Characterization of caspases from Lepidoptera and their role in apoptosis.

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Summary

Multicellular organisms need a tightly controlled regulation of cell proliferation and of cell specialization together with a controlled cell death to maintain their integrity. Apoptosis as a part of the programmed cell death mechanism is required to clear out undesirable cells which are supernumerary, malfunctioning or infected by pathogens. The importance of such a cell suicide pathway has been demonstrated numerous times since its first description. In holometabolous insects, and more specifically in Lepidoptera, which undergo a complete metamorphosis between their larval and adult stages, the importance of apoptotic events during pupation has been demonstrated as early as in the 1960's. More recently, it has been shown that apoptosis of cells infected with baculovirus can significantly reduce viral spread throughout the body. However, the molecular pathway regulating apoptosis in Lepidoptera is still poorly understood. A family of evolutionarily conserved proteases, called caspases, plays a central role in regulating apoptosis in animals. Reception of death stimuli triggers the activation of initiator caspases, which in turn activate the effector caspases, responsible for the cleavage of many other intracellular components. The discovery of p35, a baculovirus protein inhibiting caspase activity, has led to the characterization of the first lepidopteran caspase, Sf-Caspase-1, from *Spodoptera frugiperda*. Studies on the Sf-Caspase-1 mode of activation suggested that apoptosis in Lepidoptera also requires a cascade of caspase activation, as demonstrated in many other species.

In order to obtain an overview of this gene family in Lepidoptera, we performed an extensive survey of lepidopteran-derived EST datasets. We identified 66 sequences encoding putative caspases, distributed among 27 species of butterflies and moths. Phylogenetic analyses showed that Lepidoptera possess at least 5 caspases, for which we propose a unified nomenclature. According to their homology to the *Drosophila melanogaster* counterparts and their primary structure, we proposed that Lep-Caspase-1, -2 and -3 are effector caspases, whereas Lep-Caspase-5 and -6 are putative initiators. The likely function of Lep-Caspase-4 remains unclear. Lep-Caspase-2 is absent from the silkworm genome and appears to be noctuid specific, and to have arisen from tandem duplication of the Caspase-1 gene. In the tobacco hawkmoth, 3 distinct transcripts encoding putative Caspase-4 were identified, suggesting at least 2 duplication events in this species. The basic repertoire of five major types of caspases shared among Lepidoptera seems to be smaller than for most other groups studied to date, but gene

duplication still plays an essential role in lineage-specific increases in diversity, similar to what has been described in Diptera and mammals.

Despite an overall decrease in mRNA and protein biosynthesis, apoptosis requires the selective transcription and translation of several genes involved in regulation and execution. In an attempt to characterize caspase expression profiles in *Helicoverpa armigera*, we used real time quantitative RT-PCR to assess their resting levels of expression in different larval tissues and during development. Furthermore, we analyzed the effect of immune challenge and induction of apoptosis on their expression. We found that Caspase-1 and -2 have complementary expression profiles during larval development, suggesting a differential regulation. Caspase-3 and -6 are upregulated upon immune challenge, suggesting a role in the IMD pathway. Caspase-5 is upregulated during pupation and upon induction with 20-hydroxyecdysone, supporting the hypothesis of Caspase-5 playing a similar role as Dronc in developmental apoptosis in *Drosophila*.

In mammalian systems, almost 300 potential targets of caspases have been described and the expression of more than 120 proteins can be altered during apoptosis. In an attempt to obtain further insights into the molecular pathway(s) underlying apoptosis in lepidopteran insects, we used 2D differential electrophoresis to identify 31 proteins, for which relative abundance was altered during apoptosis of *H. armigera* derived cells, induced by actinomycin D. Among these 13 proteins, we identified the putative effector Caspase-1, 3 chaperone proteins and several pro-apoptotic proteins, such as actin and a voltage dependant anion channel. We also identified two anti-apoptotic proteins, a member of the aldo/keto reductase family and annexin IX.

In conclusion, the molecular pathway(s) underlying apoptosis in Lepidoptera seems to be similar to what has been described in other organisms. However, a better understanding of the apoptotic pathway(s) in lepidopteran insects would contribute to improve our knowledge in fields such as interaction between the insect and its pathogens, or between the insect and its host plant. This could lead to the discovery of deterrents that could be used as potential "environmentally-friendly" insecticides.

Zusammenfassung

Zellteilung, Zellspezialisierung und Zelltod müssen in einem vielzelligen Organismus hochgradig reguliert werden um dessen Funktionsfähigkeit zu gewährleisten. Apoptose ist ein Teil des programmierten Zelltods und wird benötigt, um überschüssige, gestörte oder mit Pathogenen infizierte Zellen zu vernichten. Die Wichtigkeit dieses ‚Zellselbstmord'-Signalweges für multizelluläre Organismen wurde seit dessen Entdeckung mehrfach belegt. Für holometabole Insekten und besonders für Lepidopteren, die in ihrer Entwicklung von der Larve bis zum Adultus eine komplette Metamorphose durchlaufen, ist die Bedeutung von apoptotischen Abläufen während der Verpuppung schon in den 1960'er Jahren bewiesen worden. Unlängst wurde gezeigt, dass durch Apoptose, in mit Baculoviren infizierten Zellen, die Verbreitung des Virus im Körper signifikant reduzieren werden kann. Allerdings sind die molekularen Signalwege, die Apoptose in Lepidopteren regulieren, immer noch wenig verstanden. Eine evolutionär stark konservierte Protease Familie, die Caspasen, spielt eine zentrale Rolle in der Regulierung von Apoptose in Tieren. Die Wahrnehmung von ‚Todes-Stimuli' lösen die Aktivierung von Initiator-Caspasen aus, die für die Spaltung vieler anderer intrazellulärer Komponenten verantwortlich sind. Die Entdeckung von p35, ein Caspase-Aktivität inhibierendes Baculovirus Protein, hat zu der Charakterisierung der ersten Caspase in Lepidopteren (Sf-Caspase-1 aus *Spodoptera frugiperda*) geführt. Studien über die Wirkungsweise von Sf-Caspase-1 legen nahe, dass Apoptose in Lepidopteren eine Kaskade von Caspase Aktivierungen benötigt, wie es bereits in vielen anderen Arten gezeigt wurde.

Um eine Übersicht über diese Genfamilie in Lepidopteren zu erhalten, haben wir eine umfassende Untersuchung der aus Lepidopteren generierten Transkriptom-Datensätze durchgeführt. In 27 Schmetterlingsarten wurden insgesamt 66 cDNA Sequenzen identifiziert, die für putative Caspasen kodieren. Phylogenetische Analysen zeigen, dass Lepidopteren über mindestens 5 Caspasen verfügen, für die wir eine vereinheitlichte Nomenklatur vorschlagen. In Übereinstimmung mit der Homologie zu Caspasen in *Drosophila* und ihrer primären Struktur, schlagen wir vor, dass Lep-Caspase-1, -2 und -3 Effektor-Caspasen und Lep-Caspase-5 und -6 putative Initiator-Caspasen sind. Die mögliche Funktion von Lep-Caspase-4 bleibt ungewiss. Lep-Caspase-2 ist nicht im Genom des Seidenspinners vorhanden und scheint daher eine Noctuiden-spezifische Caspase zu sein, die durch Tandemduplikation des Lep-Caspase-1 Gens entstanden ist. Im Tabakschwärmer wurden 3 verschiedene Transkripte für putative Caspase-4

Enzyme identifiziert, was auf mindestens 2 Duplikationsereignisse in dieser Art hindeutet. Lepidopteren besitzen 5 Hauptklassen an Caspasen und haben damit, im Vergleich zu anderen untersuchten Tiergruppen, eine relativ geringe Anzahl an Caspaseklassen. Allerdings spielen Genduplikationen auch bei Lepidopteren eine wichtige Rolle, um eine Gattungsspezifische Erhöhung der Caspase-Diversität zu erreichen, ähnlich wie es auch für Dipteren und Säugetieren beschrieben wurde.

Ungeachtet des generellen Rückgangs von mRNA Level und Proteinbiosynthese während der Apoptose, werden Transkription und Translation von einigen Genen, zur Regulierung und Ausführung der apoptotischen Prozesse selbst, benötigt. Um das Caspase-Expressionsprofil in *Helicoverpa armigera* zu charakterisieren, haben wir mittels quantitativer PCR die Expression der zur Apoptose benötigten Gene sowohl im Ruhezustand in verschiedenen Larvalstadien als auch während der Entwicklung gemessen. Weiterhin haben wir den Einfluss von Immunstimulation und Induktion von Apoptose auf die Expression dieser Gene untersucht. Wir haben festgestellt, dass Caspase-1 und -2 gegensätzliche Expressionsprofile in der Larvalentwicklung besitzen und daher wahrscheinlich unterschiedlich reguliert werden. Caspase 3 und-6 werden beide durch Immunstimulation hochreguliert und spielen daher wahrscheinlich eine Rolle im *imd* Signalweg. Caspase-5 wird während der Verpuppung und nach der Induktion mit 20-Hydroxyecdyson hochreguliert ist, was die Hypothese unterstützt, dass diese Caspase eine ähnliche Funktion in Lepidopteren wie Dronc in der entwicklungsspezifischen Apoptose in *Drosophila* besitzt.

