasitized hosts for the development of their offspring. They have developed efficient strategies in order to overcome the host immune defense (Beckage and Gelman, 2004). For instance, members of the

hymenopteran wasps *Braconidae* and *Ichneumonidae* have developed unusual symbiotic relationship with a type of viruses named polydnaviruses (PDVs) (Strand and Bruke, 2013). PDVs are double stranded DNA viruses residing in the wasp chromosome as a proviral form. Their replication is restricted to specialized calyx cells in the female ovaries and vertically transmitted to descendants (Strand and Bruke, 2013). They are injected together with the wasp's eggs into the lepidopteran host hemocoel as segmented circular DNA. The circular form of the virus is unable to replicate since it lacks replication genes. Thus, PDVs do not replicate in the lepidopteran host, but express specific proteins able to interfere with the insect's immune system (Drezen *et al*., 2014).

In order to survive inside the lepidopteran host, parasitoids alter hemocytes functions. In general, PDVs infect efficiently lepidopteran hemocytes inducing the expression of their specific immunosuppressive proteins (Asgari *et al*., 1997,Turnbull *et al*., 2004). In some host species, hemocytes dysfunction involves a dramatic reduction of the number of functional hemocytes, while in others, PDV proteins alter the behavior of hemocytes that fail to spread over the surface of the parasitoid egg (Amaya *et al*., 2005). Hemocytes alteration has been described as a result of the disruption of cytoskeleton actin arrangement (Asgari *et al*., 1997). In addition, it has been described that some PDVs proteins can inhibit the actin expression in the immune cells and so causing their dysfunction, such as the case of the glc1.8 protein expressed by *Microplitis demolitor* bracovirus parasitizing *Pseudoplusia includens* (Strand *et al*., 2006). In order to provide a sustainable expression of such immunosuppressive proteins, it has been suggested that the polydnavirus integrates part of its genome in the DNA of certain cells of the host (Drezen *et al*., 2014).

In the previous chapter, we have reported horizontal gene transfer events that have occurred from a bracovirus symbiotic to an unidentified *Cotesia sp* to the lepidopteran *S. exigua*. One of these events has resulted in the integration of a *Cotesia sp* bracovirus sequence encoding the *BV2-5* gene. *BV2-5* is a member of a specific

gene family of *Cotesia* bracovirus. Chen and colleagues (Chen *et al*., 2007) have shown that CvBV2 is expressed in parasitized *Plutella xylostella* hemocytes at a very early stage of parasitism and suggested that *BV2* can be implicated in the early protection of the parasitoid egg against encapsulation.

Based on these previous observations, we decided to further investigate the possible role of this protein in the lepidopteran immune system. Accordingly, we have found in this study that this protein, mainly expressed in hemocytes, interacts with the actin cytoskeleton of *Spodoptera* cells. Cytoskeletal actin is involved in different cellular mechanisms, such as phagocytosis and encapsulation, and it is used by baculovirus to successfully infect the lepidopteran cells. We additionally investigated these mechanisms and found that the BV2-5 presence interferes with the successful infection and productivity of baculovirus as well as the phagocytic capacity of the insect hemocytes.

2.2. Materials and methods

2.2.1. Insects and DNA samples

Three different colonies of *Spodoptera exigua* were continuously reared on artificial diet at 25± 3°C with 70±5% relative humidity and a photoperiod of 16h light: 8h dark. The FRA strain was kindly supplied by M. Lopez-Ferber, INRA (St.Christol les Alés, France). The SUI population was kindly provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland). The XEN-R strain was obtained from insects collected from cotton fields in Pattville, (USA) which were later selected for resistance to *Bacillus thuringiensis* (Hernández-Martínez *et al*., 2010, Park *et al*., 2014).

2.2.2. *BV2-5* expression analysis by reverse transcriptase quantitative-PCR (RT-qPCR)

To study the change in the expression profile of *BV2-5* after intrahemocoelic larval injection with bacteria, *S. exigua* FRA fourth instar larvae were injected with 5 µl PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 27 mM KCl; pH 7.0)(control) or with 5 µl of 10^7 cells/ml from either *Micrococcus luteus*, *Escherichia coli* or *Bacillus thuringiensis*. Bacterial preparations were previously inactivated by heat shock treatment (120°C, 30min) and resuspended in PBS. Hemocytes and fat body were collected from injected larvae, as well as non-treated larvae, 8 h and 24 h post injection. Total RNA from the treated and control larvae were collected and processed for RT-qPCR. Data are presented as fold change using the method of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) and normalized to the internal control gene, *ATP synthase* (Herrero *et al.*, 2007). To determine the effect of the injection, PBS-injected larvae were compared against non-injected control larvae. However, to determine the effect of the injection of bacteria, PBS-injected larvae were used as a control sample. The standard deviation of the ΔCt values of treated and control samples was calculated as $(s_1^2 + s_2^2)^{1/2}$, where s_1 is the standard deviation of the gene of interest and s_2 is the standard deviation of the reference gene. The standard deviation of the ΔCt was then incorporated into the fold change calculation as described in the Applied Biosystem manual "Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR".

2.2.3.Generation of recombinant baculovirus

Two types of baculovirus constructs have been employed in this study (Fig 2.1). Generation of recombinant baculovirus expressing the different forms of BV2-5 and forming viral occlusion bodies (by expressing AcMNPV polyhedrin gene downstream the ph promoter (Fig. 2.1 B) have been previously described (Chapter 1).

Another type of construct was generated to study the cellular localization of BV2-5 by expressing this protein fused to GFP. Primers containing BglII and *Eco*RI were designed to amplify the *BV2-5* gene from the pFBD-pH_BV2-5 described in the first chapter. The obtained fragment was sub-cloned into the pGEM-T Easy, double digested with BglII and EcoRI, and cloned in p166AcV5-Se8-GFP (Ijkel *et al*., 2000) in order to obtain the fusion gene *BV2-5_GFP* in the plasmid p166AcV5-Se8-BV2-5GFP. Subsequently, the *GFP* gene and the recombinant *BV2-5GFP* gene were amplified using specific primers that contained the restriction sites of NotI and PstI (Forward *BV2-5GFP*: 5' TTGCGGCCGCATGTTGCCTATTACC3'; Forward *GFP*: 5' CTGCGGCCGCATGGGCAAAGGAGAAGAACTTT3'; Reverse: 5'AGCTGCAGTTACGACCAGCCGCCGCTGGCATCT3'). Both genes were cloned under the ph promoter of pFBD to generate pFBD-GFP and pFBD-BV2-5GFP, which were used to transpose into the AcMNPV bacmid as previously described (Fig. 2.1 A).

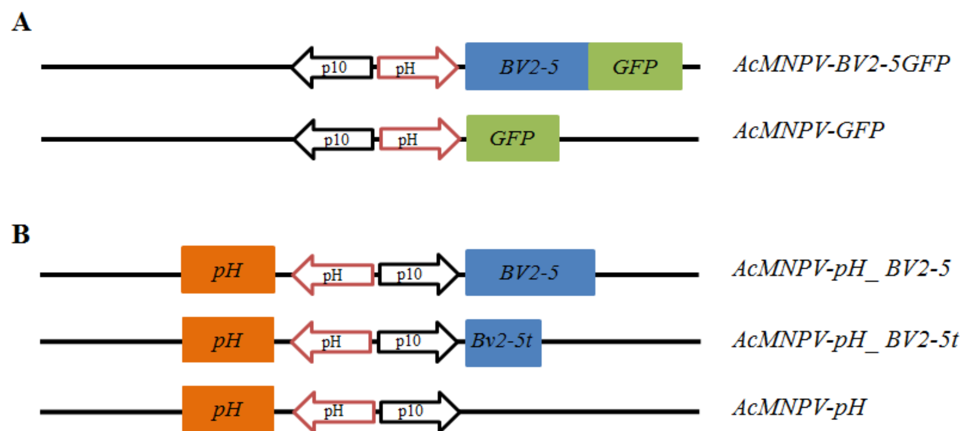

Figure 2.1. Schematic representation of the recombinant baculoviruses generated in this work.

2.2.4. Cellular localization of the BV2-5 protein by confocal microscopy

Cellular localization of the BV2-5 protein was determined using the recombinant baculovirus expressing BV2-5 fused to GFP (AcMNPV-BV2-5GFP).

Previously to the confocal analysis, Sf21 cells were maintained in Grace's medium (Invitrogen) supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin at 27°C. A first set of cells was infected with AcMNPV-BV2-5 GFP and a second set with AcMNPV-GFP at a multiplicity of infection (MOI) of 10. A third set of cells was infected with AcMNPV-GFP and treated with 5 μM latrunculin A (Sigma Aldrich) 12 hours post infection (hpi). A fourth group of cells was maintained without any treatment as a negative control. Seventy-two hpi, cells were pelleted by centrifugation for 2 min at 3000x*g* and fixed with 4% paraformaldehyde (PFA) for 20 min. Then, the cells were washed twice with PBS and permeabilized for 10 min with 0.2% Triton X-100 in PBS-BSA 10%. After another step of PBS washing, cellular actin was stained overnight at 4°C with phalloidin-tetramethylrhodamine B isocyanate TRITC (Sigma Aldrich). Finally, the cells were washed, stained with DAPI (4', 6'-diamidino-2-phenylindole) to visualize the nucleus of cells and fixed by dakocytomation fluorescent mounting medium (Dakocytomation). Mounted cells were observed under confocal microscope (FV1000, OLYMPUS).

2.2.5. BV2-5 effect on the baculovirus infectivity and production

In separate T75 flasks, about 10^6 Sf21 cells were infected with the AcMNPV-pH_BV2-5, AcMNPV-pH_BV2-5 and AcMNPV-pH viruses at MOI 2. In addition, one group of cells was infected with the control virus AcMNPV-pH and treated with 5 μM latrunculin A at 12 hpi. Four days post infection, the percentage of cells containing OBs was manually counted. Then, infected cells were collected from each flask, pelleted and treated with 1% SDS for 5 min to liberate the occlusion bodies (OBs). Recovered OBs were washed twice with water and the resulting pellets were resuspended in water. Purified OBs were used to count the number of occlusion derived viruses (ODVs) packed in each OB by electron microscopy. For transmission electron microscopy (TEM), OBs in suspension were fixed for 2 h at 4°C with glutaraldehyde 1.5%. The samples were then concentrated in agar 0.4% and washed with phosphate buffer (0.2 M, pH 7.3). Subsequently, they were postfixed with 2%

osmiol for 2 h, dehydrated and contrasted for 1 h with 2% of uranyl acetate. The contrasted samples were included in epoxy resin and let to polymerize for 48 h at 60°C. After resin polymerization, samples were cut with an ultramicrotome (Leica UC6), then transferred to the TEM grids, and finally contrasted with plumb acetate. The obtained grids were observed under an electron transmission microscope of 100KV (JEOL JEM 1010). Different fields of each sample were photographed with a digital camera (Mega View III) at a magnification of 40,000 x and the number of ODVs packed in each OB, as well as the OB diameter, were manually determined. Experiments were performed in triplicate and at least 30 OBs were counted to determine the numbers of ODVs per OB. Values for each sample were statistically compared by Dunnett's multiple comparison test.

2.2.6. BV2-5 effects on hemocyte phagocytosis and susceptibility to bacteria

The phagocytic capacity of *S. exigua* hemocytes in the presence of BV2-5 was characterized using fluorescent bacteria, *Bacillus thuringiensis* expressing the green fluorescent protein, GFP, as a target. Third instar larvae (SUI) were fed diet disks contaminated with 10^4 OBs of each virus (AcMNPV-pH_BV2-5, AcMNPV-pH_BV2-5t or AcMNPV-pH). Larvae were incubated 48 h under the conditions described before in order to allow the infection of hemocytes and recombinant protein expression. Then, 10^7 bacterial cells were intrahaemocoelically injected in each larva. Non infected larvae were also injected with bacteria as control larvae. Twelve hours post injection, hemolymph was collected from larvae from each treatment and directly observed under confocal microscopy (1000FV, OLYMPUS). A total of 32 larvae were used for each treatment and three independent replicates were performed. A group of injected larvae were not sacrificed and used to score their mortality after bacterial infection. Mortality was scored every 12 h until death or pupation of all larvae. Mortality was expressed as the percentage of dead larvae. The time to death was

assessed by comparing the mortality curves using the Kaplan Meier method. The statistical significance was determined using the log-rank analysis (Mantel-cox test).

2.2.7. Sequence comparison and motif finding

Protein sequences were aligned using CLUSTALW (Thompson *et al.*, 1994). Conserved motifs in protein sequences were identified using motif based sequence analysis tool, MEME Suite version 4.6.1 (Bailey *et al.*, 2009) using a set of 27 proteins with homology to BV2-5 retrieved by Blastp search at NCBI (nr database). For motif finding, the following parameters were adjusted: optimum width 6–200 amino acid, any number of repetitions of a motif and maximum number of motifs set to 10.

2.3. Results

2.3.1. BV2-5 tissue distribution and response to bacterial injections

Expression of *BV2-5* in hemocytes, fat body and midgut of the fifth instar larvae was evaluated by RT-qPCR. When compared among them, the highest levels were found for hemocytes, being about 10-fold more abundant than in fat body or midgut.

Gene expression of *BV2-5* after intrahaemocoelic injection with PBS or bacteria was analysed by RT-qPCR in larval fat body and hemocytes (Fig. 2.2). Interestingly, *BV2-5* expression was down-regulated in hemocytes as well as in the fat body as early as 8 hours after intrahemocoelic injection with PBS emulating a parasitic injection. Normal expression levels were recovered at 24 hours after PBS injection. In contrast, the presence of *B. thuringiensis* (Bt) cells in the injected PBS was strongly up-regulating the expression of *BV2-5* being more evident at 8 hours after injection. At 24 hours after injection, the up-regulation was observed in the hemocytes but not in the fat body. No changes in gene expression were observed after injection with the non-pathogenic bacteria *M. luteus* or *E. coli.*

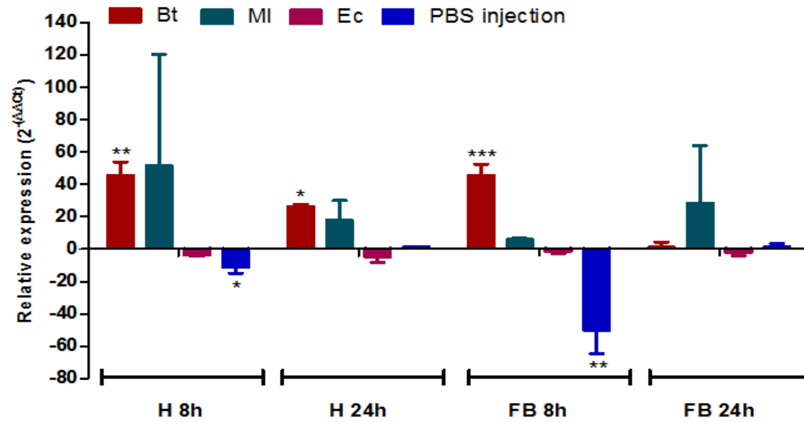

Figure 2.2. Response of *BV2-5* to Gram-positive and Gram negative bacteria (PBS injection was used as a control) in larval hemocytes (H) and fat body (FB). The results are represented as the mean ± standard deviation of three independent replicates. Bt: *Bacillus thuringiensis*, Ml: *Micrococcus luteus* and Ec: *Escherichia coli*. Means were analysed by Dunnett's statistic test. The * represents $p<0.05$ and ** represents $p<0.001$.

In order to get more information about the role of BV2-5 on the insect physiology we decided to express the functional form of BV2-5, found in the American and Asian populations of *S. exigua,* as well as the truncated form found in the European populations (Chapter 1). Initial trials on the bacterial expression system were unsuccessful due to the apparent toxicity of this protein. Therefore we decided to approach the functional study of that protein by the generation of different recombinant baculoviruses, each of them engineered to address different questions (Fig. 2.1).

2.3.2. BV2-5 physically interacts with actin

As a first approach that could provide some indication about the role of BV2-5, we decided to determine its cellular localization. Two recombinant viruses were generated: one producing BV2-5 fused to GFP and a control virus expressing GFP

(Fig. 2.1 A). Sf21 cells were infected with the recombinant viruses and localization of BV2-5 was tracked down by following the green fluorescence by confocal microscopy (Fig. 2.3).

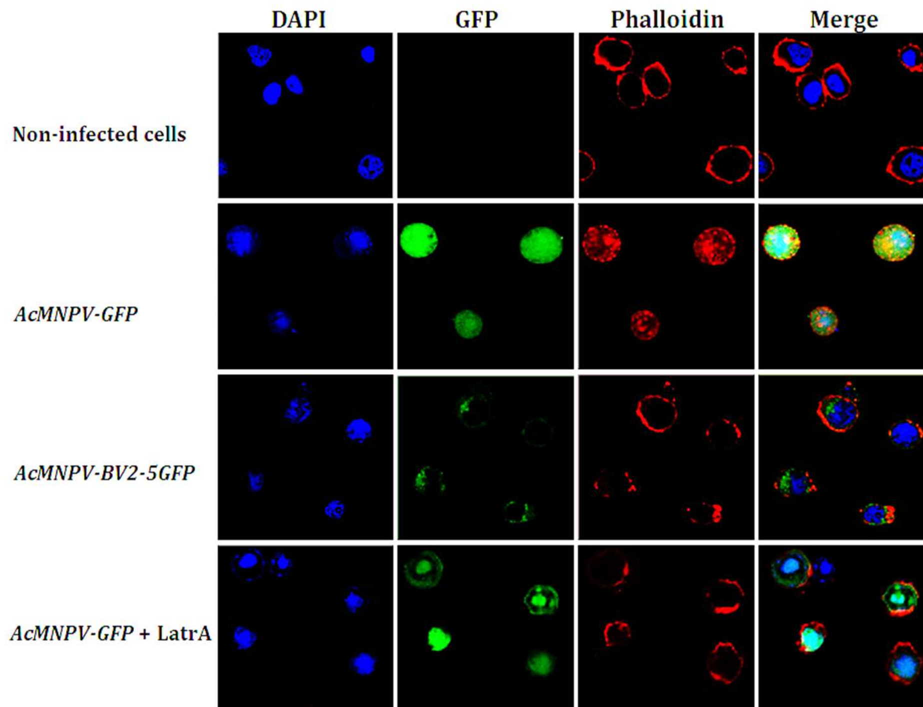

Figure 2.3. Cellular localisation of BV2-5 and its effect on actin distribution. Sf21 cells were infected with different recombinant viruses. The upper horizontal panel represents non-infected cells and the rest represent cells infected with *AcMNPV-GFP*, *AcMNPV-BV2-5GFP* and *AcMNPV-GFP* treated by latrunculin A, respectively. The fluorescence was visualized by confocal microscopy.

Confocal observations revealed that BV2-5 is localized in the periphery of the cells (channel GFP for AcMNPV-BV2-5GFP). Based on this observation, we hypothesized that BV2-5 may interact with some cellular components such as the actin cytoskeleton. In order to investigate our hypothesis, actin from non-infected cells as well as from cells infected with the different viruses, was stained with the

phalloidin-TRITC. Partial co-localization of BV2-5-GFP with phalloidin was observed suggesting that BV2-5 is co-localizing with the cytoskeleton. Interestingly, we also observed that actin distribution (phalloidin staining) in Sf21 cells infected with the BV2-5-expressing virus (AcMNPV-BV2-5GFP*)* differed from its distribution in cells infected with the control virus (AcMNPV-GFP). As previously described for baculovirus infection (Ohkawa *et al.*, 2010), polymerization of the actin and changes in its distribution into the cell was observed when cells were infected with the control virus. However, expression of BV2-5 during infection was affecting the standard distribution of actin during baculovirus infection, supporting the physical interaction of BV2-5 with cellular cytoskeleton. To support such interaction hypothesis, we treated Sf21-AcMNPV-GFP infected cells with latrunculin A, an inhibitor of the actin polymerization. A similar distribution of actin was observed in cells infected with AcMNPV-BV2-5-GFP and in cells infected with Sf21-AcMNPV-GFP and treated with latrunculin A (Fig. 2.3; Phalloidin channel).

2.3.3. Expression of BV2-5 interferes with the viral infection

In the previous chapter we have reported a negative effect of *BV2-5* expression on baculovirus multiplication. Moreover, Ohkawa *et al* (Ohkawa *et al*., 2010) have shown that AcMNPV utilizes actin-based motility for a successful collision with the nuclear envelop and translocation into the nucleus. The data described above shows differences in the distribution of the actin in the presence or absence of the BV2-5 protein. To further study the possible effect of BV2-5 on baculovirus infection, we used fully functional recombinant viruses expressing polyhedral protein as well as BV2-5 or its truncated form (Fig. 2.1 B). Microscopic observation of Sf21 cells infected by the different recombinant baculoviruses revealed that, in the presence of BV2-5, the cellular symptoms of viral infection were reduced when compared to the control virus or to the virus expressing the truncated protein (Fig. 2.4 A). That was reflected in a significant decrease in the percentage of cells showing the characteristic presence of viral occlusion bodies (Fig. 2.4 A). Four

days post infection, the presence of viral OBs was observed in about 40% of the cells infected with the control virus or infected with the virus expressing the truncated form of BV2-5 (Fig. 2.4 B). In contrast, in the presence of BV2-5 or latrunculin A, only about 20% of the cells showed the formation of OBs. We used transmission electronic microscope to visualize the occlusion bodies (OBs) produced by each recombinant virus. Remarkably, the microscopy study showed that OBs in BV2-5 expressing virus, as well as the virus in the presence of latrunculin A, had less regular shapes and smaller size in general (Fig 2.4 C, and Fig. 2.5). In addition, a dramatic reduction in the number of occlusion derived viruses (ODVs) packed in each OB was detected when compared to the control virus (Figs. 2.4 D and Fig. 2.5).

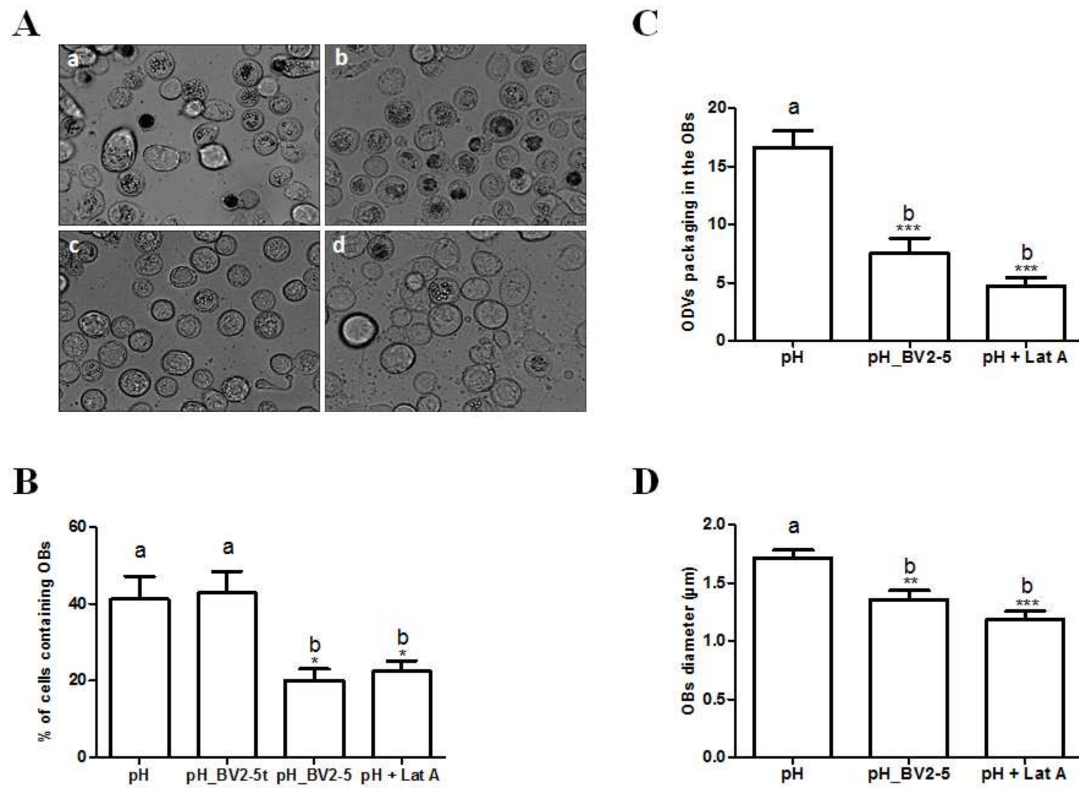

Figure 2.4. The effect of BV2-5 on the AcMNPV infective process. **A)** Microscopic observations of the OB production for each recombinant baculovirus; a: Sf21 cells infected with *Δcc-pH*; b: cells infected with *Δcc-pH_ BV2-5 t*; c: cells infected with *Δcc-pH_ BV2-5*; d: cells infected with *Δcc-pH* and treated with latrunculin. **B)** Percentage of the cells containing OBs for each treatment. **C)** The number of ODVs packaged in each OB. **D)** Comparison of the diameter of the OBs produced by each virus. Bars represents means ± SEM. Means were analysed by Dunnett's test and different letters denote significant differences. The * represent p<0.05, ** represent p<0.001 and *** represent p<0.0001.

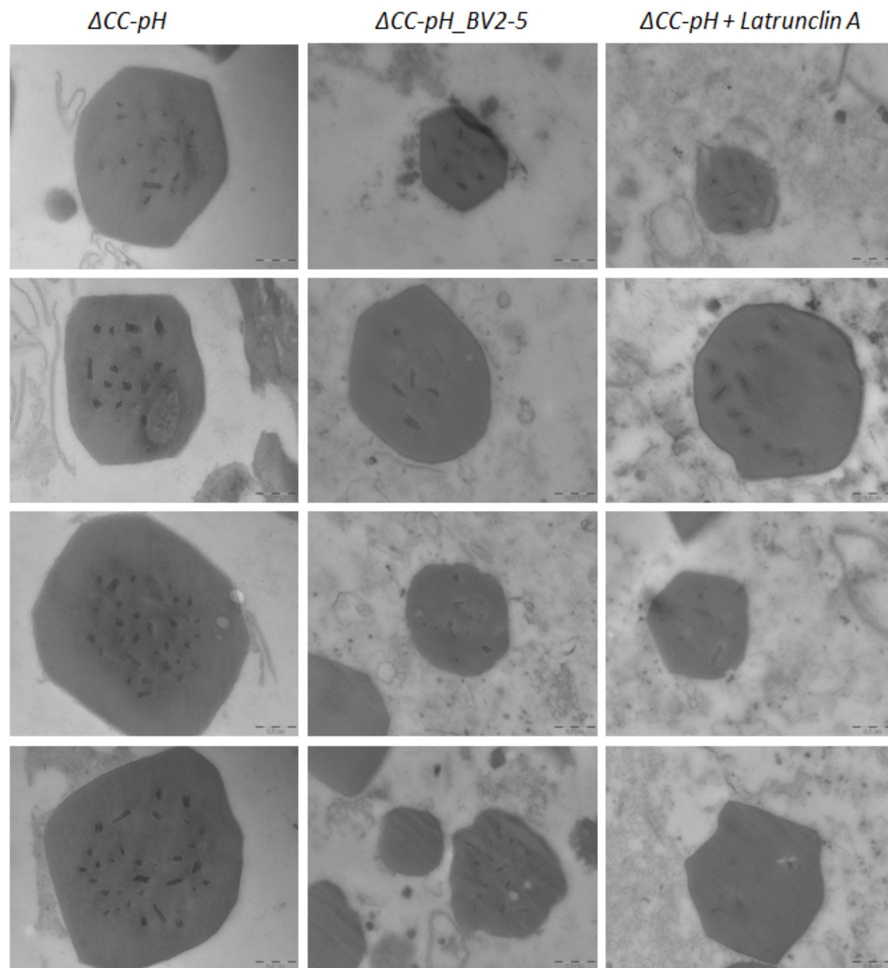

Figure 2.5. Effect of BV2-5 on polyhedra formation. Microphotographies of occlusion bodies produced by the different recombinant viruses (ΔCC-pH, ΔCC-pH_ BV2-5, ΔCC-pH + latrunclin A) observed by transmission electron microscopy.

2.3.4. Expression of BV2-5 interferes with the insect cells phagocytic activity

Previous results on the gene expression pattern of *BV2-5* revealed that, though expressed in different tissues at the larval stage, *BV2-5* was mainly expressed

in the larval hemocytes. Hemocytes are targeted by PDV-derived proteins in order to block the cellular response of the parasitized larvae and prevent encapsulation and phagocytosis of the injected eggs (Amaya *et al*., 2005). A trademark of phagocytosis is the exquisitely localized actin polymerization that takes place right underneath the phagocytic target (Castellano *et al*., 2001, Kochubey *et al.*, 2006). We thus hypothesize that BV2-5 could also interfere with the phagocytic activity of the insect hemocytes. We designed a set of experiments to test such hypothesis.

In order to investigate our hypothesis, we infected third instar *S. exigua* larvae with the recombinant baculoviruses described above. After a lapse time of 48 hours, that allowed the infection of hemocytes and, in consequence, the expression of BV2-5 and BV2-5 t, we injected the larvae with *B. thuringiensis* expressing GFP. The phagocytic ability of hemocytes was observed under confocal microscope as the presence of the GFP signal inside the hemocytes. No effect of baculovirus infection on the phagocytosis ability of the cells was observed (Fig. 2.6 A and 2.6 B). However, in the presence of BV2-5, the ability of the hemocytes to phagocytize the foreign bacteria was significantly reduced (Fig 2.6 A and 2.6 B). Again, the truncated form of BV2-5 did not show any effect on cell phagocytosis.