In Säugetiersystemen wurden fast 300 potentielle Caspase-Ziele beschrieben und die Expression von mehr als 120 Proteinen kann während der Apoptose verändert werden. Um ein genaueren Einblick in die apoptotischen Signalwege in Lepidopteren zu erhalten, haben wir 2D Elektrophorese Techniken angewandt. Wir haben 14 Proteine identifiziert, deren relative Vorkommen sich in *H. armigera* Zellen während der Apopotose, durch Actinomycin D induziert, verändert haben. Unter diesen 14 Proteinen befanden sich die putativen Effektoren Caspase-1, 3 Chaperon-Proteine und einige pro-apoptotische Proteine, wie zum Beispiel Actin und ein Membran-potential abhängiger Anionen-Kanal. Wir haben weiterhin zwei anti-apoptotische Proteine, ein Mitglied der Aldo/Keto Reduktase Familie und Annexin IX identifiziert.

Zusammenfassend erscheint der molekulare Signalweg, der apoptotischen Prozessen in Lepidopteren unterliegt, ähnlich zu den schon bekannten Signalwegen in anderen Organismen zu sein. Ein erweiterter Wissensstand von apoptotischen Signalwegen in Lepidopteren würde zu einem besseren Verständnis der Interaktionen zwischen Pflanzen und Insekten-Wirtspflanzen Interaktionen beitragen. Dies könnte zur Entdeckung von Abwehrstoffen führen, die als umweltfreundliche Insektizide einsetzbar wären.

General Introduction

The emergence of eukaryotic multicellular organisms was enabled by the evolution of new fundamental systems such as cell cycle regulation, cell specialization as well as controlled (or programmed) cell death (PCD) in order to maintain organism integrity. As Koonin and Aravind said "*From a purely systemic point of view, Programmed Cell Death in a multicellular organism appears to be just as inevitable as law enforcement in a state. In any differentiated community of specialized cells or citizens, Rogue elements will necessarily emerge and to protect the community, these need to be subdued or destroyed by specialized agencies*" (Koonin and Aravind, 2002) Programmed Cell Death is thus useful to eliminate malfunctioning cells, or cells the organism no longer requires; PCD is also involved in fighting pathogenic invaders.

The basic features of Programmed Cell Death were first described in the 1960's, however, in 1972 the term apoptosis was used for the first time by Kerr and colleagues, to describe a type of cell death which involved a kind of self regulation (Kerr, *et al*., 1972)

For many years, apoptosis and PCD were considered synonymous. However, recent discoveries have shown that PCD is a more general phenomenon comprising not only apoptosis, but also including autophagic cell death, cornification (a form of PCD specific to the skin upper layer), and "necroptosis" (which is a regulated necrosis). These distinct phenomena are described in the Classification of Cell Death proposed by the Nomenclature Committee of Cell Death (NCCD), (Kroemer, *et al*., 2008) In plants, only two forms of PCD have been described, autophagic cell death and one similar to apoptosis, called apoptosis-like-PCD (Reape, *et al*., 2008)

Apoptosis versus Necrosis

Apoptosis, a distinctive and highly regulated type of cell death, differs considerably from necrosis, which can be described as a "chaotic" form of cell death.

Necrosis is usually induced by processes having a radical effect on cell metabolism and affecting a large amount of cells simultaneously, such as extreme temperature or pH, physical trauma, or metabolic poisons. Cells then lose their cytoplasmic membrane integrity (Fig. 1) sodium ions and water accumulate in the cytoplasm causing the swelling of the cells, whereas calcium ions leak out. Cell metabolism stops, DNA is degraded in a random fashion, and internal membranes are also affected, releasing potentially dangerous enzymes into the

cytoplasm. At a later stage, due to permeability of the plasma membrane, cytoplasmic components are released into the extracellular matrix. The released components can attack and damage surrounding cells, creating a death chain reaction and triggering an inflammatory response.

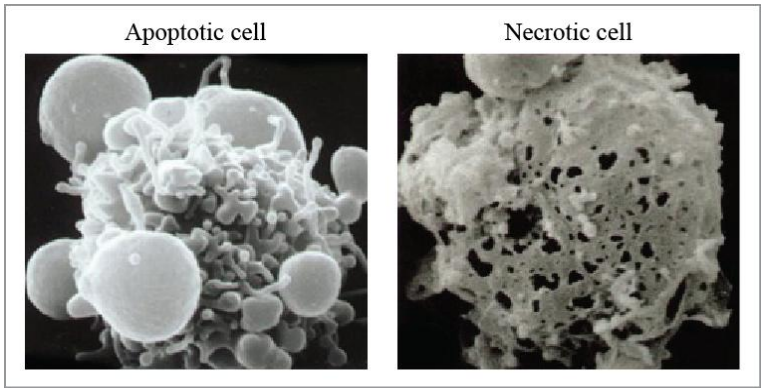


Figure 1. SEM pictures of an apoptotic and necrotic cell (magnification x5000) (Immunologia's, 1998)

On the other hand, apoptosis is triggered by a specific and highly regulated signal and can affect single isolated cells. Typically, apoptotic cells keep their cytoplasmic membrane intact but display a distinctive morphology. The cleavage of lamin and actin filaments leads to cell shrinkage and a loss of cell-cell contact. The breakdown of chromatin between histones, in regular intervals of 180 to 185 bp (also referred as DNA laddering) leads to nuclear condensation. Despite cytoplasmic condensation, all the organelles remain intact and potentially functional until very late in the process. The plasma membrane also starts to bleb, leading to the formation of apoptotic bodies, vesicles containing cytoplasmic material (Fig. 1). Apoptotic bodies can be recognized because they expose on their outer surface phosphatidylserines which are normally exposed on the internal face of the plasma membrane. The apoptotic bodies are then phagocytosed by surrounding cells or by specialized cells (phagocytes or macrophages). Until the end of the process, the plasma membrane remains intact; therefore there is no inflammatory reaction.

Apoptotic pathways

In mammalian cells, apoptosis is triggered via intrinsic or extrinsic pathways depending on the origin of the "death signal" (Fig. 2). The intrinsic pathway is activated in response to intracellular signals such as DNA damage, respiratory chain malfunction or protein misfolding. The apoptotic signal induces a permeabilization of the mitochondria outer membrane which allows the release in the cytosol of several proteins such as Smac / Diablo and Cytochrome c.

Cytochrome c forms a protein complex with Apaf-1 called the apoptosome, which recruits the initiator Caspase-9 leading to its subsequent activation.

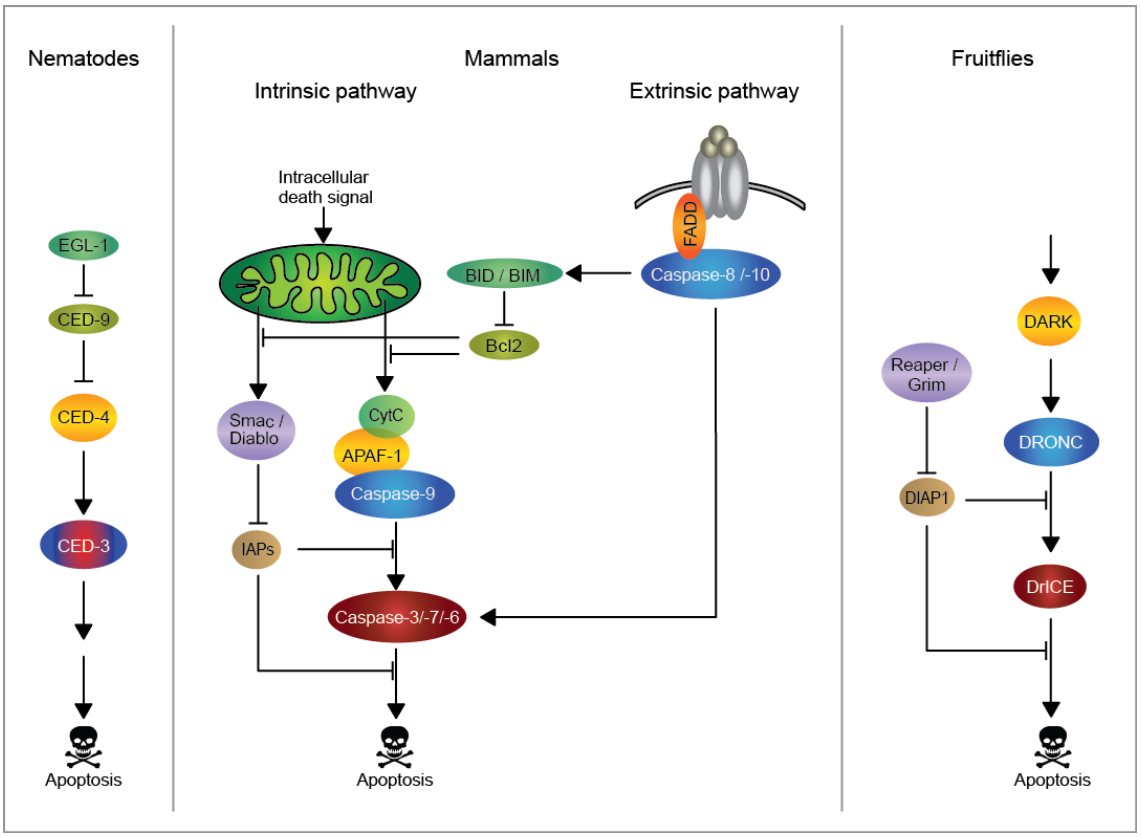


Figure 2. Apoptotic pathway in nematodes, mammals and fruitflies.
Homologues of caspases and caspases regulators across species are indicated by the same color (modified from Riedl and Shi (2004)).