During the course of this study, mortality of the larvae after baculovirus infection and bacterial injection was also measured. At the injected dose, no differences in the median survival time of larvae infected only with the different types of viruses were observed, 84 hours for all of them (Fig. 2.6 C). However, when the baculovirus-infected larvae were exposed to Bt, the presence of BV2-5 resulted in increased susceptibility of the insects to *B. thuringiensis* reflected in the reduction of the median survival time of the larvae , from 60 hours to 36 hours (Fig 2.6 D) (Mentel-cox test, $n=3$, $p<0.0001$). These results suggest the disruption of actin polymerization by BV2-5 may lead to the loss of bacterial endocytosis by hemocytes, increasing the insect susceptibility to bacterial infections.

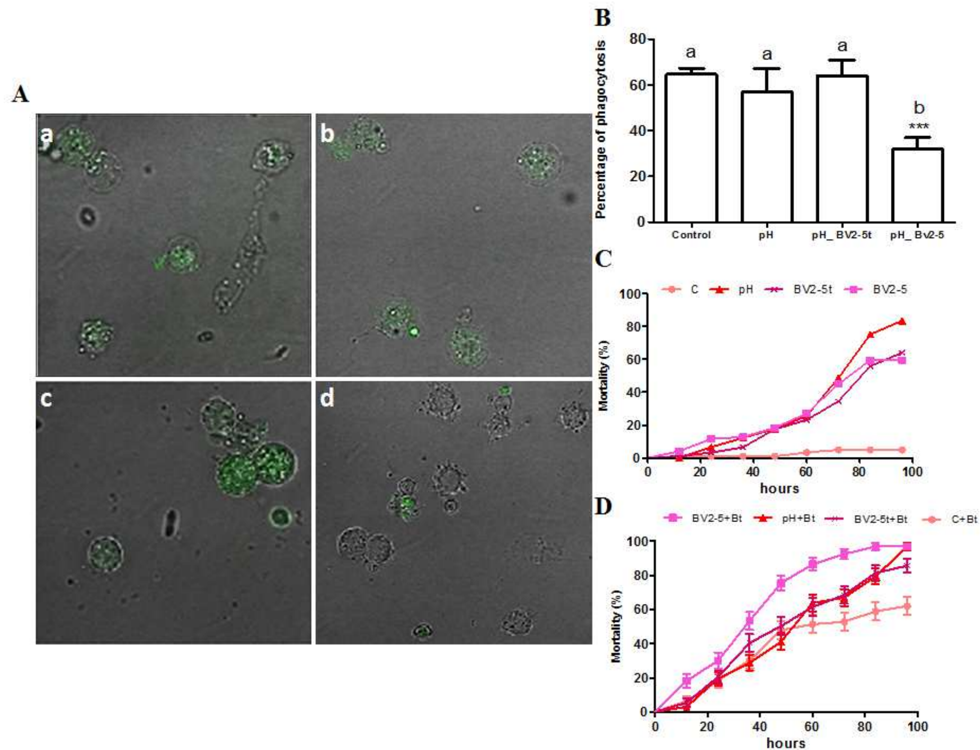

Figure 2.6. Effect of BV2-5 on the insect-bacteria interaction. **A)** Phagocytic activity of *S. exigua* hemocytes against *Bacillus thurigiensis*. a: control larvae; b: larvae infected with *AcMNPV-pH*; c: larvae infected with *AcMNPV-pH_ BV2-5 t*; d: larvae infected with *AcMNPV-pH_ BV2-5*. **B)** Percentage of phagocytosis in presence or absence of BV2-5. The bars represent the mean ± SEM. Means were analysed by Dunnett's test and different letters denote significant differences. The *** represent $p<0.0001$. **C)** Survival curves of larvae treated by *B. thuringiensis* in the presence or absence of BV2-5. The survival curves were plotted using the Kaplan-Meier method. Statistical significance was determined using Mantel-Cox test ($p<0.0001$). **D)** Survival curves of larvae treated with the different baculoviruses in absence of *B. thuringiensis* injection.

2.4. Discussion

BV2-5 was initially identified as a PDV-related gene in the transcriptome of *S. exigua* larvae exposed to different types of pathogens (Pascual *et al.*, 2012).

Further studies revealed the early acquisition of this gene by horizontal transfer between PDVs and an ancestral *Spodoptera* sp, suggesting the host domestication of that gene in order to reduce insect susceptibility to pathogens such as baculovirus. It is well known that polydnavirus manipulate the lepidopteran host immunity by expressing immunosuppressive proteins able to interfere with the hemocytes activity (Asgari *et al*., 1996, Hu *et al*., 2003 ,Ibrahim and Kim, 2006, Strand *et al*., 2006, Turnbull *et al*., 2004). In agreement with that, we have observed the highest levels of expression of BV2-5 in hemocytes. However, we have found that intrahaemocoelic injection of the larvae (somehow resembling the wasp injection), instead of activating the expression of *BV2-5* reduced its expression. It is possible that host domestication of *BV2*-5 and its repression in response to parasitism is a mechanism to counteract the parasitism. In contrast to its down-regulation after injection with PBS, we have found strong up-regulation when larvae were injected with pathogenic bacteria such as *B. thuringiensis.* Such regulation was bacteria-specific since no up-regulation of *BV2-5* was found when larvae were injected with non-pathogenic bacteria, independently of being a Gram positive (*M. luteus*) or Gram negative (*E. coli*) bacterial species. *B. thuringiensis* is a Gram positive entomopathogenic bacterium that produces a set of virulence factors that have detrimental effects against the host cells. Given the absence of response against another Gram positive species (*M. luteus*), it is more likely that the regulation observed on *BV2-5* was produced in response to the damage produced by the bacteria than to the presence of bacterial elicitors (pathogen-associated molecular patterns, PAMs). Further studies focusing on the expression pattern of *BV2-5* during the parasitic process or other type of pathogenic attacks would contribute to better understanding of the biological significance of BV2-5 acquisition and may confirm the latter hypothesis.

In this work we have focused on the functional aspects of BV2-5 trying to discover its molecular role that could contribute to explain the horizontal transfer of *BV2-5* into the host and its further domestication. In that sense, our results suggest

that BV2-5 is an actin-interacting protein interfering with multiple cellular processes. Actin is one of the most abundant and highly conserved proteins in eukaryotes being one of the major components of the microfilament network of the cytoskeleton (Pollard and Cooper, 2009). Tight regulation of filamentous actin (F-actin) assembly and disassembly at specific sites in the cell is crucial for the cell life and functions. It has been shown that dynamic actin networks are involved in cell migration, cytoplasmic organization, ingression of the cytokinesis, tissue morphogenesis, cellular endocytosis, and other cellular mechanisms (Cardamone *et al.*, 2011, Holubcová *et al.*, 2013, Pollard and Cooper, 2009). It has been also proved that the entry of many viruses into the cell is actin dependent (Cudmore *et al.*, 1997). Such is the case of the baculovirus nucleocapsids movement towards the nucleus (Ploubidou and Way, 2001). Immediately after baculovirus entrance into insect cells, cables of polymerized actin (F-actin) can be detected within the cytoplasm in association with viral nucleocapsids which are transported from the cytoplasmic membrane to the nucleus (Wang *et al.*, 2010, Ohkawa *et al.*, 2002). In the nucleus, another arrangement of actin occurs and F-actin appears within the central virogenic stroma, where viral DNA synthesis and nucleocapsids assembly occurs (Goley *et al.*, 2006, Ohkawa *et al*., 2002). The data presented in this study confirms that baculovirus infection affects the actin distribution in Sf21-infected cells. This actin arrangement was disrupted in the presence of BV2-5 as well as with the inhibitor of actin polymerization; latrunculin A. Accordingly, the negative effect of BV2-5 in virus infectivity, multiplication, and OB formation and assembly is likely due to the disruption of the actin dynamics during the viral infection.

In this and in the previous chapters, we have observed that the presence of functional BV2-5 could increase the larval tolerance to baculovirus. In contrast, the opposite effect has been observed regarding the insect interaction with bacterial pathogens. BV2-5 expression on larval hemocytes inhibited its phagocytic ability. In the presence of the functional form of this protein, the hemocytes lose their ability to phagocytize *B. thuringiensis* which is reflected in an increase in the larval

susceptibility to the bacterial infection when compared to larvae exposed to the truncated form of the protein. These results are in agreement with the role of BV2-5 in disrupting actin rearrangement. It is well known that polydnavirus manipulates the lepidpteran host immunity by expressing immunosuppressive proteins able to interfere with the hemocytes activity (Asgari *et al.*, 1996, Hu *et al.*, 2003, Ibraham and Kim, 2006, Strand *et al*., 2006, Turnbull *et al*., 2004). The main mechanism described to cause the loss of hemocytes' activity is that PDVs express a number of their proteins in the hemocytes that interfere with the cytoskeletal actin. The F-actin cytoskeleton appears to be disrupted by many polydnaviruses which leads to the failure of hemocytes to adhere to the parasitoid eggs (Turnbull *et al.*, 2004).

So far, closest homologs to BV2-5 in non *Spodoptera* spp are members of the family 2 proteins from *Cotesia congregata* bracovirus (CcBV f2) (Espagne *et al.*, 2004) (Fig 2.7A). This family is composed by six proteins of unknown function in CcBV and orthologs have also been found in other species of polydnaviruses and parasitic wasps. Given their sequence homology with BV2-5, it is likely that those proteins are able to interact with actin compromising the host immune competence by inhibiting the adherence of hemocytes to the parasitoid eggs. These and 21 additional homolog proteins from other PDVs and wasp species at NCBI were analyzed for motif discovery using MEME (Multiple Em for Motif Elicitation) software (Bailey *et al*., 2009). Two conserved motifs of 77 and 41 amino acids were identified in all the proteins (Fig. 2.7 B). Further studies in the interaction of the conserved domains with cytoskeleton proteins could contribute to delimit the functional domain in this family of proteins. Based on the results reported here on the BV2-5 interaction with actin we tentatively proposed to name this protein as SeGasmin.

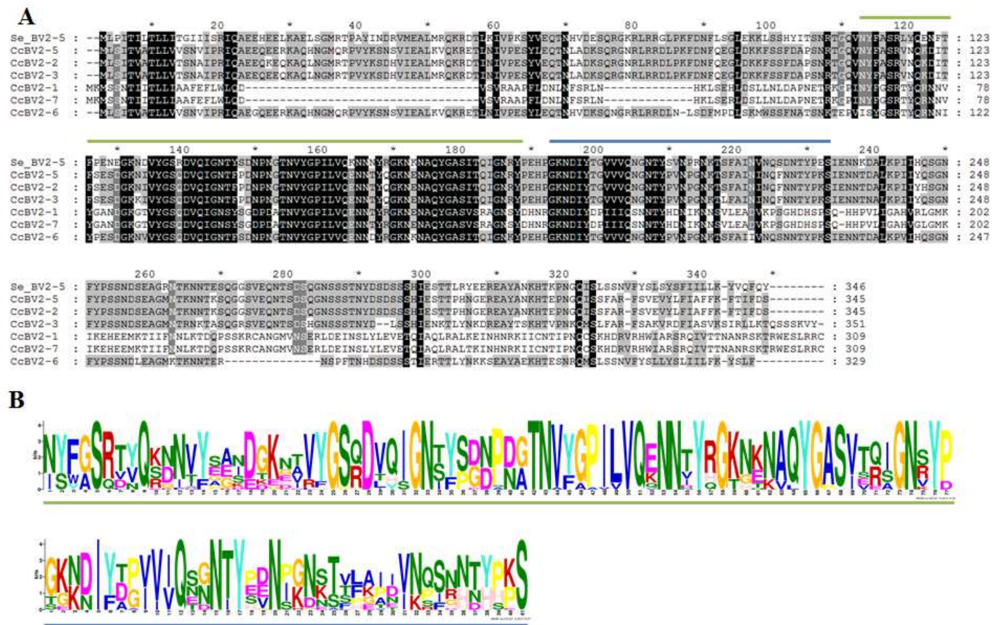

Figure 2.7. BV2-5 homologs in other bracoviruses and wasps. **A)** ClustalX alignment of the deduced amino acid sequences from BV2-5 and its homologs in CCBV. **B)** Conserved motifs identified by MEME analysis in homologs to BV2-5 found in bracoviruses and wasps. The location of the motifs in the aligned proteins is indicated by the color lines.

Although American and Asiatic populations have conserved the *BV2-5* gene, a point mutation has occurred in European populations and a non-functional truncated form of the protein has been conserved. This mutational distribution can be caused by neutral evolution but it is likely to be indicative of an ancient integration and further host adaptation to the different environments. Indeed, horizontal gene transfer of bacterial-derived genes to the lepidopteran host germ line can confer adaptive advantages to the host which can increase its survival and fecundity (Sun *et al*., 2013). According to our results and the pathogens tested, the presence of a functional form of *BV2-5* has a different impact on the host fitness. The expression of BV2-5 has a positive impact on the host interaction with baculovirus, but a negative impact on its interaction with *B. thuringiensis.* In this context,

American and Asiatic populations have conserved the functional form of the protein that is more likely helping the insect to escape baculovirus infection. However, the same protein apparently increases the susceptibility of the insect to bacterial infection. It is tempting to speculate that this allele distribution could be the result of an evolutionary trade-off modulated according to type of pathogens and its selection pressure. In this study we have only tested the effect of BV2-5 on insect susceptibility to two types of pathogens, though the number and types of pathogens and parasitoids, and their selective pressure under field conditions is very diverse. Further studies on the incidence of the main pathogen in each geographic region may contribute to explain the geographic distribution of the functional/non-functional protein.

In summary, by a combination of different approaches we have found that SeGasmin, a PDV-derived protein, is able to interact with the cellular cytoskeleton interfering with the normal actin arrangement and affecting in different ways the insect's interaction with pathogens. Sequence similarity to a family of proteins present in several species of PDVs suggest a related role for those proteins and sheds light about the mechanisms of host immunosuppression and hemocytes dysfunction occurring during wasp parasitism.

CHAPTER 3

Lectins from *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), a protein family involved in the immune response of the insect

3.1. Introduction

Insects are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites. In order to survive in such environment, they have developed an efficient immune system (Gillespie and Kanost, 1997). Although they lack an adaptive immunity, the insects are able to survive surrounded by a wide range of pathogens due to the innate immune system which is the only defence system available for invertebrates. A crucial component of the innate immune system is a mechanism for surveillance by which an organism can detect the presence of foreign invaders and defend themselves. In order to recognize the "non-self-molecules", they utilize proteins known as pattern recognition proteins (PRPs) or receptors which are able to bind to common molecules of the membrane of microorganisms (Eleftherianos *et al*., 2007). These molecules are referred to as pathogen-associated molecular patterns (PAMPs). They are unique to microbes, not produced by the host and are invariant among different classes of microorganisms. They include microbial surface molecules or cell wall components such as bacterial lipopolysaccharides (LPS), lipoteichoic acid (LTA) and peptidoglycan, as well as fungal β-1,3-glucan (Luig and Yu, 2007; Janeway and Medzhitov, 2002).