The extrinsic pathway involves the reception of the "death signal" via Death receptors on the cell surface. Binding of the ligand to its receptor changes the receptor conformation, allowing the recruitment of an adaptor protein such as FADD and initiator caspase-8. Formation of this complex allows the auto-activation of caspase-8. Cross-talk between the two pathways is possible via the cleavage of the pro-apoptotic factor BID by Caspase-8.

In both pathways, activated initiator caspases then cleave and subsequently activate the effector caspase-3, -7 and -6 which in turn cleave numerous nuclear and cytoplasmic proteins, thereby leading to the characteristic features of apoptotic cells (Cohen, 2000; Fuchs and Yang, 2000; Fan *et al.*, 2005).

Despite an overall conservation of the apoptotic pathways, their complexity differs greatly across animal species. In *Caenorhabditis elegans*, only four proteins are involved in the apoptotic pathway, functioning sequentially. This species also possesses only a single apoptotic

caspase which takes on simultaneously the initiator and effector functions (Hengartner and Horvitz, 1994). In *Drosophila*, Cytochrome c is not required for the successful activation of the intrinsic pathway (Dorstyn, *et al*., 2004).

Consequences of apoptosis

Apoptosis is fundamental to various biological processes such as development (Amundson, *et al*., 1998; Creagh, *et al*., 2003; Penaloza, *et al*., 2008), tissue homeostasis, DNA damage response (Amundson, *et al*., 1998) and immune response (Creagh, *et al*., 2003).

Apoptosis and development

The first organism in which developmental apoptosis was extensively studied is *C. elegans*, in which the lineage of every cell in the organism is known. Exactly 131 cells out of the 1,090 generated between fertilization and the adult hermaphrodite die from apoptosis in *C. elegans*. The first wave occurs shortly after fertilization, then the second wave during the L2 larval stage, when some of the new neurons are removed, and the third wave in the adult germ line, where about half of the oocytes are removed (Lettre and Hengartner, 2006). During embryonic development of vertebrates, apoptosis is required to shape the morphology and structure of the developing organs and to remove transient embryonic tissues and organs (Penaloza, *et al*., 2008). One of the best characterized developmental event in which apoptosis plays a central role is the morphogenesis of digits. During limb development of animals with free digits, the interdigital webbings are degraded by apoptosis, giving the digits their shape (Montero and Huber, 2010). *A contrario*, no apoptosis of the interdigital webbings was observed during the digit morphogenesis of animals with webbed digits (Weatherbee, *et al*., 2006). In members of the ancient phylum Cnidaria, apoptosis has been shown to be involved not only in metamorphosis (Seipp, *et al*., 2001) but also during asexual reproduction. The sea anemone *Halpiplanella lineata* commonly uses longitudinal fission as a mode of reproduction. As the body of the sea anemone stretches, apoptosis is triggered in the fission axis, degrading the different tissues and allowing the sea anemone to split into two equal halves (Mire and Venable, 1999).

Apoptosis and homeostasis

During cell division, the fidelity of the genetic information transmitted to the daughter cells is monitored by several surveillance mechanisms, therefore when DNA replication or mitotic spindle assembly are not successfully completed, further progression of the cell cycle is placed on hold to provide the opportunity to repair DNA and complete proper chromosome segregation. In mammals, one of the most famous genome guardian proteins, p53, plays a central role in maintaining genome integrity by promoting cell cycle arrest and DNA repair mechanisms. But when DNA damage is beyond repair, p53 promotes the transcription of pro-apoptotic members of the BAX family (e.g. BAX, PUMA) and therefore triggers the apoptotic intrinsic pathway. Apoptosis induced by p53 can also be triggered by oxidative stress, metabolic compromises and cellular Ca^{2+} overload (Chipuk and Green, 2006). A well known and well studied case of a malfunctioning maintenance of cell integrity is cancer. In almost all, if not all, cancers a deficient apoptosis is involved leading to accumulation of deleterious mutations. If cancers are diseases where apoptosis is suppressed, some neurodegenerative and autoimmune diseases, such as Parkinson or rheumatoid arthritis are characterized by an over activated apoptosis, targeting healthy cells.

Apoptosis and immunity

To thwart pathogen infections, mammals have developed a powerful defense system composed of both innate and adaptive immune responses. On the other hand, insects only manifest an innate immune response, which is nevertheless effective. In an attempt to prevent viral replication and dissemination, many of the protective measures triggered by the innate immune response involve apoptosis of the host cell.

In mammalian systems, T lymphocytes detect non-self antigens at the surface of infected cells and release lytic granules composed of perforin, granulysin and granzymes. Perforin forms pores in the plasmamembrane, allowing massive entry of salt and water as well as the entry of granzyme B, an enzyme sharing the same substrate specificity as caspase-8 and therefore able to activate the effector caspase-3. T lymphocytes also express FAS ligand at their surface, which, in close contact with a contaminated cell, can bind to the FAS receptor, leading to the activation of extrinsic pathways (Fig. 2).

Importance of apoptosis in holometabolous insects

Development of holometabolous insects is characterized by a complete metamorphosis between the wingless larval stage, mostly dedicated to nutrient acquisition and growth, and the winged adult form, dedicated to reproduction. These drastic modifications in appearance and physiology require massive histolysis and histogenesis. The importance of apoptotic events during development of holometabolous insects and more specifically in Lepidoptera has been shown as early as the 1960's. In wild silkmoths and the Tobacco hawkmoth, the first ecdysone peak during metamorphosis induces apoptotic degeneration of the larval intersegmental muscles, proleg motoneurons, and labial glands (Lockshin and Williams, 1964; Lockshin and Zakeri, 1994; Weeks and Truman, 1985). The decrease in ecdysone titer shortly before hatching induces apoptotic degeneration of abdominal neurons and intersegmental muscles (Truman, 1984). Important changes in food habits between larval and adult stages also imply extensive remodeling of the digestive tract. For example, in *Galleria mellonella*, the larval midgut undergoes apoptosis during metamorphosis (Uwo, *et al*., 2002). Similarly, in *Heliothis virescens*, apoptosis of the larval midgut has been correlated with higher caspase expression shortly before and after pupation (Parthasarathy and Palli, 2007).

Among the different protective measures triggered by the insect immune system to thwart pathogen infection, host cell suicide through apoptosis can significantly reduce viral replication, dissemination and infectivity (Clem, 2007). Studies of the interactions between baculoviruses and lepidopteran species have led to the discovery of several inhibitors of apoptosis (IAPs). The protein p35, first described in the *Autographa californica* MNPV baculovirus, is a universal suicide inhibitor of effector caspases (Clem, *et al*., 1991), whereas p49, from *Spodoptera litura* NPV is a potent inhibitor of initiator caspases (Jabbour, *et al*., 2002; Zoog *et al*., 2002). Members of the p35 family are expressed only in some baculoviruses, but other inhibitors of apoptosis, the IAP proteins are expressed in all baculoviruses. Homologs of viral IAPs have been identified in many other organisms ranging from yeast to humans, meaning that IAPs are ubiquitous in eukaryotes and part of the 'normal' apoptotic pathway. However, their exact function remains so far unclear (Callus and Vaux, 2006).

Aims of this work

As described above, apoptosis is of crucial importance for the development of Lepidoptera and for their defense against pathogens. However the molecular pathway underlying apoptosis in Lepidoptera is still poorly understood.

As the caspase gene family plays a major role during apoptotic events, but only one effector caspase has been characterized in Lepidoptera so far, I focused my research on characterizing this enzyme family in lepidopteran insects. Using the overall conservation of caspase sequences and especially the conserved active site ($Q^A/_RC^R/_QG$) and binding site ($^L/_S{}^T/_SHG$) motifs, I used the Sf-Caspase-1 protein sequence as a query to search publicly available and "in house" lepidopteran EST databases, and retrieve the putative caspase sequences from 27 lepidopteran species. I then determined their phylogenetic relationships and proposed a naming nomenclature derived from the one established for vertebrates. In an attempt to characterize these lepidopteran caspases, I determined their primary structure, as well as their intron-exon structure and chromosomal localization (Chapter 1).

To further characterize the putative physiological functions, I used real time quantitative RT-PCR to analyze their resting mRNA expression profiles in various larval tissues as well as their expression profiles during the development of *Helicoverpa armigera*. Finally, I studied the effects of "classical" apoptotic inducers as well as immune challenge on their expression (Chapter 2).

Caspases occupy a core role of the apoptotic machinery. However, it has been shown in mammals that during apoptosis more than 200 other proteins can be altered, not only targets of the apoptotic processes but also regulators and executioners. In order to identify other potential key players of the apoptotic events in Lepidoptera, I used an unbiased comparative proteomic approach using a *H. armigera*-derived cell line exposed to the apoptotic inducer, actinomycin D (Chapter 3).

Chapter 1.
Characterization of the Caspase gene family in Lepidoptera

Introduction

A major family of evolutionarily conserved cysteine-dependent aspartate-specific proteases, called caspases, plays a central role in apoptosis. Studies on prokaryotes, closely related species of the free-living ancestor of mitochondria, have revealed the presence of genes similar to caspases, suggesting that ancestor of eukaryotic caspases may be derived from mitochondrial endosymbionts (Koonin and Aravind, 2002). Ancestors of caspase genes then evolved into "metacaspases" in plants, yeast, parasitic protozoans and fungi; "paracaspases" in slime molds and animals and "true caspases" in animals.

Metacaspases and Paracaspases

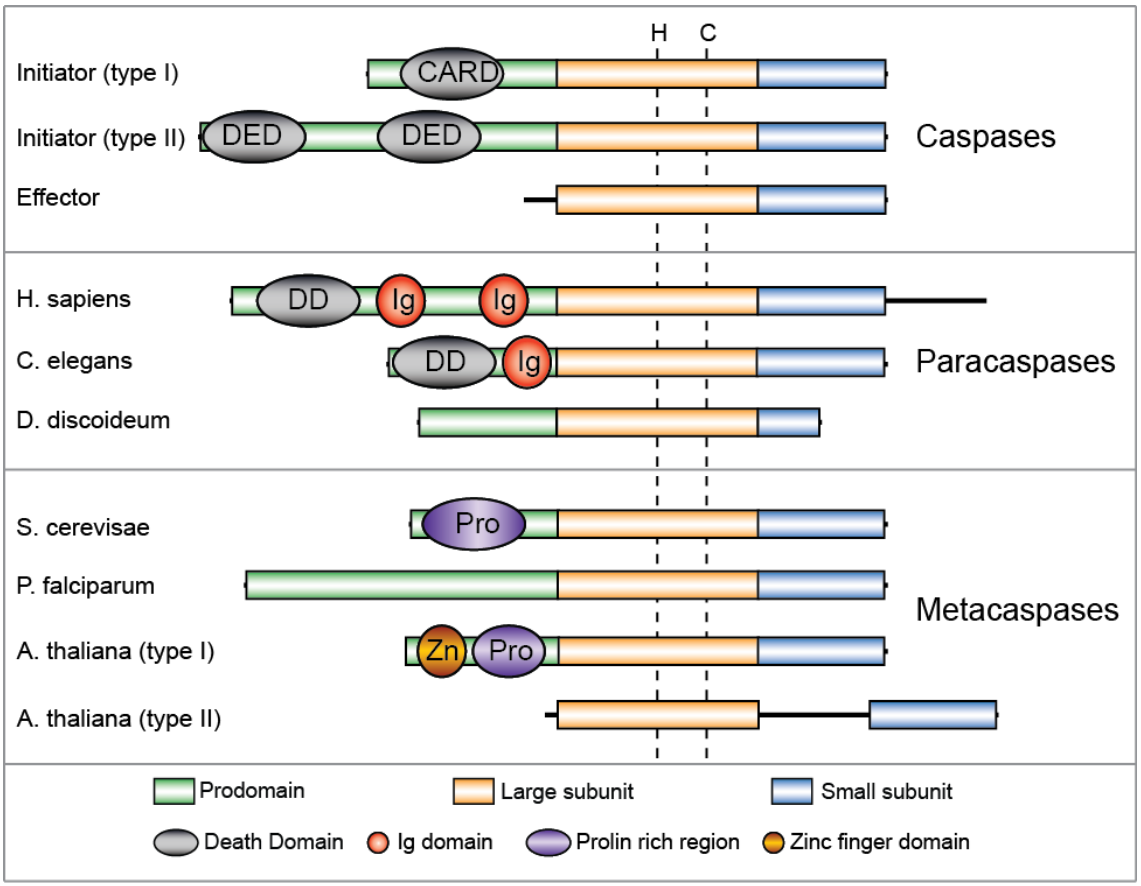


Figure 1. Structure of Caspases, Paracaspases and Metacaspases, adapted from Uren et al. (2000)

Metacaspases and paracaspases were first identified as “caspase-related” proteins with a distant but nevertheless statistically significant similarity to caspases. They bear the characteristic Caspase/Hemoglobinase fold and possess the conserved catalytic histidine/cysteine dyad. The prodomain of paracaspases has a death domain followed by one or two immunoglobulin (Ig) domains (Fig. 1). Metacaspases are divided into type I and type II according to their overall sequence similarities and structure (Fig. 1). Type I proteins harbor a proline rich region in their prodomain. In addition, plant type I proteins harbor a zinc finger domain. Type II proteins seem to be restricted to plants and do not have any obvious prodomain but have a conserved insertion of 180 amino acids between the large and small subunit (Uren, *et al.*, 2000).

The exact role of metacaspases and paracaspases remains unclear. Their substrate specificity toward an arginine or lysine residue in P1 , instead of an aspartate for caspases, suggest that they might have a completely different role than caspases (Vercammen, *et al.*, 2007). However several other studies show that metacaspases are involved in PCD in some plants (Woltering, 2010). Study on the human paracaspase showed that in the case of MALT lymphoma, it interacts with Bcl10 and activates NF-κB (Uren, *et al.*, 2000).

The True caspases

The number of “true” caspase genes differs greatly among animal lineages with as few as four genes in *Caenorhabditis elegans* (Shaham, 1998), five in the sea anemone *Nematostella vectensis*, seven in the fruitfly *Drosophila melanogaster* (Kumar and Doumanis, 2000), ten and eleven in the mosquitoes *Anopheles gambiae* and *Aedes aegypti* respectively (Bryant, *et al.*, 2008), fifteen in human (Chowdhury *et al.*, 2008) to up to 31 in the sea urchin *Strongulocentrotus purpuratus* (Robertson, *et al.*, 2006). It has been hypothesized that host pathogen interactions have been one of the major evolutionary forces shaping the apoptotic machinery and therefore the caspase repertoire (Aravind, 2002; Robertson, *et al.*, 2006).

All members of the caspase family share a canonical structure consisting of a prodomain and a catalytic domain named “peptidase C14” (Caspase domain pfam PF00656) (Fig. 2). The catalytic domain is composed of 2 subunits, a large one of about 20 kDa, also known as p20, and a small one of about 10 kDa, also referred as p10 (Fig. 2A). Upon proteolytic processing of the proenzyme, the two subunits form a heterodimer and the active caspase enzyme is made up of the association of two of these heterodimers (Fig. 2B).

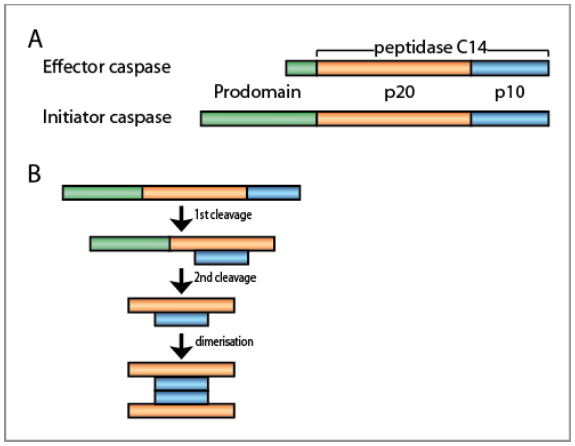


Figure 2. Schematic representation of caspase activation.
(A) Canonical structure of apoptotic caspases
(B) Caspase activation cascade. The first cleavage occurs at an Asp residue between the two subunits which form a dimer. After prodomain cleavage at an Asp residue, two dimers associate to form an active caspase.

The enzymatic activity of caspases shows strong cleavage site specificity towards a four amino-acid motif ending with an aspartate residue (Garcia-Calvo, *et al*., 1999; Nicholson, 1999; Talanian, *et al*., 1997). In contrast to the catalytic domain, which is structurally conserved, the prodomain varies significantly among caspases. The characteristic binding (LVTD/GHG) and active sites ($Q^A/_RC^R/_QG$) are conserved in all caspases except in *D. melanogaster* Dronc (Dorstyn, *et al.*, 1999a).

The "true" caspases are divided into two groups, the inflammatory caspases and the apoptotic caspases.

Inflammatory caspases

To date, dedicated inflammatory caspases seem to be restricted to mammals. Inflammatory caspases are encoded by five main genes in human (Caspase-1, -4 and -5), four in chimpanzee (caspase-1, -4, -5, and -12) and three in mouse (caspase-1, -11 and -12) and it is worth noticing that they are organized as a single locus in the genome, resulting from several tandem gene duplication events in the course of evolution. These caspases are named "inflammatory" because identified substrates for caspase-1 are pro-interleukin-1β (IL-1β), proIL-18 and proIL33, which are critical cytokines involved in inflammation (Martinon and Tschopp, 2006).

Apoptotic caspases

Apoptotic caspases are themselves divided into two subgroups, the effector and initiator caspases (Chang and Yang, 2000). The targets of initiator caspases are generally effector caspases, which are activated after cleavage; and the targets of effector caspases are other cellular proteins, which can be destroyed after cleavage, or alternatively activated and/or translocated to different cellular compartments. Whereas effector caspases have a short prodomain of about 30 amino acids, initiator caspases have a prodomain of 80 or more amino acids (Fuentes-Prior and Salvesen, 2004) (Fig. 1 & 2). Long prodomains usually harbor structures belonging to the Death Domain superfamily, such as Death Effector Domain (DED) or Caspase Recruitment Domain (CARD) (Fig. 1), which are involved in the recruitment and subsequent activation of initiator caspases by death receptors or by the apoptosome (Park *et al*., 2007).