Lectins are among the best studied and diversified protein families of PRPs. They can be defined as proteins that recognize glycoconjugates in microbial cell surface. In 1988, Drickamer proposed to classify animal lectins into different categories; among them Ca^{2+}-dependent lectins were classified as the C-type lectin group. C-type lectins are the first described and the most diverse family of animal lectins. Pathogen agglutination activity of the snake venom C-type lectins was reported as early as 1860 (Kilpatrick, 2002). The members of this protein family are known to contain carbohydrate recognition domains (CRDs) homologous to each other although they mediate variable interactions by binding specifically to diverse sugars (Weis *et al*., 1998). Even though insect lectins are not well characterized, a set of C-type lectins which contain one or two carbohydrate recognition domains have been identified. Analyses of the whole genome of the insect *Drosophila melanogaster*

have shown that the C-type-lectin family is as abundant and diverse as it is in the vertebrates (Wang *et al.,* 2012). Due to the absence of antibody based immunity, insect lectins may have a crucial role in the recognition of pathogens. If lectins act in an analogous way to antibodies, a multitude of lectins that have different carbohydrate-binding specificity have to be present in the insect circulatory system. In lepidopteran species, lectins have been shown to be widely distributed in the tissues and body fluids (Mosson *et al*., 2000). The best studied lectins in Lepidoptera are hemolymph lectins and fat body lectins. In addition to be involved in various developmental events including regeneration, moulting, metamorphosis and embryogenesis (Mosson *et al.,* 2000), they have been shown to play a major role in the insects' immune system. They are involved in diverse immune responses such as phagocytosis, encapsulation, nodule formation, hemolymph coagulation and prophenoloxidase cascade (Chai *et al.,* 2008; Yu *et al*., 2002; Yu *et al*., 2003).

In a previous study carried out by our group, the *Spodoptera exigua* transcriptome was characterized (Pascual *et al*., 2012), which allowed the generation of a large public set of ESTs (ncbi accession number: TSA GAOQ00000000). Blast analyses showed the presence of large number of unigenes encoding proteins that contained lectin-like domains (at least thirty-two). Due to the large number of these proteins in the *S. exigua* transcriptome and to the important role that they may play in the immune system of lepidopterans, we decided to further study this family of proteins.

In the present study we have chosen ten *S. exigua* unigenes with homology to C-type lectins. Sequence analysis allowed dividing the chosen lectins into two groups according to their phylogenetic origin, which were subsequently compared to other lectin-like genes. We investigated the possible roles of the lectins in the insect's immune system and we provided for the first time clear evidence about the role of some lectins as antiviral proteins.

3.2. Materials and Methods

3.2.1. Insect rearing

Spodoptera exigua colonies FRA, SUI and MESUI were reared continuously on artificial diet at 25± 3°C with 70±5% relative humidity and a photoperiod of 16h light: 8h dark. FRA insects were kindly supplied by M. Lopez-Ferber, INRA (St.Christol les Alés, France), and SUI insects were kindly provided by Andermatt Biocontrol AG (Grossdietwil, Switzerland). MESUI is maintained in our laboratory and is the result of the crossing of SUI population and a Mexican population kindly provided by P. Caballero (Universidad Publica de Navarra).

3.2.2. Sequence analysis

ORFs derived from the selected *S. exigua* unigenes with homology to C-type lectins were predicted using the EditSeq program (DNAstar package, Madison, WI) and the search for homologous proteins in other species was conducted using BLASTp (http: //www.ncbi.nlm.nhi.gov/). The predicted amino acid sequences were aligned using the ClustalX software (Thompson *et al*., 1997) and visualized with the GenDoc program (Nicholas *et al*., 1997). Evolutionary distance was calculated for aligned sequences by Maximum-likelhood method (Saitou and Nei, 1987) and the phylogenetic trees were constructed using MEGA5 program (Tanura *et al*., 2011). 1000 replicates were analyzed by the bootstrap analysis.

3.2.3. Analysis of the expression pattern and the response to pathogens

The presence and abundance of mRNA of the studied lectins in different larval tissues were analyzed by quantitative reverse transcription PCR (qPCR) as described previously (Chapter1). Briefly, total RNAs from fat body, midgut and hemocytes were isolated from untreated 5th instar FRA larvae using the RNAzol reagent (Molecular research center, INC) as described in the manufacturer's protocol. A total of 1 µg RNA was reverse transcribed into cDNA with oligo-(dT) primer using SuperScript™ II reverse transcriptase (Invitrogen). cDNAs were used

to determine the level of transcripts for each gene by qPCR. Reactions were carried out using an ABI Prism 7700 thermocycler from Applied Biosystems. SYBR green Ex Taq™ master mix (Clontech) was employed in a total volume of 20 µl. Specific primers for each gene were designed by Primer Express Software (Applied Biosystems) (Table 3.1). For each gene, at least three biological replicates were employed. Data are presented as fold change using the method of $2^{-\Delta\Delta Ct}$ and normalized to the internal control gene, ATP synthase. Expression enrichment in the analyzed tissues was observed by estimation of the expression level from the three studied tissues.

To study the change in expression profile of the lectins after intrahaemocoelical injection with different bacterial pathogens, *S. exigua* FRA fourth instar larvae were immunized with 5 µl of 10^7 cells/ml from *Micrococcus luteus*, *Escherichia coli* or *Bacillus thuringiensis*. Bacterial preparations were previously inactivated by heat shock treatment (120°C, 30min) and resuspended in PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 27 mM KCl; pH 7.0). Control larvae were injected with the same volume of PBS. In order to check the effect of PBS injection in gene expression and additional control without injection was employed. Hemocytes, midgut and fat body were collected at 8 h and 24 h post injection.

The effect of viral infection on the expression pattern of the lectins in the larval midgut was also determined. For baculovirus infection, Third instar larvae (SUI colony) were orally infected with *S. exigua* nucleopolyhedrovirus SeMNPV. Each larva was fed with 10^4 occlusion bodies (OBs). Midguts of treated and untreated larvae were collected 72 h after treatment. For densovirus infection, fourth instar larvae of MESUI population were used to investigate the effect of *Janonia coenia* densovirus JcDNV on the lectins' expression pattern. Each larva was fed with JcDNV-contaminated food. 10 µL from a solution of 10^{10} copies of JcDNV per mL were added to a diet disk. 72 hours post infection, hemocytes, midgut and fat body were collected from infected and non-infected larvae that were used as control. Total RNA from the treated and control larvae were collected as described above. cDNA was reverse-

transcribed and the presence and abundance of the mRNA was determined using qPCR as described above.

Table 3.1. Primers used in qPCR reactions and for the cloning of *Se-BLL2* (*Se-BLL2* ORF).

Gene name	Forward primer	Reverse primer
Se-LL1	GAATAGCGGCTCCTTCATCAGA	TCTCACACACAAACATGGATTTCTT
Se-LL2	AGGATTCTTGTACCCGGACAAC	GGCACGATTCGTCTTCAGTTTT
Se-LL3	ACCGAGGGCTGCCATTC	AAACATTGCAGTGTCGGTCGTA
Se-LL4	ACGCTCGCAGTAGCACAGAA	TTTGGTCTGCTAGCGCATTTC
Se-BLL1	ACAACCCAGCAACCCAACA	CCATGGCTCCTTGTAGCAACA
Se-BLL2	TCCAGGACCTCAGAACACAGAA	GGGCTTCATCAAATGTTGCA
Se-BLL3	GTGGAAATCTTGCCGTCGTT	TCCACAGAGCCAGCATTTCA
Se-BLL4	GGAAGTGGGCTATGGCAAAC	GGTTGTACCCCCATCCAGTTG
Se-BLL5	AATGCTGGCCCTGTGGAA	GCTTGAGCATTCAATCCATGTG
Se-BLL6	ACAGCCAAAGTGGGCAATG	CGTTGCGACTCCAGTTAAGGT
Se-BLL2 ORF (cloning)	GGTCTAGAATGATAAAATCAATTTATTTAAT	CTCGAGCTAATGGTGATGGTGAACATCGCAATCACAAGGTTGGAC

3.2.4. Recombinant expression and purification of Se-BLL2

Specific primers (Table 3.1) were designed to amplify the complete ORF of *Se-BLL2* from the cDNA obtained from the fat body of fifth instar *S. exigua* larvae. Those primers were designed to add *Xba*I restriction site at the 5' end of the gene and *Xho*I site at the 3' side. A sequence for 6xHis-tag was also included in the reverse primer (C-terminal part of the expressed protein). After digestion of PCR product with *Xba*I and *Xho*I, the DNA was ligated into the expression vector pET-16b and transformed into *E. coli* XL-Blue competent cells. After selection of positive clones, recombinant pET-6b_BLL2 was transformed into *E. coli* BL21 competent cells. For production of recombinant protein, the overnight culture (15 ml) of an individual colony was inoculated to 1.5 L of LB medium and incubated at 37°C in an orbital shaker until the OD_{600} reached 0.8-1, then isopropyl β-D-1-thiogalactopyranoside

(IPTG) was added at a final concentration of 1 mM and incubated overnight with agitation. Bacterial cells were harvested by centrifugation, suspended in 20 mM Tris-HCl (pH 8) and lysed by sonication (five times for 60 s with a 10 s pause in between). After centrifugation (15 min at 17000 x*g*), the pellet was resuspended in solubilisation buffer 1 (20 mMM Tris-HCl, 2 M urea, 2% Triton X-100 and 0.5 M NaCl, pH 8). A second step of sonication was performed and the pellet obtained after centrifugation was resuspended in the solubilisation buffer 2 (20 mM Tris-HCl, 6 M guanidine hydrochloride, 0.5 mM β-mercaptoethanol, 5 mM imidazole and 0.5 M NaCl, pH 8). After incubation for 30 min at room temperature, the mixture was centrifuged at 17000 x*g* and the supernatant was collected and filtered through a 0.22 μm filter. This lysate supernatant was used for further purification.

Affinity chromatography purification of Se-BLL2 from the second step of sonication was carried out using the HiTrap™ Chelating HP column (GE Healthcare). The solution was loaded on the column and proteins were refolded with a decreasing urea gradient (6 M to 0 M urea in 20 mM Tris-HCl, 20 mM imidazole, 0.5 M NaCl and 0.5 mM β-mercaptoethanol, pH 8). The retained proteins were eluted with a discontinuous imidazole gradient (50 mM to 500 mM imidazole in 20 mM Tris-HCl, 0.5 M NaCl, 0.5 mM β-mercaptoethanol). Aliquots of the elution fractions were analysed by 12% SDS-PAGE and the gel was stained with Coomassie blue. Fractions containing the purified Se-BLL2 protein was dialyzed overnight against 20 mM Tris-HCl, pH 8, and quantified by the Bradford's method.

3.2.5. Effect of Se-BLL2 on larval mortality

S. exigua third instar larvae were infected with SeMNPV using the drop feeding method. 5×10^5 OBs were added to a solution containing sucrose and phenol red colorant (10% and 0.05% respectively) in presence or absence of purified Se-BLL2 (Chapter 1). The same experiment was performed infecting third instar larvae of MESUI population with the densovirus JcDNV using a concentration of 10^8 viral copies/mL. In both bioassays, mortality was recorded every 12 h until death or

pupation of all the larvae. Sixteen larvae were used for each treatment and three independent replicates were performed. Mortality was expressed as the percentage of dead larvae. The time to death was assessed by comparing the mortality curves using the Kaplan Meier method (GraphPad Prism 5). The statistical significance was determined using the log-rank analysis (Mantel-cox test).

3.3. Results:

3.3.1. Sequence analysis and tissue distribution

Phylogenetic analysis of the selected *S. exigua* lectins has showed that they clustered in two different groups (Fig. 3.1 A). The first group consisted of four lectins highly homologous to other lepidopteran lectins, whereas the second group consisted of six proteins with high homologies to lectins from bracovirus. We named the proteins in the first group *S. exigua* lepidopteran-like lectins (Se-LL) and those in the second group *S. exigua* bracovirus-like lectins (Se-BLL). Interestingly, their phylogenetic clustering agree with their expression pattern in the main larval tissues: fat body, hemocytes and midgut. (Fig 3.1 B and C). The lepidopteran-like lectins were mainly expressed in fat body, while the bracovirus-like lectins were mainly expressed in hemocytes. In general, the expression level in the midgut was the lowest for the tested lectins (Fig. 3.1 A). The differences in the expression pattern, together with their phylogenetic distribution may reflect their different origins or functions.

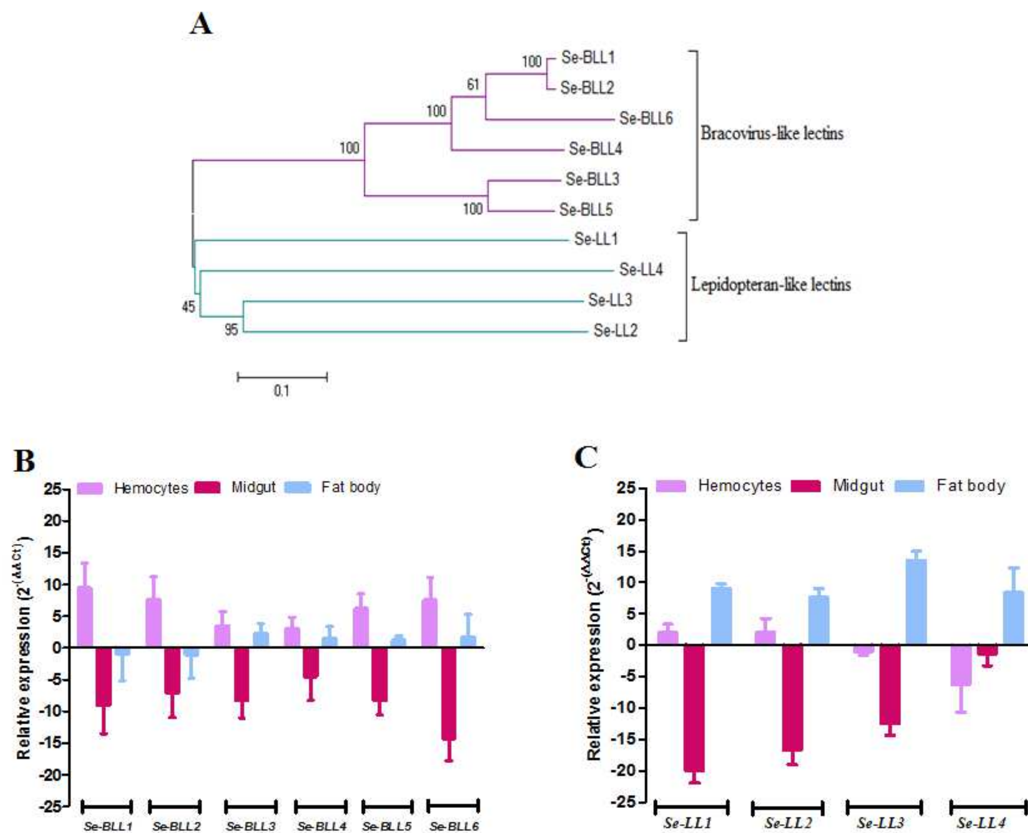

Figure 3.1. Phylogenetic analysis and larval tissue distribution of selected *Spodoptera exigua* lectins. **A)** Phylogenetic relationship of the studied *S. exigua* lectins. **B)** Relative expression on the main larval tissues of BLLs. **C)** Tissue distribution of LLs. Expression level for each tissue was normalized against the average expression in all the tissues. The results are represented as the mean ± standard deviation of three independent replicates.