Caspases in Lepidoptera

In Lepidoptera, very little is known about apoptotic molecular pathways. The discovery of p35, a baculovirus pan-caspase inhibitor in the early 1990's (Clem, *et al*., 1991), has led to the characterization of the first lepidopteran caspase, caspase-1, from the Sf9 cell line derived from the noctuid moth *Spodoptera frugiperda* (Ahmad, *et al*., 1997). Later, the likely orthologs from *S. littoralis* (Liu, *et al*., 2005), *Helicoverpa armigera* (Yang, *et al*., 2007) and *Trichoplusia ni* (Hebert, *et al*., 2009) were also characterized. Members of this caspase-1 group have been shown to act as effector caspases, so far no other class of caspase has been described in Lepidoptera. Nonetheless, the existence of an "apical" caspase responsible for the activation of Sf-caspase-1 has been suggested (Manji and Friesen, 2001). Furthermore, 2 other baculovirus proteins, p49 and IAP (Seshagiri and Miller, 1997; Zoog *et al*., 2002), have been shown to inhibit apoptosis in Sf9 cells by blocking the processing of caspase-1, suggesting that the likely target of these two inhibitors may be an apical caspase, tentatively named Sf-caspase-X. So far, this caspase X has not been characterized, but these results suggest that, in Lepidoptera, apoptosis requires a cascade of caspase activation, similar to what has been described in other organisms.

In an attempt to characterize the caspase gene family in lepidopteran insects, we first surveyed the recently sequenced genome of the silkworm *Bombyx mori* (Xia, *et al*., 2008) as well as publicly available and 'in-house' EST datasets. From these, we retrieved 6 transcripts

encoding putative caspases from 27 species of butterflies and moths. Phylogenetic analyses showed that these genes clustered into six distinct clades (Caspase-1 to -6), three of which are classified as putative effector caspases, two corresponding to putative initiator caspases and one that could not be readily classified as either. We proposed a naming convention for these genes based on our phylogenetic analyses. Finally, we discovered that one of the caspase subfamilies (Caspase-2) is absent from the genome of the silkworm, has evolved from a tandem gene duplication of Caspase-1, is under purifying selection which however is more relaxed than for Caspase-1, and is likely to be restricted to species of the family Noctuidae.

Methods

Preparation of cDNA libraries and EST sequencing

Generation of cDNA libraries for *T. ni* whole larvae (Freitak, *et al*., 2007); *Plutella xylostella, Pieris rapae, Colias eurytheme* and *Anthocharis cardamines* whole larvae (Fischer, *et al*., 2008) and for *H. armigera* midgut and *S. littoralis* whole larvae (Pauchet, *et al*., 2009) were previously described. TRIzol Reagent (Invitrogen) was used to isolate total RNA from whole larvae or dissected larval tissues of *Pontia daplidice, Eucheira socialis, Lymantria dispar, Lymantria monacha, G. mellonella, H. virescens, Mamestra brassicae*, and *Spodoptera exigua*. Normalized, full-length, enriched cDNA libraries were generated using both the Creator SMART cDNA library construction kit (BD Clontech) and the Trimmer Direct cDNA normalization kit (Evrogen), generally following the manufacturer's protocol but with several important modifications. In brief, 2 µg of poly(A)+ mRNA was used for each cDNA library generated. Reverse transcription was performed with a mix of several reverse transcriptases for 1 h at 42°C and 90 min at 50°C. cDNA size fractionation was performed with SizeSep 400 spun columns (GE Healthcare) that resulted in a cutoff at ~300 bp. The full-length, enriched cDNAs were cut with SfiI and ligated to the SfiI-digested pDNR-Lib plasmid vector (Clontech). Ligations were transformed into *Escherichia coli* ELECTROMAX DH5α-E electro-competent cells (Invitrogen). Plasmids from bacterial colonies grown in 96 deep-well plates were prepared using the Robot 96 Plasmid isolation kit (Eppendorf) on a Tecan Evo Freedom 150 robotic platform (Tecan). Single-pass sequencing of the 5' termini of cDNA libraries was carried out on an ABI 3730 xl Automatic DNA Sequencer (PE Applied Biosystems). Vector clipping, quality trimming, and sequence assembly were done with the Lasergene 8 software package (DNAStar Inc.). BLAST searches were conducted on a local server using the National Center for

Biotechnology Information (NCBI) blastall program. Sequences were aligned using ClustalW software (Thompson, *et al*., 1997).

Database mining for putative caspase sequences

The protein sequence of Sf-caspase-1 (AF548387) (Ahmad, *et al*., 1997) was used as a query to perform TBLASTN searches of lepidopteran EST databases publicly available on NCBI dbEST, on InsectaCentral (http://insectacentral.org) (Papanicolaou, *et al*., 2009) or in-house (Table S1). Sequences were accepted as being "caspases" when presenting the characteristic binding site ($^{L}/_{S}$ $^{T}/_{S}$ H G) and/or active site (QA$^{R}/_{Q}$G). Some exceptions were made when partial amino acid sequences presented a strong amino acid identity with sequences previously accepted as caspases.

Caspase gene amplification and sequencing

Specific primers (Table 1) were designed in order to re-amplify BmCasp-4, and -5, as well as HaCasp-1, -2, -3 and -4 using total RNA prepared respectively from *B. mori* and *H. armigera* whole larvae as starting material. In order to get full-length cDNAs, 5'- and 3'-RACE were performed for HaCasp-5, and -6, BmCasp-4, MsCasp-1, -4-2 and -6 as well as EaCasp-1 and -3 using the SMART RACE cDNA amplification kit (Clontech), following the manufacturer's instructions. Total RNA from *H. virescens*, *H. subflexa* and *H. armigera* larvae was extracted using TRIzol, according to the manufacturer's specifications. Forward and reverse specific primers designed according to the HvCasp-6 sequence (Table 1) were used to amplify HaCasp-6 from *H. armigera* cDNAs. Forward and reverse specific primers designed according to the HaCasp-1 and -2 sequences (Table 1) were used to amplify Casp-1 and -2 from *H. virescens* and *H. subflexa* cDNAs. Finally, a *H. armigera* draft genome assembly generated by 454 pyrosequencing (unpublished data) was searched using BmCasp-5 as query to identify a homolog in *H. armigera*. Forward and reverse primers (Table 1) were designed based on the sequences of contigs 28382 and 105066 in order to amplify HaCasp-5 sequence from cDNA extracted from *H. armigera* HaAM1 cells (McIntosh, *et al*., 1983) treated with 75 µM ecdysone. All amplicons were then ligated in pCR2.1-Topo® vector (Invitrogen). Ligations were transformed into *E. coli* TOP10 chemically competent cells (Invitrogen) and plasmid preparation and sequencing were performed as mentioned above.

Table 1. Primer sequences used for amplification of caspase transcripts

Gene	Primer name	Primer sequence
Bm-Caspase-4	BmC4R1	CAGCATATCCGCTTCAATTGGC
Bm-Caspase-5	BmC5F1	GATACTAGTATCACACCTCG
	BmC5R1	CGGGAGGTCCGTGAAGTT
	BmC5R2	TCACTCGTACAGACCGGGGTG
	BmC5F3	CACTACAACTGCGAGCCAGA
	BmC5F4	GTGGACTGCGACAAGTTGAAG
	BmC5R3	GCACAGGACCTGGATGTACC
Bm-Caspase-6	BmC6F2	TATGATATAGATGTAAGCGGG
	BmC6R1	ATTTTGGATTTGTTTCATTGGC
Ea-Caspase-1	EaC1RACEF	TGCCTGTAGACAGGAACGCACCGTTT
	EaC1RACER	CGCCATGAGTCAGCACCGCAAT
Ea-Caspase-3	EaC3RACEF	TGCCAAAAGTGGAACCTGACGCAGT
	EaC3RACER	AGGCCTGTCGGCTGCGAAAAGA
Ha-Caspase-1	HaC1F1	ATGTTGGACGGTGATGTTCAAG
	HaC1R1	CTTCCTACCAAACACAAGCAA
Ha-Caspase-2	HaC2F1	ATGGAAAATATGGACGAAACTT
	HaC2R1	AAATTTCAGCAGCTTTGTCAGC
Ha-Caspase-5	HaC5F1	ATGGAGCAGAAACACAAAGAAGC
	HaC5F2	CACAGACCTGGACTCTGTTG
	HaC5F3	GTAGACACACTGGGTGAACTG
	HaC5R3	CTCGATGGTGACGTCACAGG
	HaC5R5	GTAGTCGATGATGTCCATGAGG
Hv-Caspase-6	HvC6F1	CTGATCGCAAGCAGTTAGACATTG
	HvC6R1	CAAAGTCACATTGTCTGTAAC
Ms-Caspase-1	MsC1RACEF	GGCGCTACGCCAAGATGCCTGT
	MsC1RACER	TCAGCACAGCGACGAGCAAGCA
Ms Caspase-4-1	MsC4-1F	ATGGACAGTGAACTTCAGGATAC
	MsC4-1R	TTAAATTCTCTTAATGTATACAAATTTG
Ms-Caspase-4-2	MsC4-2RACEF	TCCAAGGGCGTTACCCAAAGAATCTGA
	MsC4-2RACER	GCCATGCGTCAGGACCGCAATA
Ms-Caspase-6	MsC6RACEF	CAGCGTTAAAACAAAGGGCGTAGCTCA

Sequence alignment and phylogenetic analysis

Deduced amino acid sequences from caspase transcripts retrieved from the various databases searched were aligned together with the *Drosophila* caspases (Drice , Dcp-1, Decay, Dronc, Dredd, Damm and Strica) and with *C. elegans* caspase Ced3 as an outgroup, using the FFT-Ns-i strategy implemented in the MAFFT alignment program (Katoh and Toh, 2008). After removing the prodomain, the amino acid alignment was then used for the phylogenetic analyses. Only sequences containing the complete open reading frame (ORF) or partial sequences containing at least the part encoding the complete large and small subunits were used to build the phylogenies.

The phylogenetic reconstruction was done by Bayesian inference using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The prior was set for the amino acid model to mix, thereby allowing model jumping between fixed-rate amino acid models. Markov Chain Monte Carlo runs were carried out for 10,000,000 generations after which log likelihood values showed that equilibrium had been reached after the first 400 generations in all cases, and those data were

discarded from each run and considered as 'burnin'. Two runs were conducted for the dataset showing agreement in topology and likelihood. To determine which clusters particular caspase sequences belong to, amino acid sequences were aligned with MAFFT using the G-INS-i strategy, and a phylogenetic tree was generated by Neighbor-joining (1000 replicates) using MEGA4 software (Tamura, *et al*., 2007).

Relative-rate tests were applied to compare rates of evolution in the Casp-1 and Caspase-2 subfamilies. Synonymous and non synonymous substitution rates were determined by the Nei and Gojobori algorithm (Nei and Gojobori, 1986) as implemented in MEGA4. Statistics were performed using the Codon-based Z-test, which corresponds to a t-test with an infinite degree of freedom (LaCount, *et al*., 2000). The coefficient of functional divergence θ was calculated using the program DIVERGE 2.0 (Gu and Vander Velden, 2002). This coefficient ranges between 0 and 1 and measures the overall degree to which site-specific substitution rates differ; values significantly greater than zero indicate rate differences among homologous sites in the two subfamilies.

In order to characterize the primary structure and domain architecture of the different caspases, their predicted amino acid sequences were submitted to ExPASy ScanProsite (http://www.expasy.ch/tools/scanprosite).

BAC library screening and sequencing

Nylon filters from a *H. armigera* BAC library established from the strain Toowoomba were washed, blocked, and hybridized with horseradish peroxidase-labelled DNA fragment containing part of the *H. armigera* Casp-1 and Casp-2 genes. Labeling, hybridization, and probe detection were done according to specifications in the ECL DNA labeling and detection kit (GE Healthcare). Positive clones were isolated from glycerol stocks, grown in Terrific Broth, and BAC DNA was isolated with the Nucleobond Xtra Maxi Kit according to the manufacturer's instructions (Macherey-Nagel). BAC genomic DNA quantities were estimated spectrophotometrically on a Nanodrop ND 1000 (Peqlab biotechnologie GmbH). Positive clones were digested by EcoR1 and HindIII, blotted and rehybridized first with HaCaspase-1 probe then with HaCaspase-2 to identify positives clones for the 2 probes. BAC DNA was sheared into two different size ranges (5-12kb and 4-5kb) with a hydroshear device (Molecular devices), blunted with the Quick blunting kit (New England Biolabs), isolated from agarose gel, column purified, and ligated into the pUC-19 SmaI vector (Fermentas). Ligations were transformed into

E. coli ELECTROMAX DH5α-E electrocompetent cells (Invitrogen). Plasmid preparation, sequencing, and assembly were performed as mentioned above.

Intron-Exon structure

Bombyx mori caspase cDNA sequences were compared to the genomic sequences retrieved from KAIKObase (Shimomura *et al*., 2009) (http://sgp.dna.affrc.go.jp/KAIKObase/; Caspase-1: BGIBMGA006940, Caspase-3: BGIBMGA006131, Caspase-4: BGIBMGA004420, Caspase-5: BGIBMGA002841, Caspase-6: BGIBMGA006726) using the Spidey software available at NCBI. The same comparison was done using *H. armigera* cDNA sequences and the genomic sequences retrieved from the *H. armigera* draft genome assembly mentioned above.

Results and discussion

Lepidopteran caspases cluster into six distinct clades

As the genome of the silkworm *Bombyx mori* is the sole fully sequenced genome for insects of the order Lepidoptera to date, we first searched it for the presence of putative caspase genes (Fig. 3). We identified a total of 5 genes, one on chromosome 4 (Caspase-3), three at various places on chromosome 10 (Caspase-1, -5 and -6) and one on chromosome 20 (Caspase-4) (Fig. 3).

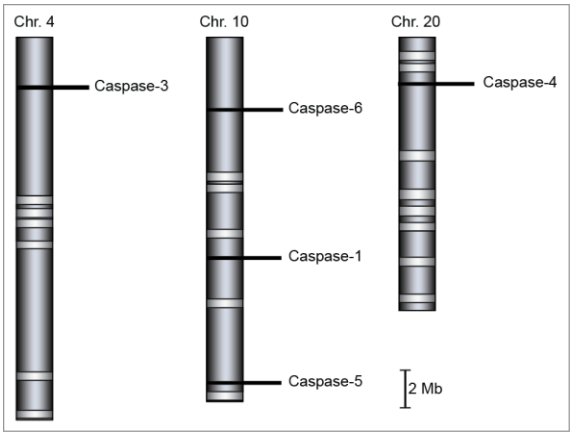


Figure 3. Schematic representation of the chromosomal localization of caspase genes on the Silkworm genome, according to Kaikogaas.

To get further insight into this gene family, we then mined publicly available as well as "in-house" EST datasets for transcripts encoding putative caspase enzymes (Supplementary Table S1). We retrieved a total of 66 transcripts corresponding to 31 different genes, from 27 species of moths and butterflies, spanning 11 lepidopteran families (Supplementary Table S1).

Caspase sequences were accepted if they fulfilled the following criteria: presence of the characteristic binding site (L/F/S T/S H G) and/or active site (QAC[R/Q]G), and level of amino acid identity (for complete amino acid alignments, see Supplementary Figures S1 to S6).

A Bayesian inferred phylogenetic analysis clustered these sequences into six distinct clades (Fig. 4), revealing an extra caspase gene family that was not found in *B. mori* genome (Caspase-2). This observed number of caspase genes in Lepidoptera is close to the seven genes found in *Drosophila* species (Drice, Dcp-1, Decay, Damm, Dronc, Dredd and Strica) (Kumar and Doumanis, 2000). To facilitate our analysis at this stage, we decided to establish a naming convention for these genes based on the results of the phylogenetic analyses. The first caspase fully characterized for a lepidopteran insect has been named Casp-1 preceded by the initials Sf, corresponding to the name of the species *Spodoptera frugiperda* (Ahmad, *et al*., 1997). We extended this convention by naming each caspase starting with the initials of the species from which it has been isolated, followed by "-Caspase" and by a number depending on which clade the sequence clustered into. Clades were arbitrarily numbered Casp-1 to Casp-6. The term "Lep" was added to the cluster name to differentiate lepidopteran caspases from sequences derived from other organisms. When splicing variants were found (see page 36), we used the nomenclature proposed by Alnemri et al in which each variant is identified by a letter (Alnemri, *et al*., 1996).