To examine the relationship between *S. exigua* lepidopteran-like lectins and other lepidopteran lectins, they were aligned with selected lectins representative from different orders of Lepidoptera such as *Manduca sexta, Bombyx mori, Helicoverpa armigera, Mamestra configurata,* and *Ostrinia furnacalis*. The results of

the alignment were used to construct a maximum-likelihood tree (Fig. 3.2 A, Annex 1.4 A). The phylogenetic tree revealed that the *S. exigua* lepidopteran-like lectins did not cluster in a single group but instead were distributed with their orthologs in different species. Only Se-LL4 did not cluster with any of the aligned lectins being specific from *S. exigua*.

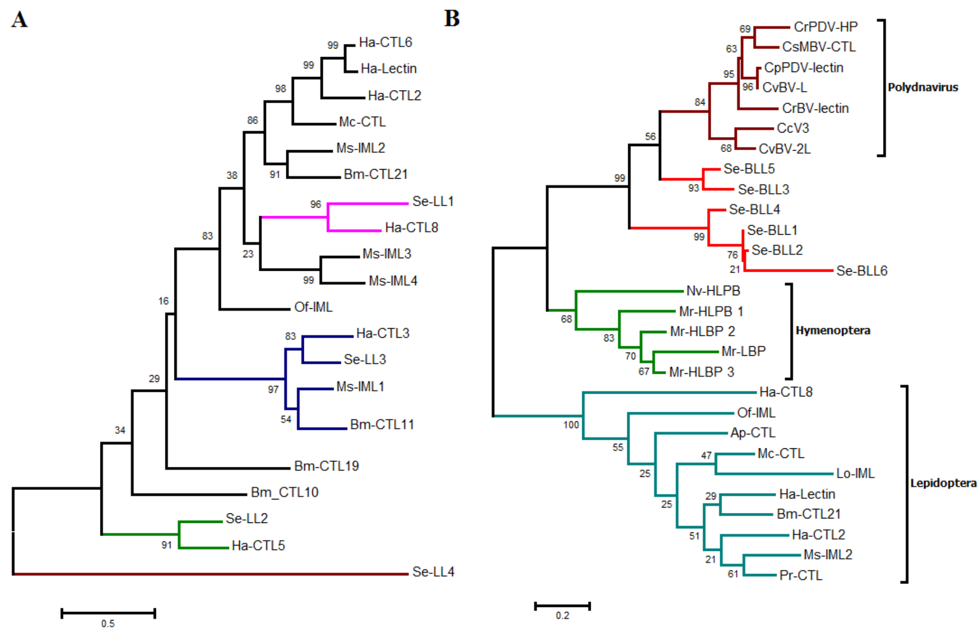

Figure 3.2. Phylogenetic trees derived from the ClustalX alignment of C-type lectins. The evolutionary distance was calculated for aligned sequences by Maximum likelihood method. The numbers on the branch represent bootstrap values. **A)** Phylogenetic analysis of *S. exigua* lepidopteran-like lectins compared with lepidopteran lectins from different species. **B)** Phylogenetic analysis of *S. exigua* bracovirus-like lectins aligned with lectins from Polydnavirus, Hymenoptera and Lepidoptera species.

The *S. exigua* bracovirus-like lectins were aligned with polydnaviral lectins, lepidopteran lectins and lectins from parasitic hymenopteran. The resulting

maximum-likelihood tree revealed that the six Se-BLLs clearly clustered together with the polydnaviral lectins (Fig. 3.2 B, Annex 1.4 B). They are more closely related to the hymenopteran lectins than to their closest lepidopteran lectins available in the public databases. These observations could be explained by the bracoviral origin of these lectins.

Analyses of the deduced amino acid sequences of Se-LL1, Se-LL2, Se-LL3 and Se-LL4 (Fig 3.3 A, Annex 1.4 A) showed that they belong to the C-type lectins with dual carbohydrate recognition domains (CRDs). All of them contain a short-form CRD in the N-terminal part and a long-form CRD in the C-terminous. In general, lepidopteran lectins contain four conserved cysteine residues in the N-terminal CRD and six in the C-terminal CRD. Remarkably, only Se-LL3 contains all the cysteine residues. Se-LL1 lacks one cys residue in the N-terminal CRD and three in the C-terminal part, Se-LL2 lacks two cys residues in each CRD. Se-LL4 contains only two cysteine residues, in the long-form CRD.

Most animal C-type lectins bind D-mannose, D-glucose and related sugars (Man-type ligands), or to D-galactose and its derivates (Gal-type ligands). Accordingly, the Man-type ligands contain in their sequences a conserved EPN motif and the Gal-type ligands - a QPD motif (Kolatkar and Weis, 1996). The C-terminal CRD of Se-LL1 contains a QPD (Gln-Pro-Asp) motif characteristic for galactose binding CRD, whereas, Se-LL3 contains a specific mannose binding motif EPN (Glu-Pro-Asn). In addition to those two lectins, the corresponding sites of Se-LL4 and Se-LL2 include GTS (Gly-Thr-Ser) and QNG (Glu-Asn-Gly) motifs, respectively. Moreover, Se-LL2, Se-LL3 and Se-LL1 show a conserved PXXC motif at the end of the short-form CRD and a conserved FXCE motif in the long-form CRD. Interestingly, besides the fact that Se-LL4 lacks the majority of the cysteine conserved residues, it also lacks both conserved motifs (Fig. 3.3 B, Annex 1.4 B).

Figure 3.3. ClustalX alignment of the deduced amino acid sequences of *S. exigua* lepidopteran-like lectins aligned with other lepidopteran lectins (A) and ClustalX alignment of the deduced amino acid sequences of *S. exigua* bracovirus-like lectins compared with other lectins from Polydnavirus, Hymenoptera and Lepidoptera species (B). Identical amino acids for all the aligned sequences are shaded in black and amino acids with lower identity matches are shaded in gray. The carbohydrate recognition domain (CRD) are boxed. Inverted triangles indicate the conserved cysteine residues in C-type lectins. Putative specific carbohydrate binding motifs are indicated by a horizontal line. Numbers on the right represent the position of the last amino acid at the end of the row for each protein (see clearer images in Annex 1.4.).

3.3.2. Transcriptional response of *S. exigua* lectins to bacterial and viral pathogens

In order to further explore the possible function of *S. exigua* lectins, we investigated the change in their expression levels after challenge with different microorganisms (Fig. 3.4, Annex 1.5, and Annex 1.6). *S. exigua* fourth instar larvae were intrahaemocoelic injected with *E. coli* as representative of Gram-negative bacteria and *M. luteus* and *B. thuringiensis* as representatives of non-pathogenic and pathogenic Gram-positive bacteria, respectively. In general, the injection with only PBS emulating an injury induced the down-regulation of most of the lectins in midgut and hemocytes (Fig 3.4 A, Annex 1.5). Such down-regulation was more obvious for all the bracovirus-like lectins at 8 hours post injection (hpi). However, no common pattern of regulation was detected for the different tissues, treatments, and lectin groups. (Fig. 3.4 A). Among the changes observed in hemocytes, *Se-BLL1* was down regulated in response to *E. coli*, *Se-BLL6* was up regulated against *M. luteus* and *Se-BLL4* was up regulated in response to the three bacterial species (Annex 1.6 A). In fat body, *Se-BLL2* was up regulated in response to the three bacteria (Annex 1.6 B). In the midgut, *Se-BLL1* and *Se-BLL2* expression was induced in response to *M. luteus* (Annex 1.6 C). Regarding the lepidopteran-like lectins, no significative changes were detected in any of the conditions.

Moreover, we tested the response of the insect lectins to viral infections with two entomopathogenic viruses, baculovirus and densovirus. Three lectins, *Se-BLL2*, *Se-BLL6* and *Se-LL3*, were induced in the midgut in response to the SeMNPV infection (Fig. 3.4 B). In addition, *Se-BLL1*, *Se-BLL2*, *Se-BLL4* and *Se-LL2* were significantly repressed in response to JcDNV in hemocytes Whereas, *Se-BLL1* and *Se-BLL4* were induced in fat body and midgut respectively (Fig. 3.4 C). These results suggest that *S. exigua* lectins could be involved in the immune response to viral infection.

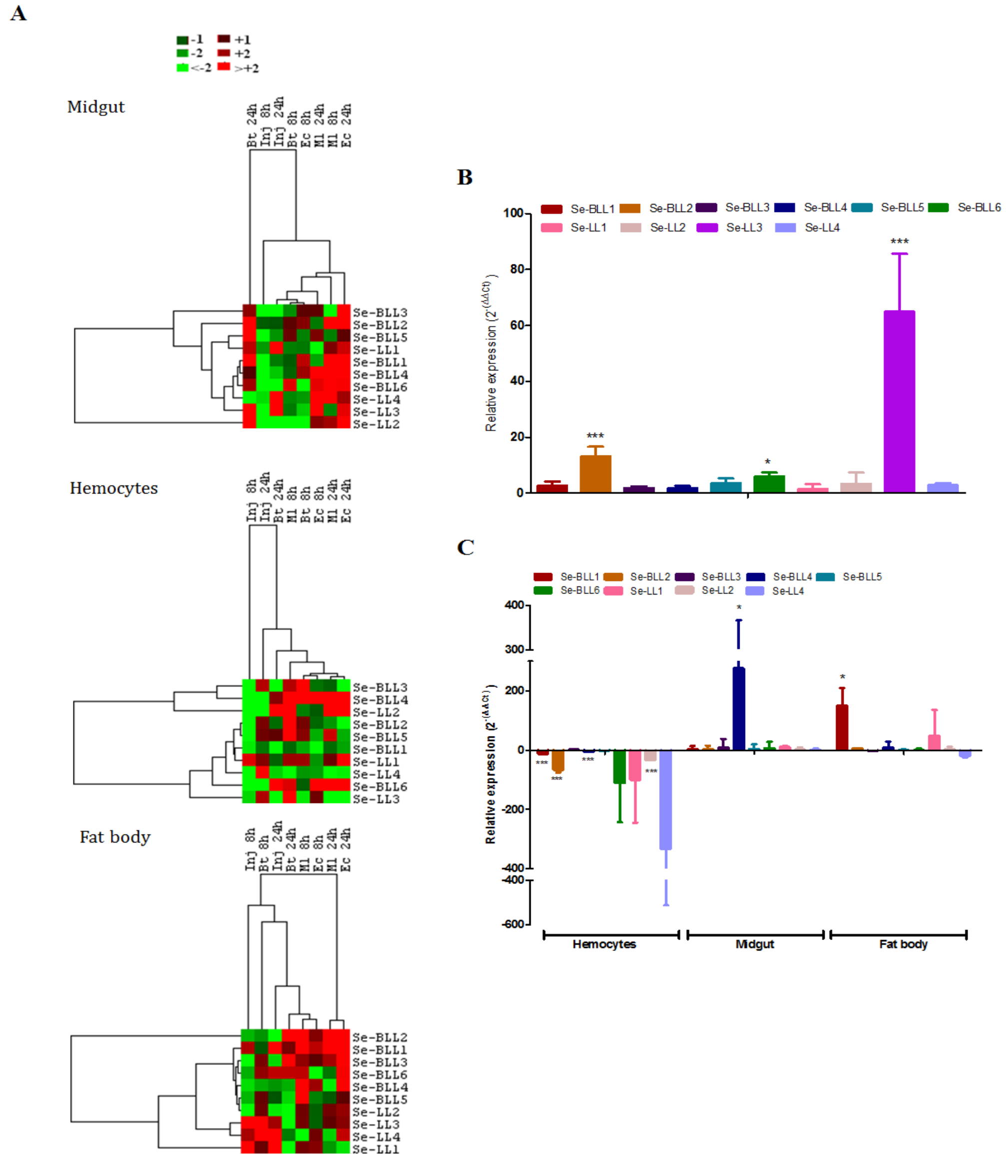

Figure 3.4. Response of *S. exigua* lectins to bacterial and viral pathogens. **A)** Heatmap summarizing the response to the tested pathogens of the studied lectins in the main larval tissues (more detailed values are reported in Annex 1.6). Regulation of the different lectins in response to the baculovirus SeMNPV in the larval midgut (B) or to densovirus JcDNV in the main larval tissues (C). For (B) and (C) the results are represented as the mean ± standard deviation from three independent replicates. The * represent $p<0.05$, ** represent $p<0.001$ and *** represent $p<0.0001$.

3.3.3. Effect of Se-BLL2 administration on viral infectivity

Se-BLL2 responded to both baculovirus and densovirus infections but in different ways. This gene was induced in response to the infection with baculovirus, however it was repressed in response to densovirus. Thus, further investigation of the antiviral potential of this protein would be interesting. To do that, Se-BLL2 was recombinantly expressed in *E. coli* and further purified by affinity chromatography. Then insect susceptibility to SeMNPV was tested in the presence or absence of purified Se-BLL2. The joint administration of this lectin with SeMNPV increased significantly ($P < 0.05$) the larvae tolerance to the viral infection (Fig. 3.5 A) (Chapter 1). This result confirms that this lectin can act as an antiviral protein protecting the insect larvae from SeMNPV infection. In the other hand, the administration of Se-BLL2 with the densovirus JcDNV did not affect this virus infectivity (Fig. 3.5 B). Both results indicate specific response of the studied protein.

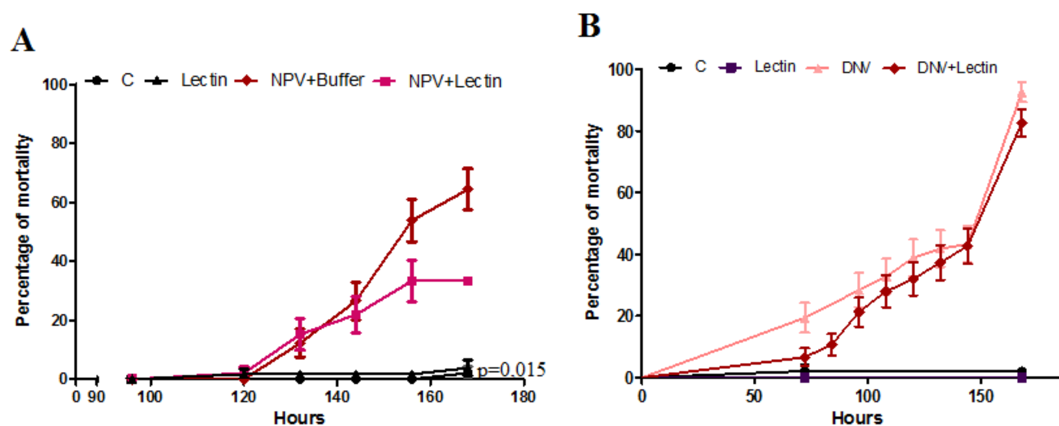

Figure 3.5. The effect of oral ingestion of Se-BLL2 in the infectivity of different viruses. **A)** Mortality curves in response to *S. exigua* baculovirus SeMNPV in presence and absence of Se-BLL2. (Data from chapter1) **B)** Mortality curves in response to the densovirus JcDNV in presence or absence of Se-BLL2.