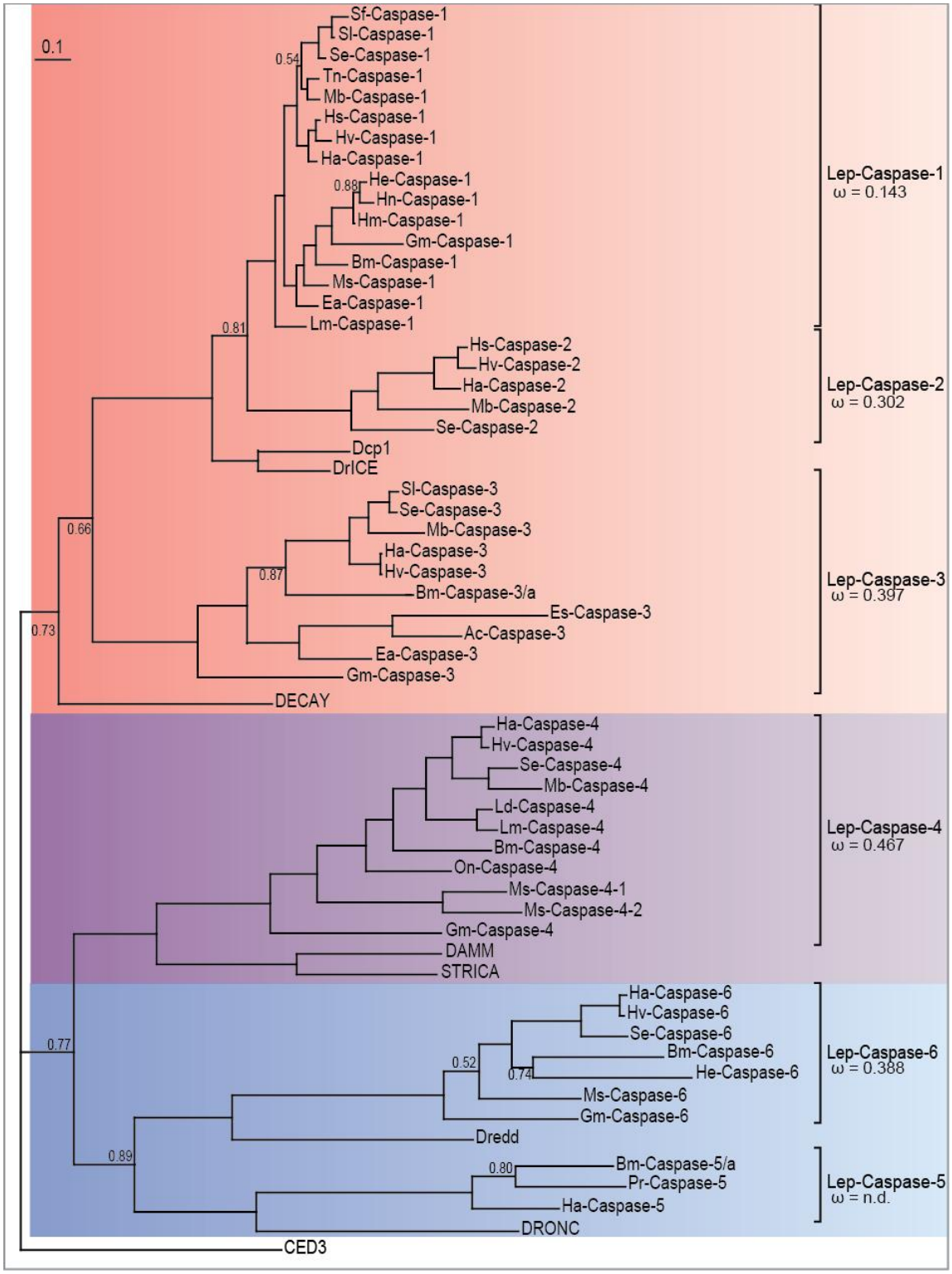


Figure 4. Phylogenetic relationships between caspase sequences found in Lepidoptera in relation with their *Drosophila* counterparts.
Amino acid alignments of caspase sequences without their prodomain were used to build an unrooted phylogenetic tree using a Bayesian inferred method with the *C. elegans* caspase Ced3 as an outgroup. Lepidopteran caspase clades were determined by branching patterns and are represented by brackets on the right. Posterior probabilities are shown only for nodes below 0.9. dN/dS ratio (ω) for each clade is mentioned below the clade name, except for Lep-Caspase-5 for which the number of sequences was too low to determine it.

Classification of the lepidopteran caspases

The phylogenetic analysis clustered Lep-Caspase-1, -2 and-3 with Dcp-1, Drice and Decay(Fig. 4). These3 *Drosophila* caspases have been classified as effectors due to their short prodomain, their substrate specificity towardthe tetrapeptide DEVD and their ability to cleave the Poly (ADP-ribose) polymeraseand/or p35(Dorstyn, *et al*., 1999b; Fraser and Evan, 1997; Fraser, *et al*., 1997; Song*et al*., 1997). Lep-Caspase-1 and-3 also harbor a short prodomain, characteristic of effector caspases (Fig. 5).

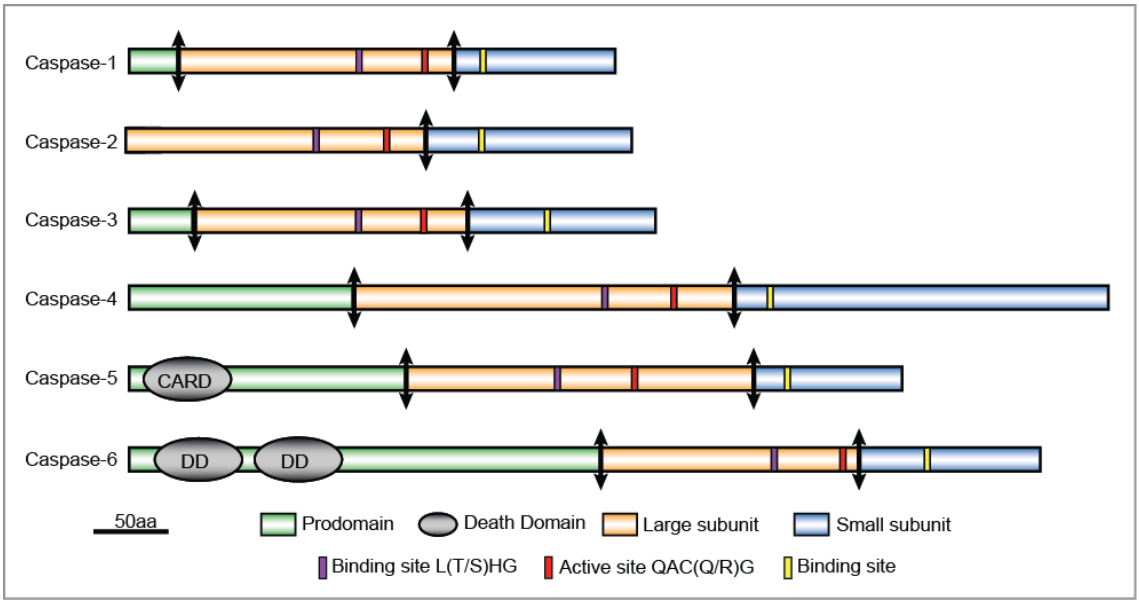


Figure 5. Predicted domain structure of lepidopteran caspases.
Conserved domains were predicted using ExPASy ScanProsite. Active and binding sites are indicated. Arrows represent the putative cleavage sites between the prodomain and the large subunit, and between the large and small subunits. Note that no suitable cleavage site between prodomain and large subunit could be found in Lep-Caspase-2 sequences.

However, no suitable cleavage site between the prodomain and the large subunit could be found in Lep-Caspase-2, and an amino acid alignment of Lep-Caspase-1 and-2 sequences suggests that the latter lacks a prodomain (Fig. 6). Caspase-1 has already been characterized in *S. frugiperda* and *S. littoralis*. These studies demonstrated substrate specificity toward DEVD (Liu, *et al*., 2005) and the ability to cleave p35, similar to the *Drosophila* effector caspases (Ahmad, *et al*., 1997). Although Drice and Dcp-1 are highly similar in sequence, several studies have shown that Drice, and not Dcp-1, is the main effector caspase in *Drosophila* (Fraser, *et al*., 1997; Muro, *et al*., 2006). A study comparing the effect of single and double mutants for Drice and Dcp-1 showed that these two caspases have an overlapping function (Xu, *et al*., 2006). The role of Decay has still to be clarified.

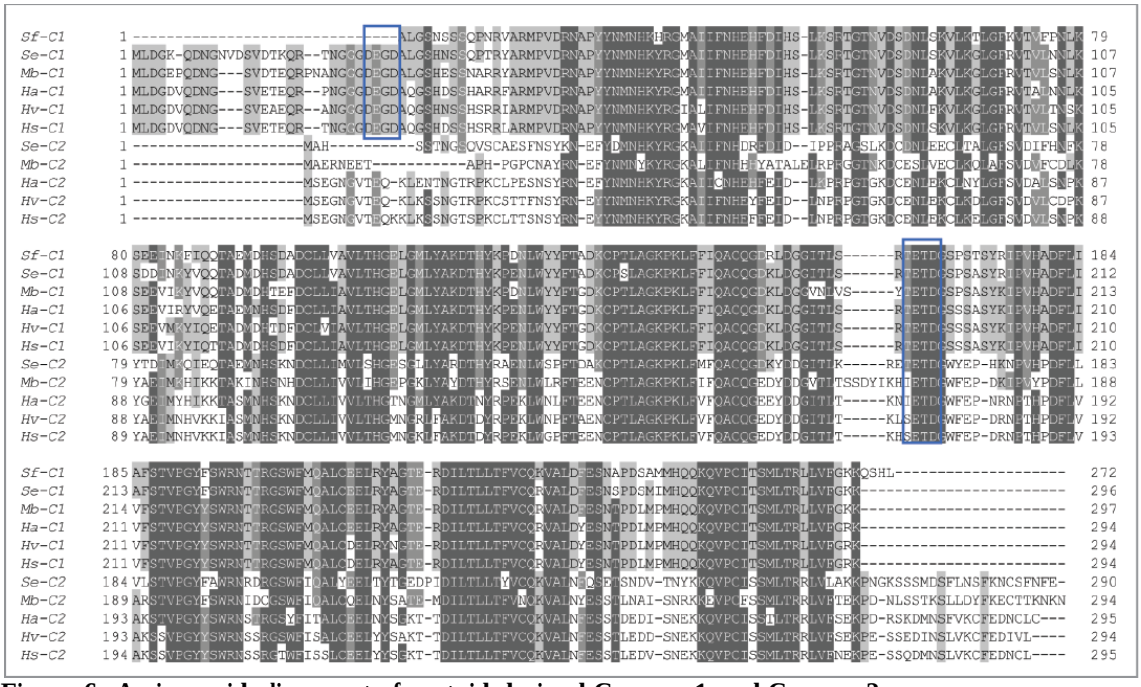


Figure 6. Amino acid alignment of noctuid-derived Caspase-1 and Caspase-2 sequences.
Identical residues are shaded in dark grey and conserved residues in lighter grey. Blue frames indicate putative cleavage sites. Note the apparent lack of cleavage site between the prodomain and the large subunit in Caspase-2 sequences.