3.4. Discussion

Due to the lack of antibody-based immunity in invertebrates, lectins may play a crucial role in the recognition of the non-self. The increasing number of insects' genomic sequences reveals that the insects' genomes contain a large number of lectins. For example, *D. melanogaster*, *A. gambiae* and *B. mori* genomes contain more than 30, 20 and 15 genes encoding C-type lectin domains, respectively (Dodd and Drickamer, 2001; Christophides *et al*., 2002). We have found that the genome of *S. exigua* carries at least 32 unigenes encoding proteins that contain lectin-like domains, being the majority of these lectin-like homologous to those of C-type lectins (Pascual *et al*., 2012). Moreover, unlike most animal C-type lectins that contain unique carbohydrate-binding domain (CRD), a large number of *S. exigua* lectins contain two CRDs, as the majority of other lepidopteran lectins (Wang *et al*., 2012). The presence of a large number of lectins in the transcriptome of *S. exigua* and other insects, in addition to the presence of more than one recognition domain, can increase the ability of the insect to recognize a wide variety of invading microorganisms.

Zelensky *et al.* (2005) reported that the C-type-lectin domain (CTLD) fold has a double loop. The conserved cysteine residues of the CRDs form disulphide budges at the bases of the formed loops. Hence, it has been suggested that this conformation is very important for the CRD function (Zelensky *et al*., 2005). Thus, deletions or mutations affecting these cys residues may affect their function. Remarkably, some of the lectins in the present study lack a number of the cys residues. The lack of some cys residues has also been described in *C. echinata* CEL-I lectin (Hatakeyoma *et al*., 2002) and in *H. armigera* lectins Ha-CTL7 and Ha-CTL8 (Wang *et al.*, 2012). In addition to the conserved cys residues, two additional conserved motifs, PXXC and FXCE, have been detected in *S. exigua* lectins. It has been shown that the conserved PXXC motif plays an important role in disulphide bond formation in many seed storage proteins (Kowagoe *et al*., 2005). Moreover, Wang *et al*. (2012) suggested that in the lepidopteran CRDs, the conserved PXXC and FXCE

motifs might contribute to the formation of disulphide bonds. Interestingly, Se-LL4 and Se-BLL6 do not have these motifs. It would be interesting to further investigate whether the deletions/mutations that have occurred affecting these conserved motifs and the conserved cysteine residues have an effect on the conformation and/or function of these proteins.

The two groups of *S. exigua* lectins described in this work are phylogenetically distant likely as consequence of their different origins. They also differ in the number of the carbohydrate recognition domains and in their expression patterns. Se-LLs are mainly expressed in the fat body and Se-BLLs are mainly expressed in hemocytes. In fact, the majority of insect lectins have been detected in the hemolymph and in most cases they are thought to be synthesized by the insect fat body and/or hemocytes (Mosson *et al.*, 2000). Furthermore, Se-LLs and Se-BLLs encoding genes are regulated in different tissues in response to different invading pathogens. We suggest that Se-LLs are synthesized by the fat body and Se-BLLs are synthesized by the hemocytes, then are secreted in the insect circulation to be delivered to different tissues involved in the insect defense. Indeed, several tissues participate in the protection of the insect against alien microorganisms (Lemaitre and Hoffman, 2007).

Lepidopteran immune related genes such as *H. armigera* lectin Ha-CTL4 (Wang *et al*., 2012) and two *B. mori* immunolectins (Liu *et al*., 2009) are up-regulated in response to PBS injection, since they play a role in the response to injury. In contrast, the six *Se-BLLs* are repressed in hemocytes and the midgut in response to the injection. This fact, along with the distribution of *S. exigua* lectins into two different genetically distant groups, may shed light on the divergence of such a large number of lectins in the lepidopteran genome and the evolution of the insect. In the first chapter, we showed that *Se-BLL2* is a polydnavirus gene horizontally transmitted to the host insect which was integrated and maintained in *S. exigua* genome. At this point it is tempting to speculate that this may be the case for all the *S. exigua* bracovirus-like lectins. In general, PDVs lectins are able to recognize

specifically the carbohydrate on the surface of the endoparasitoid eggs and, thus, inhibit the recognition of the eggs by the lepidopteran host recognition proteins (Lee *et al*., 2008). Therefore, the possible up-regulation of these PDV-originated genes in response to the wasp's eggs injection could be detrimental for the insect host in favour of the parasitoid wasp. However, the induction of these genes is necessary for the insect to face other pathogens. We propose that the insects have adquired and domesticated those genes to be able to protect themselves from a larger number of pathogens, such as bacteria and virus, whereas in response to the injection of the wasp's eggs, the insects repress those genes to counteract the inhibition of recognizing wasp eggs.

Among the ten *S. exigua* lectins, *Se-BLL2* responds to two different viruses, baculovirus and densovirus. Although little is known about the response of C-type lectins to viral invaders, crustacean lectins have been reported to be related to the antiviral defence (He *et al.,* 2005; Liu *et al*., 2007; Pan *et al*., 2005). In lepidopterans, the only example of antiviral response of C-type lectins was reported by Chai *et al.* (2008). A C-type lectin from the cotton bollworm *H. armigera* was up-regulated in hemocytes after injection of *H. armigera* polyhedrosis virus HaSNPV. In the present study, *Se-BLL2*, in addition to *Se-BLL6* and *Se-LL3*, were highly up-regulated in the midgut in response to SeMNPV. Moreover, different lectins also responded to densovirus infection. These results suggest that lectins are involved in the antiviral response of *S. exigua*. The administration of *Se-BLL2* together within SeMNPV to *S. exigua* larvae increased larvae survival, whereas its administration with the densovirus did not affect JcDNV infectivity. *Se-BLL2* might specifically recognize SeMNPV which facilitate the immune response of the insect to this pathogen. To date, this is the first clear report on the C-type lectins antiviral activity. It would be interesting to study in more detail the role of insect lectins in the response to viral infection and to take it into consideration when formulating new virus-based biopesticides.

GENERAL DISCUSSION

Horizontal gene transfer from Bracovirus to the Lepidoptera *Spodoptera exigua*

The exchange of genetic information between distantly related organisms outside of reproduction is known as horizontal gene transfer (HGT) or lateral gene transfer (Sun *et al*., 2013). This process has been long considered as a central mechanism of evolutionary adaptation for prokaryotes (Acuña *et al*., 2011). HGT has been widely described as a mechanism to gain foreign genetic materials in prokaryotes, but remained largely undocumented in eukaryotes (Huang, 2013). Ultimately, cases of HGT in eukaryotes are being described. The majority of them involve endosymbionts where the association between a microorganism, mostly bacteria, and host cells is particularly intimate and can occur in germ cells (Acuña *et al*., 2011). In this context we report evidences of horizontal gene transfer to the lepidopteran *Spodoptera exigua* mediated by a virus in the context of an intimate relationship: endoparasitoid wasp, bracovirus and lepidopteran host. In this system, the three genomes are continuously in contact. The hymenopteran parasitoid which has domesticated a symbiotic virus inject its eggs along with the virus in the lepidopteran host hemocoel where the hymenopteran larvae develop.

At least seven unigenes highly homolog to *Cotesia congregata* bracovirus and *Cotesia vestalis* bracovirus were detected in transcriptome of the beet armyworm *Spodoptera exigua* (Pascual *et al*., 2012). Six genes encode lectin proteins and the seventh gene encode a protein homolog to BV2-5, a member of *Cotesia* bracovirus protein family. Despite all of them are expressed in different tissues of the lepidopteran which suggest that they are functional genes, they are mainly expressed in hemocytes. Indeed, during oviposition, the wasp female injects its symbiotic bracovirus (BV) into the lepidopteran host hemocoel where the virus genes are expressed (Drezen *et al*., 2003). The high homology of these genes to bracovirus genes and their expression preference in hemocytes point to possible integration of bracoviral DNA into *S. exigua* germ line. To confirm that, we have

obtained the genomic sequences of two of these genes, *BV2-5* and *Se-BLL2*, and compared them with genomic sequences of closed related species. Sequence analysis of *BV2-5* revealed the presence of fragments of about 2 Kb highly similar to CcBV C19 and CcBV C25 flanked by the lepidopteran sequences. Similarly, genomic sequence of *Se-BLL2*, one of the bracovirus-like lectins, showed the presence of a fragment of 715 bp related to *CcV2-like* gene of CcBV C30 that was also flanked by sequences with homology to the corresponding *S. frugiperda* genomic BAC.

Remarkably, *BV2-5* sequence revealed the presence of the circle junction or direct repeat junction (DRJ) conserved in CcBV circles. DRJs are specific DNA circularization sequence motif flanking polydnaviral segments and are conserved to all bracovirus (Herniou *et al.,* 2013). The process of excision and circularization of viral segments from the parasitoid genome has been proposed to occur through juxtaposition of these repeats (Dupuy *et al.,* 2012). The presence of such DRJ in *BV2-5* sequence unambiguously point out to the viral origin of this gene. Orthologs of this sequence was detected in *S. litura* and *S. littoralis* genomes suggesting an ancient horizontal transfer from *Cotesia* bracovirus to an ancestor of these species, but no homolog was found in *S. frugiperda*. Based on the phylogenetics of *Spodoptera* species performed by Kergoat and collaborators (2012) the four species should have common ancestor and so *S. frugiperda* should contain *BV2-5* sequence. The presence of *BV2-5* in *S. exigua*, *S. litura* and *S. littoralis* but not *S. frugiperda* can be explicated by separated integration events. Alternatively and more likely, the absence of this sequence in *S. frugiperda* may be a result of gene loss after an ancient HGT event.

As for *Se-BLL2*, no DRJ has been detected and the integrated sequence represent the open reading frame. However, we suspect that this sequence as well as the other bracovirus-like lectins are viral genes integrated in the lepidopteran germ line. *BLL2* integration can be the result of more ancient integration event that has been the subject of selective pressures resulting in the deletion of the DRJ. Another explanation would be that this acquisition has been mediated by a broader mechanism such as DNA repair. A third explanation can be that the integration might

be the result of the integration of cDNA after retrotranscription of the viral transcript. Similar integration events was detected in the genome of *Bombyx mori* which was the recipient of bacterial genes (Sun *et al*., 2013). Remarkably, more than six bracovirus-like lectins are present in *S. exigua* genome, two in *S. frugiperda*, one in *S. litura* and one in *S. littoralis* genomes. Homology analyses indicate that all those genes were transferred from the same donor and are homologous to the same gene, suggesting a unique HGT event has occurred in the common ancestor of these species followed by different duplication events following their speciation.

Several reports suggest that HGT events can allow the recipient to broaden or change its diet, colonize new habitats, or survive in previously lethal conditions. They are relevant in the context of parasitic or pathogenic relationships or in the context of adaptation to new environmental conditions (Sun *et al*., 2013). In addition, gene duplication itself may be an important evolutionary mechanism that benefits the organism. Duplications are involved in the emergence, maintenance and evolution of genes (Huguet *et al*., 2012). Thus, the viral genes integrated in *S. exigua* genome and therefore duplicated may confer new functional advantages to the insect.

BV2-5 disturbs the cellular actin arrangement affecting the insect's immune response

BV2-5 sequence was detected in different *S. exigua* populations obtained from diverse geographic locations. American and Asiatic populations have conserved the full form of the protein. However, a punctual mutation occurred in European populations has generated a truncated protein. This finding leads us to wonder about the biological significance of *BV2-5* integration in the lepidopteran and about the reason of the mutation in the European populations. First experiments showed that BV2-5 disturbs the cellular actin arrangement. Tight regulation of filamentous actin assembly and disassembly at specific sites in the cells is crucial for the cell life and function. It has been documented that dynamic actin networks are

involved in a multitude of cellular mechanisms such as endocytosis, phagocytosis and encapsulation (Cardamore *et al*., 2011; Holubcova *et al.,* 2013; Pollard and Cooper, 2009). Moreover, in the last decade, numerous reports demonstrated that the actin is presented as well in the nucleus and that it is required for the chromatin remodelling and gene movement (Cibulka *et al*., 2012; Gandhi *et al*., 2012).

It is well documented that many viral pathogens use the actin cytoskeleton of their hosts in order to facilitate their transport, assembly and replicative process (kasman and Volkmann, 2000; Taylor *et al.*, 2011). For instance, the ability to command and direct the transport of actin from the cytoplasm to the nucleus and then to regulate the nuclear actin arrangement is characteristic of baculovirus infection (Gandhi *et al*., 2012; Kasman and Volkman, 2000). In fact two major actin arrangements follow *Autographa californica* multiple nucleopolhydrosis (AcMNPV) entry to the cytoplasm of infected cells. During the initial stages of infection, thick actin cables (F-actin), stretching from the plasma membrane to the nucleus, appear in the cytoplasm of the host cells (Cudmore *et al.,* 1997). These actin cables direct the nucleocapsids to the nucleus where another arrangement of actin occurs mainly in the area bordering the virogenic stroma where the morphogenesis of new nucleocapsids takes place (Kasman and Volkman, 2000). Therefore, the disruption of cellular actin arrangement may affect negatively baculovirus spread in the host cells. Indeed, we have observed cytoskeletal actin arrangement in Sf21-infected cells facilitating AcMNPV movement inside the cells. This arrangement has been disturbed in presence of BV2-5 as well as latrunculin, an inhibitor of the actin polymerization. This actin disruption has dramatically affected the virus infectivity, multiplication, occlusion bodies' formation and nucleocapsids morphogenesis and assembly. Accordingly, BV2-5 disturb the actin arrangement of host cells which affect negatively the transport of AcMNPV to the nucleus and then the morphogenesis of new nucleocapsids.

In another hand, BV2-5 affects the insect response to bacterial pathogens. Actually, it is well known that phagocytosis requires actin polymerization

(Castellano *et al.,* 2011). Therefore, BV2-5 interfering with the actin arrangement caused an important decrease in the ability of *S. exigua* hemocytes to phagocytize bacterial infections. This reduction was traduced by an increase in the susceptibility of larvae to bacteria. Regarding this protein origin, we suppose that the corresponding bracoviral protein impairs the lepidopteran hemocytes function.

In general, once inside the host larva, the bracovirus express a set of proteins implicated in causing physiological alteration in favour to the wasp development (Strand, 2012). Among the major alterations, some viral proteins inhibit the encapsulation which is the major lepidopteran response to endoparasitoid wasp (Amaya *et al.,* 2005). This inhibition is the result of hemocytes dysfunction in the majority of the described cases. Turnball and col. (2004) demonstrated that *Heliothis virescens* hemocytes are efficiently infected with *Camplotis sonorensis* PDV and they failed to adhere rapidly and to spread which is the result of the disruption of filamentous actin. Similar findings were described in other studies (Amaya *et al*., 2005; Asgari *et al*., 1996; Hu *et al*., 2003; Ibrahim and Kim, 2006; Luo and Pang, 2006). All of them reported dysfunction of the lepidopteran host hemocytes caused by the expression of polydnaviral proteins that alter the actin arrangement or even inhibit the actin expression.

In conclusion, BV2-5 is a bracovirus originated protein that disturb the cellular actin arrangement which affects two different mechanisms. It alters the baculovirus morphogenesis and multiplication which is in favour of the insect. In contrast, the actin disruption increased the susceptibility of the lepidopteran larvae to bacterial infection. Interestingly, the truncated form present in the European populations is non-functional, it has no effect on actin arrangement. Such mutation as well as the absence of the gene in *S. frugiperda* can be explained by the fitness cost that the integration impose. A positive correlation between the geographic distribution of each *Spodoptera* species or population and their pathogens distributions is suspected. However, more studies are required to confirm such hypothesis. For this purpose, we generated an hybrid population, MESUI, that

contains homozygotes for the full form of BV2-5, homozygotes for the truncated form and heterozygotes. We intend to study the effect of parasitism and bracovirus infection on the different genotypes of MESUI larvae.

Spodoptera exigua lectins: two different groups that respond to bacterial and viral infections

In addition to *BV2-5*, six genes encoding lectin proteins were found to be highly homologous to bracovirus genes. Those proteins belong to the C-type family of lectins (Pascual *et al.,* 2012). In fact, transcriptome of *S. exigua* revealed the presence of more than thirty unigenes coding for lectins. The majority of these lectins belong to the C-type family. C-type lectins (CTL) family comprises calcium dependent carbohydrate-binding proteins that bind to glycoproteins and glycolipids on the surfaces of microorganisms (Chai *et al*., 2008; Wang *et al.*, 2012). In general, lectins are carbohydrate-binding proteins that represent a major group of pattern recognition receptors (PRRs) that recognize and bind to terminal sugars in molecules on the surface of microorganisms, thereby able to agglutinate the recognized microbe (Yu *et al.,* 2005). CTLs are the most abundant and most studied compared to other lectin families. In lepidopteran species, these lectins have key role in the first line of defence against pathogens due to the lack of antibody-based immunity. Accordingly, *S. exigua* genome like other insects genomes contains a large number of lectins. Among those lectins, some are highly homologous to bracovirus lectins (Se-BLL1-6), whereas the others are similar to other lepidopteran lectins. Moreover, although members of animal C-type lectin family usually contain a unique carbohydrate recognition domain (CRD), almost all lepidopteran C-type lectins including *S. exigua* lepidopteran-like lectins contain dual CRDs (Watanabe *et al.,* 2006; Yu *et al*., 2005). The large number of lectins as well as the increasing number of carbohydrate recognition domain adding to them the acquisition of viral lectins permit the insect to recognize and respond to wide range of microorganisms. The majority of insect lectins described to date have been detected in the hemolymph

and are generally implicated in various immune mechanisms such as phagocytosis, nodule formation, encapsulation and prophenoloxidase activation (Dorrah *et al.,* 2009; Jomori *et al*., 1990; Zelensky and Gready, 2005).

Remarkably, *S. exigua* lepidopteran-like lectins reported in this study are mainly expressed in fat body in difference to the bracovirus-like lectins that are mainly expressed in hemocytes. Moreover, the two groups differ in their sequences organization: the lepidopteran-like proteins have dual CRDs while the bracovirus-like ones have unique CRD. It appears that those lectins are synthetized either in fat body or in hemocytes and directed afterwards to different tissues in order to defend the insect from the invading pathogens. Actually, no common pattern was detected for the studied proteins in response to gram positive bacteria, gram negative bacteria, baculovirus and densovirus infections. However, all the lectins respond to at least one pathogen confirming that each lectin recognizes specifically a unique microorganism or a group of pathogens that share the same surface epitopes. It has been described that lepidopteran lectins recognize preferentially gram-negative bacteria and then induce the ability of the insect hemocytes to clear the invading bacteria (Shin *et al*., 2000; Watanabe *et al*., 2006; Yu *et al.,* 2005). In addition, some studies showed that a number of these lectins recognize gram-positive bacteria (Madanagopal and Kim, 2007; Watanabe *et al*., 2006; Yu *et al.,* 2005). Finally, there was only one report for possible antiviral potential of the C–type lectins (Chai *et al.*, 2008). In this work, we are showing that the beet armyworm lectins respond to gram negative as well as gram positive bacteria. Moreover, an important number of these proteins responded to viral infection using baculovirus or densovirus. Indeed, the administration of *S. exigua* bracovirus-like lectin 2 (Se-BLL2) together with the baculovirus SeMNPV to *S. exigua* larvae decreased their susceptibility to the viral infections which represent the first direct report of antiviral properties of an insect lectin.

The present thesis started with the surprising finding of bracoviral homologous genes in the lepidopteran *Spodoptera exigua* transcriptome. We have

demonstrated that those genes are the result of horizontal gene transfer of bracovirus DNA to the insect germ line. Those genes belong to C-type lectin family except *BV2-5* which belongs to a bracoviral protein family with unknown function. Preliminary experiments revealed that BV2-5 interferes with actin polymerization. Afterwards, we were able to show that due to that, baculovirus and bacterial infections of *S. exigua* larvae are affected. Disruption of actin arrangement has caused a dramatic decrease in AcMNPV morphogenesis, multiplication and infectivity. In contrast, bacterial infection was more effective. In conclusion, it seems that BV2-5 presence can affect the lepidopteran negatively or benefits it, which can correlate with the fact that even though the full form of the protein is present in American and Asiatic population, a non-functional truncated protein is detected in European populations. Except of *BV2-5*, the other genes are C-type lectins. Those lectins differ from other *S. exigua* lectins that are homologous to lepidopteran lectins. Both groups respond to bacterial and viral pathogens. The most interesting thing is that we were able to demonstrate that *S. exigua* lectins have an important antiviral potential.

The genes transfer from *Cotesia* bracovirus to *Spodoptera exigua* reported in this study seem not to be a unique event since other lepidopteran species has been recipients of polydnaviral genes. In fact, horizontal gene transfer from *Cotesia congregata* bracovirus to *Danus plexipellus* and *Bombyx mori* genomes were detected (Gasmi *et al*., submitted). In addition, and as a result of an extensive bioinformatics search for PDV sequences in eukaryoic genomes, Schneider and Thomas (2014) detected a number of candidates of HGT from polydnavirus to *B. mori* genome. The increasing number of polydnavirus and lepidopteran sequenced genomes may reveal the presence of new transferred genes from the virus to the lepidopteran host. However, the interesting question here is the biological significance of such integration and whether it is the virus strategy to invade the host or it is a domestication of viral genes that benefit the lepidopteran host. Further investigations can provide more information to answer such question.

CONCLUSIONES

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1. Al menos siete secuencias de ADN de bracovirus se han integrado y mantenido en el genoma del lepidóptero *Spodoptera exigua*. A excepción del gen *BV2-5* que pertenece a la familia de proteínas BV2 de *Cotesia congregata bracovirus*, las otras secuencias codifican para las lectinas de tipo C. El análisis de estas secuencias indica que se han producido varios eventos de transferencia de genes desde bracovirus al lepidóptero huésped.

2. BV2-5 (Gasmin) perturba la distribución de la actina celular, lo que provoca una disminución en la capacidad de los hemocitos de *S. exigua* esencial para fagocitar *Bacillus thuringiensis*. BV2-5 en cambio, afecta negativamente a la morfogénesis y la formación de los cuerpos de oclusión virales de AcMNPV. Como resultado, la expresión de Gasmin en las larvas de *S. exigua* las hace más susceptibles a la infección bacteriana, aunque más resistentes a la infección por baculovirus.

3. A pesar de que las poblaciones americanas y asiáticas han conservado Gasmin funcional, una mutación puntual ha generado una proteína truncada y no funcional en las poblaciones europeas. Este hecho, así como el papel divergente de esta proteína en respuesta a diferentes patógenos reflejan posible adaptación al tipo de patógeno que es más abundante en cada localización geográfica.

4. El rastreo del transcriptoma de *S. exigua* ha revelado la presencia de más de treinta genes que codifican para proteínas tipo lectina. Diez lectinas de tipo C fueron elegidas en este estudio: cuatro de ellas son homólogas a otras lectinas de lepidópteros de tipo C (Se-LLs) y las otras seis son homólogas a lectinas relacionadas con bracovirus (Se-BLLs). Los dos grupos tienen diferentes patrones de expresión y organización de secuencia.

5. Cada lectina, o grupo de lectinas, responde específicamente a los agentes patógenos que comparten los mismos epítopos moleculares. Esta respuesta diferenciada frente a bacterias Gram-positivas o Gram-negativas, frente a

baculovirus, densovirus y endoparasitoides, su gran número y la presencia de dominios duales de reconocimiento de carbohidratos (CRD) y la domesticación de las lectinas viricas indica que éstas están involucradas en la respuesta a una amplia gama de patógenos.

6. Se-BLL2 responde a todos los patógenos probados, incluyendo la infección virica. La administración de esta proteína purificada junto con el baculovirus de *S. exigua*, SeMNPV aumenta la resistencia de las larvas de *S. exigua* a la infección por baculovirus. Este resultado está de acuerdo con el efecto negativo que BV2-5 (Gasmin) provoca en la multiplicación e infectividad de baculovirus. Por lo tanto, la transferencia horizontal de genes que se produjo a partir del genoma(s) de bracovirus al genoma de *Spodoptera* tiene un efecto beneficioso sobre el insecto, contribuyendo a su tolerancia a patógenos naturales.

Conclusions

1.At least seven bracovirus DNA sequences have been integrated and maintained into the genome of the lepidopteran *Spodoptera exigua*. Except of BV2-5 which belongs to the BV2 protein family of *Cotesia congregata* bracovirus, the other sequences codify for C-type lectins. Sequence analysis indicate that several gene transfer events have occurred from bracovirus to the lepidopteran host.

2.BV2-5 (Gasmin) disturbs the cellular actin arrangement, which caused critical decrease in the ability of *S. exigua* hemocytes to phagocytize *Bacillus thuringiensis* and negatively affected AcMNPV nucleocapsids morphogenesis and formation of the viral occlusion bodies'. As a result, *S. exigua* larvae became more susceptible to bacterial infection, but more resistant to baculovirus infection.

3.Even though American and Asiatic populations have conserved Gasmin, a punctual mutation generated a non-functional truncated protein in the European populations. This fact, as well as the divergent role of this protein in response to different pathogens reflect possible adaptation to the type of pathogen that is more abundant in each geographic localization.

4.Transcriptome mining of *S. exigua* have revealed the presence of more than thirty genes coding for lectin proteins. Ten C-type lectins were chosen in this study: four of them are homolog to other lepidopteran C-type lectins (Se-LLs) and the other six are homologous to bracovirus-related lectins (Se-BLLs). The two groups have different expression patterns and sequence organisation.

5.Each lectin or group of lectins respond specifically to pathogens sharing the same molecular epitopes. This differentiated response against Gram-positive or Gram-negative bacteria, baculovirus, densovirus and endoparasitoides, their large number, the presence of dual carbohydrate recognition domains (CRDs) and the

domestication of viral lectins indicate that they are involved in the response to a wide range of pathogens.

6.Se-BLL2 responds to all the tested pathogens including viral infection. The administration of this purified protein together with *S. exigua* baculovirus SeMNPV increases the resistance of *S. exigua* larvae to baculovirus infection. This result is in agreement with the negative effect that BV2-5 (Gasmin) causes on baculovirus multiplication and infectivity. Hence, the horizontal gene transfer that occurred from bracovirus genome(s) to the *Spodoptera* genome has beneficial effect on the insect, contributing to its tolerance to natural pathogens.

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ANNEX

Supplementary Figures

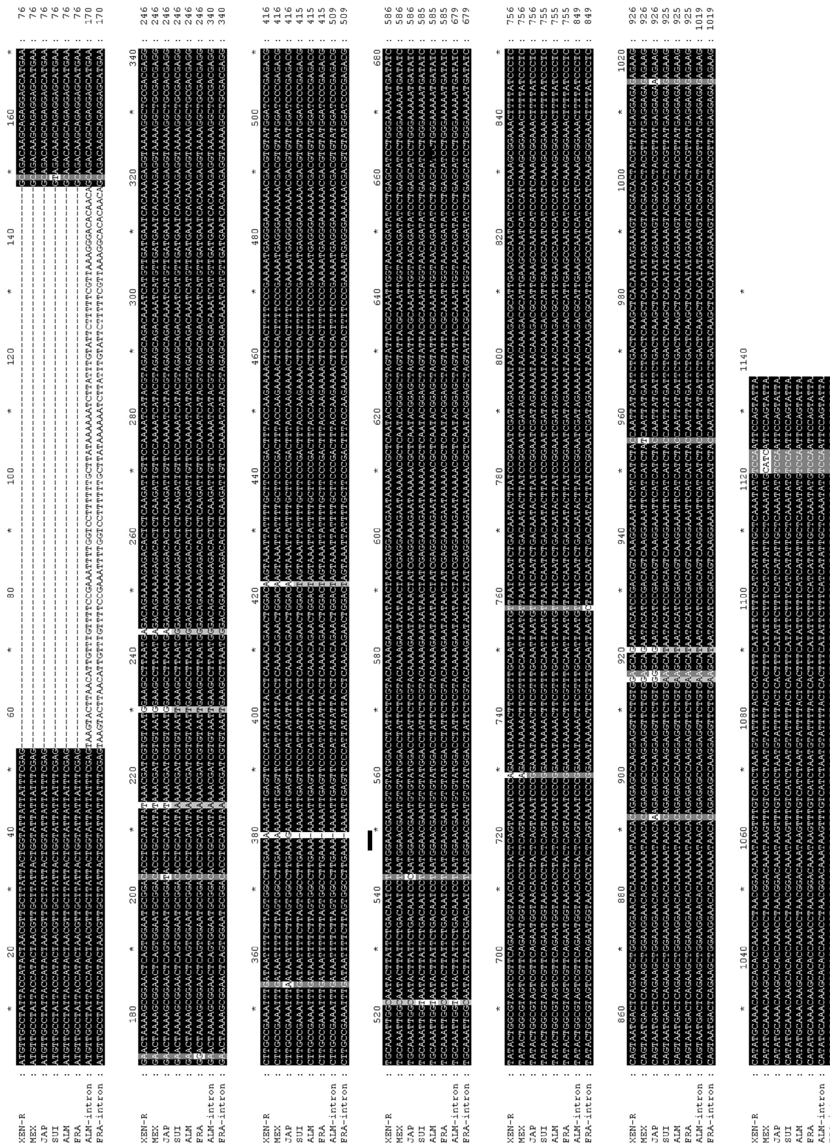
Annex 1.1. Names and access numbers of the sequences used in the different phylogenetic analysis.

Species	Protein name	Access number (NCBI)
Spodoptera frugiperda	Lectin 3-1	(spodobase)
Spodoptera frugiperda	Lectin 5-1	(spodobase)
Spodoptera littoralis	C2971	
Spodoptera littoralis	Sl_BV2-5	-
Spodoptera litura	Lectin like	
Spodoptera litura	Slit_BV2-5	
Spodoptera exigua	Se_BV2-5	KP406767
Spodoptera exigua	Se_BV2-5	KP406768
Spodoptera exigua	Se-BLL1	KP406770
Spodoptera exigua	Se-BLL2	KP406769
Spodoptera exigua	Se-BLL3	KP406771
Spodoptera exigua	Se-BLL4	KP406772
Spodoptera exigua	Se-BLL5	KP406773
Spodoptera exigua	Se-BLL6	KP406774
Spodoptera exigua	Se-LL1	KP406775
Spodoptera exigua	Se-LL2	KP406776
Spodoptera exigua	Se-LL3	KP406777
Spodoptera exigua	Se-LL4	KP406778
Cotesia congregata bracovirus	BV2-2	CCQ71078.1
Cotesia congregata bracovirus	BV2-3	CCQ71074.1
Cotesia congregata bracovirus	BV2-5	CCQ71080.1
Cotesia congregata bracovirus	C-type lectin CcV3 like	CCQ71085.1
Cotesia vestalis bracovirus	Conserved hypothetical protein	AEE09556.1
Cotesia vestalis bracovirus	Conserved hypothetical protein	AEE09527.1
Cotesia vestalis bracovirus	Lectin (CvBV 2L)	AEE09562.1
Cotesia vestalis bracovirus	Lectin (CvBV L)	AEE09593.1

Cotesia sesamiae Monmbasa bracovirus	C-type lectin (CsMBV CTL)	AGO14401.1
Cotesia ruficrus polydnavirus	Hypothetical protein (CrPDV HP)	BAC55179.1
Cotesia plutellae polydnavirus	Lectin (CpPDV lectin)	AAS10157.1
Cotesia rubecula bracovirus	Lectin (CrBV lectin)	AAO74641.1
Glyptapanteles indiensis	L1_00460	ABK57032.1
Glyptapanteles indiensis	L1_00290	ABK57015.1
Glyptapanteles flavicoxis	L7_0360	ACE75094.1
Glyptapanteles flavicoxis	L7_03570	ACE75115.1
Nasonia vitripennis	Hypothetical lipopolysaccharide binding protein	XP_001599898.2
Megachile rotunda	Hemolymph lipopolysaccharide binding protein like	XP_003708137.1
Megachile rotunda	Hemolymph lipopolysaccharide binding protein	XP_003701025.1
Megachile rotunda	Hemolymph lipopolysaccharide binding protein like	XP_003706756.1
Megachile rotunda	Hemolymph lipopolysaccharide binding protein like	XP_003704952.1
Bombyx mori	C-type lectin 10	ADB12587.1
Bombyx mori	C-type lectin 19	NP_001165396.1
Bombyx mori	C-type lectin 11	NP_001037076.1
Bombyx mori	C-type lectin 21	NP_001037056.1
Manduca sexta	Immunolectin 4	AAV41237.2
Manduca sexta	Immunolectin 3	AAV41236.1
Manduca sexta	Immunolecin 2	AAF91316.3
Manduca sexta	Immunolectin 1	ADD13530.1
Helicoverpa armigera	C-type lectin 8	AFI47453.1
Helicoverpa armigera	C-type lectin 6	AFI47451.1
Helicoverpa armigera	C-type lectin 5	AFI47450.1
Helicoverpa armigera	C-type lectin 3	AFI47448.1
Helicoverpa armigera	C-type lectin 2	ACI32834.1
Helicoverpa armigera	Lectin	ABF83203.1

Ostrinia furnacalis	Immunolectin	ABZ81710.1
Lomonia oblique	Lectin 3	AAV91450.1
Lomonia oblique	Immunolectin	AAV91436.1
Antheraea pernyi	C-type lectin	AGN70857.1
Mamestra conFigurata	C-type lectin	AEA76325.1
Pieris rapae	C-type lectin	AEO52696.1
Aanopheles stephensi	C-type lectin galactosidase binding	ACP43727.1
Aedis aegypti	C-type lectin	ABF18196.1
Musca domestica	LOC101901048	XP_005189940.1
Drosophila virilis	GJ17272	XP_002051932.1
Drosophila melanogaster	C-type lectin 27KD, isoform B	NP_001260046.1
Drosophila melanogaster	C-type lectin 27KD, isoform A	NP_608858.3
Drosophila erecta	GG24353	XP_001968708.1
Drosophila yakuba	GE14680	XP_002087961.1
Drosophila willistoni	GK23915	XP_002064562.1
Drosophila mojavensis	GI15343	XP_002001743.1

Annex 1.2. Sequence comparison of *Se_BV2-5* gene derived from different populations of *S. exigua* showing the presence of two forms of the expressed protein.



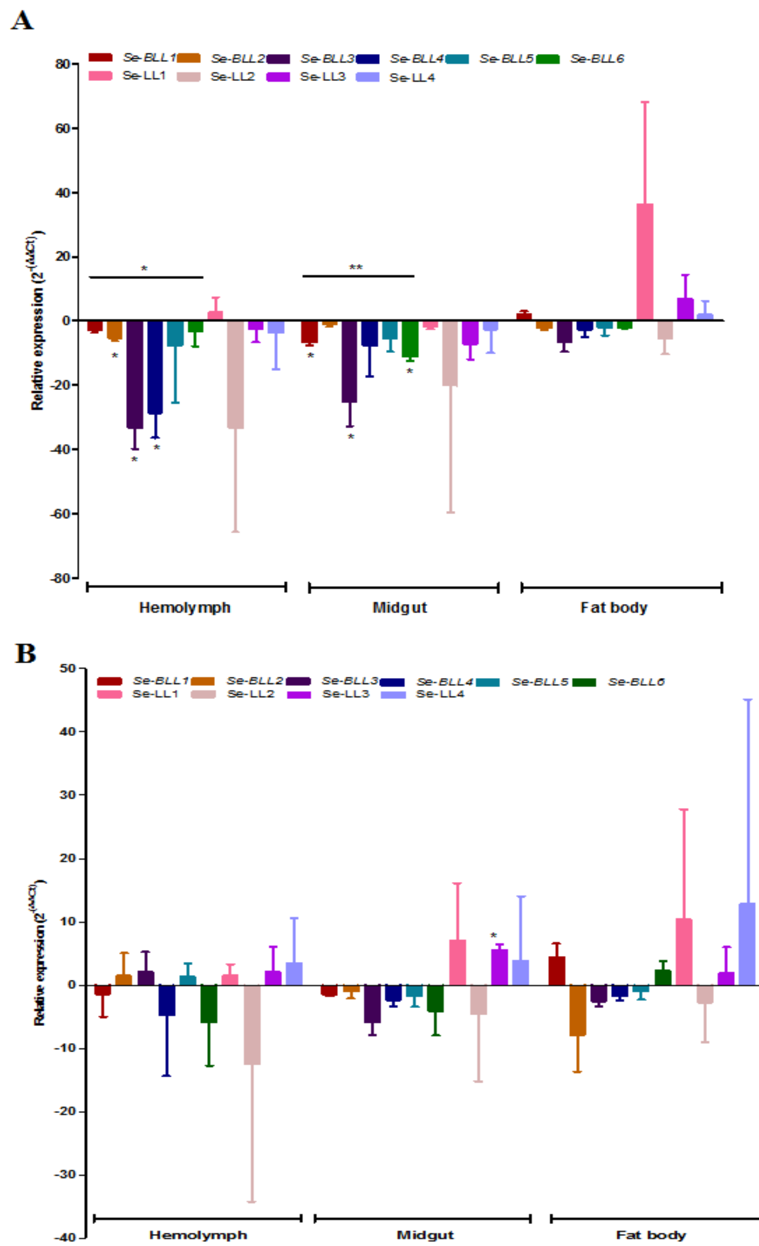
Annex 1.3. Alignment of the deduced amino acid sequences of bracovirus-lectin like proteins from different *Spodoptera* species and their homologs from polydnavirus, hymenopteran, lepidoptera and diptera species.

Annex 1.4. ClustalX alignment of the deduced amino acid sequences of *S. exigua* lepidopteran-like lectins aligned with other lepidopteran lectins (A) and ClustalX alignment of the deduced amino acid sequences of *S. exigua* bracovirus-like lectins compared with other lectins from Polydnavirus, Hymenoptera and Lepidoptera species (B).

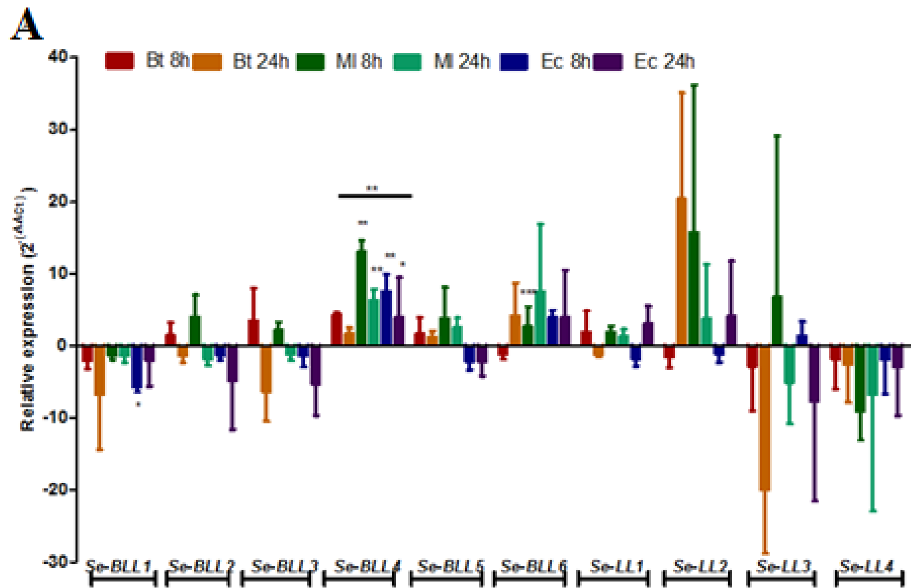
A

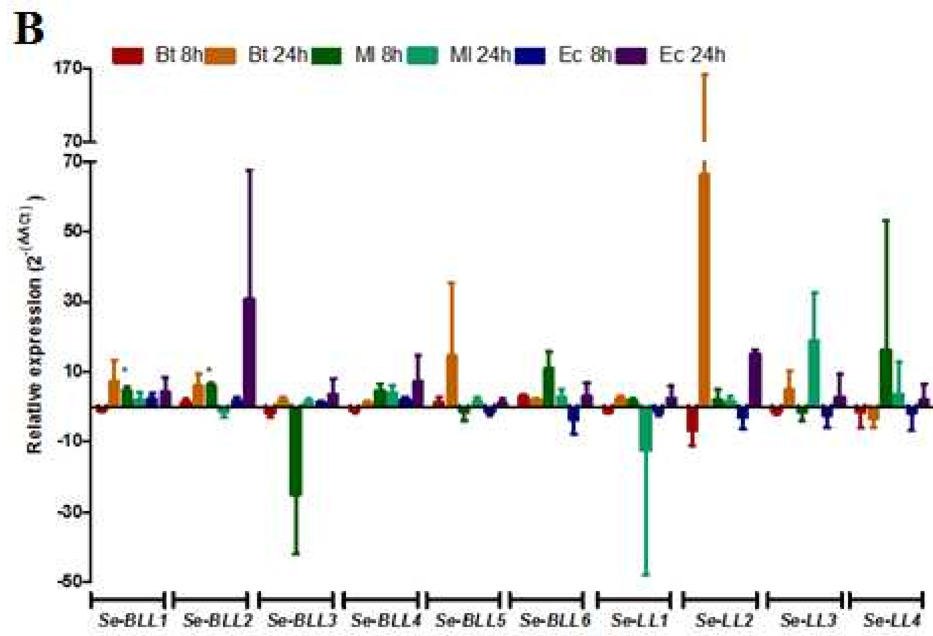
B

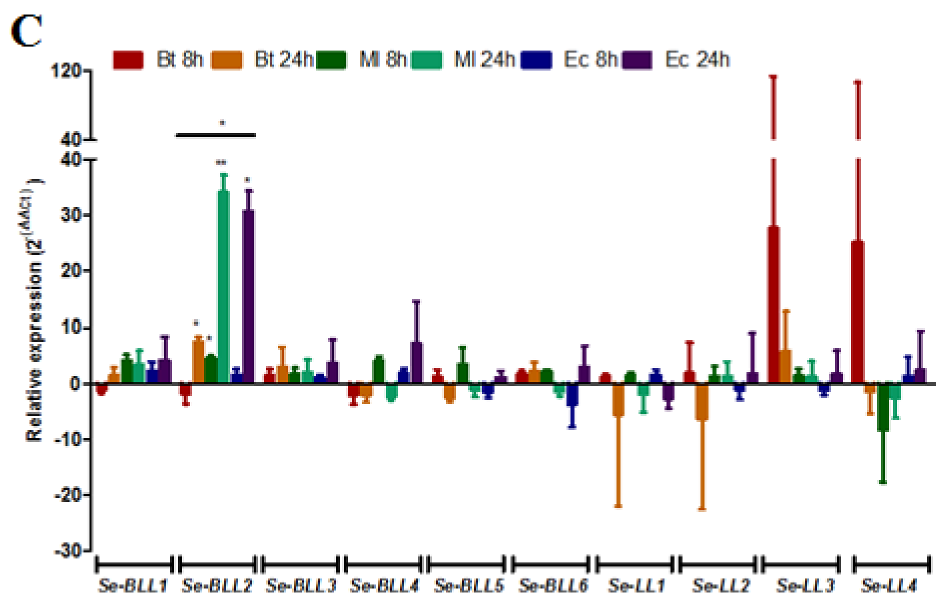
Annex 1.5. Expression profiles of the different *S. exigua* lectins in response to PBS injection in different tissues of *S. exigua* larvae, measured at 8 (A) and 24h (B) after injection. The results are represented as the mean ± standard deviation.



Annex 1.6. Expression profiles of the different *S. exigua* lectins in response to Gram positive bacteria (*B. thuringiensis* and *M. luteus*) and Gram negative bacteria (*E. coli*) in different tissues of *S. exigua* larvae, measured at 8 and 24 h after injection. The results are represented as the mean ± standard deviation . (A) hemocytes, (B) midgut and (C) fat body




B
Bt 8h
Bt 24h
Ml 8h
Ml 24h
Ec 8h
Ec 24h
Relative expression (2^(ΔΔCt))
Se-BLL1
Se-BLL2
Se-BLL3
Se-BLL4
Se-BLL5
Se-BLL6
Se-LL1
Se-LL2
Se-LL3
Se-LL4


C
Bt 8h
Bt 24h
Ml 8h
Ml 24h
Ec 8h
Ec 24h
Relative expression (2^(ΔΔCt))
Se-BLL1
Se-BLL2
Se-BLL3
Se-BLL4
Se-BLL5
Se-BLL6
Se-LL1
Se-LL2
Se-LL3
Se-LL4