Like *Drosophila* Dronc, to which it is closely related, and human Caspase-9, Lep-Caspase-5 harbors a CARD domain within its long prodomain (Fig. 5). CARD domains are involved in the interaction of initiator caspases with Ark/Apaf1, to form the apoptosome which is required for the activation of Dronc/human pro-Caspase-9 (Muro, *et al*., 2004; Snipas, *et al*., 2008). It has been shown that the *Dronc* gene is ubiquitously expressed during the development of *Drosophila* and that its expression is stimulated by ecdysone during metamorphosis (Dorstyn, *et al*., 1999a). Lep-Caspase-6 is closely related to *Drosophila* Dredd. The presence, in the long prodomain, of 2 motifs composed of 6 alpha helices, forming a three-dimensional structure characteristic of the Death Domain family (Fig. 5), strongly supports its classification as an initiator caspase (Park, *et al*., 2007). Despite the first description of Dredd as a potential initiator caspase (Chen, *et al*., 1998), it now appears to be more important in activating the innate immune response upon infection by Gram negative bacteria (Leulier, *et al*., 2000). Despite a relatively long prodomain, Lep-Caspase-4 does not harbor any known Death Domain which would have supported its classification as an initiator caspase (Fig. 5). Furthermore, the phylogenetic analyses show a relationship with the *Drosophila* caspases Damm and Strica, and the roles of these have not been clearly defined so far. Although Damm and Strica are very similar in sequence, they appear to play different roles: Damm seems to be an effector caspase

(Harvey, *et al*., 2001) whereas based on genetic studies (Baum, *et al*., 2007) Strica seems to be an initiator caspase, exhibiting redundant activity with Dronc during oogenesis. In addition, the small subunit of Lep-Caspase-4 is unique. It is twice as long as any other small subunit described so far, which usually ends 60 to 80 amino acids after the conserved serine residue (Ser^{428}) involved in substrate binding (Supplementary Figures S1 to S6). In addition, alignments (Supplementary Figure S4) show that the sequence corresponding to the last 140 residues of the small subunit is more variable compared to the rest of the subunit. However some residues are conserved in all sequences, including cysteine residues potentially involved in the formation of secondary structures. These features indicate that Lep-Caspase-4 is a peculiar caspase, the function of which cannot be solely assessed by sequence comparisons.

Alternative splicing of Caspase-3 and -5 in B. mori

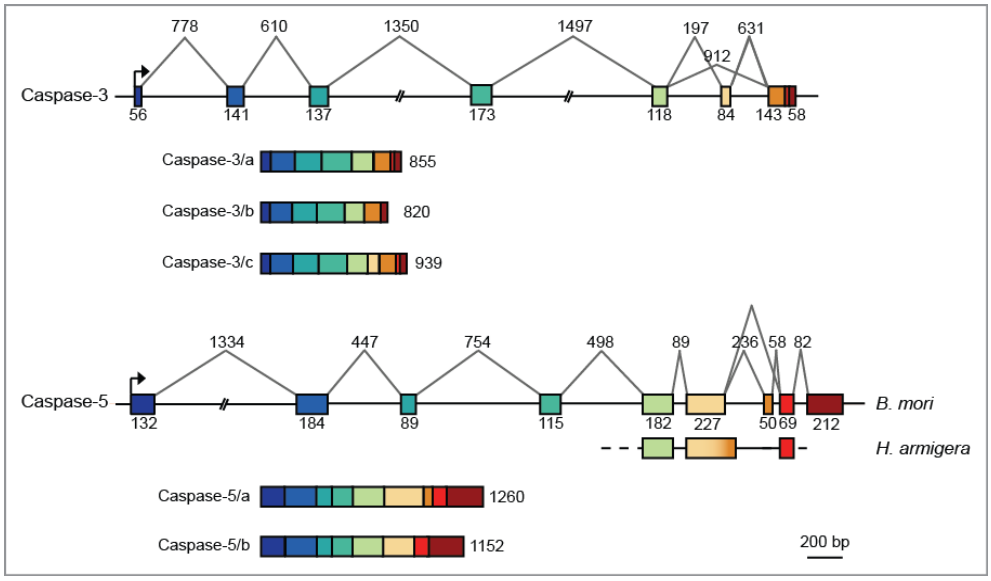


Figure 7. Intron-exon structure of lepidopteran Caspase-3 and -5.
For each gene, genomic structure is presented first with exons represented by colored boxes and introns by lines. Exon lengths in bp are indicated under the boxes, intron lengths above the lines

In *B. mori*, two isoforms of Caspase-5 have been identified, a long form (-5/a) and a short form (-5/b) in which the exon encoding the sequence corresponding to the catalytic site is spliced out (Fig. 7). The short form still possesses an intact prodomain and small subunit. In human, Caspase-9 splicing variants have been identified. In one of them, Caspase-9S, the catalytic site is missing and this isoform has been shown to be a dominant-negative inhibitor of apoptosis, by blocking the Caspase-9/Apaf1 interaction (Seol and Billiar, 1999). The short form of Bm-Caspase-5 missing the catalytic site could play a similar role as human Caspase-9S. Although several isoforms of Caspase-5 were observed in *B. mori*, only a single transcript could be

detected in *H. armigera*. In addition, the sequences corresponding to exon 6 and 7 in *B. mori* are part of a single exon in *H. armigera*.

Three splicing variants were detected for Ha-Caspase-3 (Fig. 7 and S3), all of them harboring both catalytic and binding sites. Alternative splicing was not observed for Ha-Caspase-3. It has been suggested that catalytically active isoforms may differ in specificity and efficiency, which could fine-tune caspase activity through heterodimerization, resulting in amplification or inhibition of apoptosis (Ng, *et al*., 1999).

A noctuid-specific caspase gene arose from duplication of Caspase-1

Thorough mining of lepidopteran EST datasets for putative Caspase-2 led us to find the corresponding genes only in species of the family Noctuidae (Fig. 4, Table S1). No ortholog of Caspase-2 could be found in the silkworm genome (Fig. 3). Taking into account that the amino acid identity between Lep-Caspase-1 and Lep-Caspase-2 sequences is relatively high (ranging between 47 and 53 %), we hypothesized that both genes may have arisen, in Noctuidae, from a tandem gene duplication event. To test this hypothesis, we screened a *H. armigera* BAC library using probes designed according to either the Ha-Caspase-1 or the Ha-Caspase-2 sequence. A BAC clone (clone P3E13) hit by both probes was identified and selected for sequencing. The Caspase-1 and -2 genes were found, in a 44-kb fragment of this BAC, to be organized in tandem, separated by only 3kb of non-coding DNA, were in the same orientation (Fig. 8) and had no intron, confirming our hypothesis.

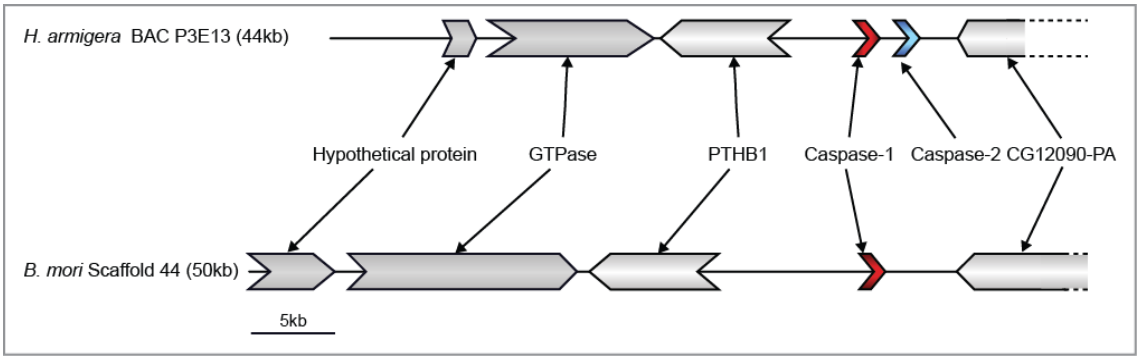


Figure 8. Representation of the genomic region surrounding Caspase-1 in *B. mori* and *H. armigera*. Deduced from a *B. mori* scaffold and the sequencing of a *H. armigera* BAC clone. The Caspase-1 and Caspase-2 genes are found in tandem on the *H. armigera* genome. Gene annotation was performed using Kaikogaas.

We then compared the organization of the genes surrounding Caspase-1 between *H. armigera* and *B. mori* (Fig. 8), we found a high degree of micro-synteny in this region of the

genome between the two species, similar to what has been reported in recent studies (d'Alencon *et al.*, 2010; Pauchet *et al.*, 2009).

To examine the relative rates of sequence evolution within the Caspase-1 and -2 subfamilies since divergence, pairwise comparisons of sequences obtained from noctuid species were performed by applying the Nei-Gojobori algorithm (Nei and Gojobori, 1986). The mean synonymous substitutions per synonymous site (d_S) was 0.480 and 0.549 within Caspase-1 and Caspase-2 respectively; mean d_S between Caspase-1 and -2 is 0.646, indicating saturation. Mean nonsynonymous substitutions per nonsynonymous site (d_N) was 0.047 for Caspase-1 and 0.170 for Caspase-2. We used the Codon-based Z test (Nei, 2000) to test the null hypothesis $d_S = d_N$ which would indicate that both genes are neutrally evolving. We could reject this null hypothesis as we found that d_N is significantly smaller than d_S (Caspase-1: Z = -17.000, p<0.001; Caspase-2: Z = -14.138, p<0.001), indicating a strong purifying selection on both genes. We also tested whether synonymous and nonsynonymous substitution rates differ between the two genes ($d_{Scasp1} = d_{Scasp2}$ and $d_{Ncasp1} = d_{Ncasp2}$, same Z test as before) we could not reject that $d_{Scasp1} = d_{Scasp2}$ (Z = -2.03, p>0.05), but the nonsynonymous substitution rate in Caspase-2 was significantly higher compared to the one in Caspase-1 (Z = -8.853, p<0.001). The coefficient of functional divergence (Gu, 1999), which measures the overall difference between site-specific substitutions among the two subfamilies, θ is = 0.57, which is significantly greater than zero (likelihood ratio test; $\chi^2 = 7.31$, P < 0.01). These results strongly suggest that Caspase-2, although also under purifying selection following the duplication event, has evolved under much more relaxed selective constraints than Caspase-1. It has been well documented that gene duplication is often followed by a period of relaxed selective constraints on one of the duplicate, allowing it to accumulate more mutations (Conant and Wagner, 2003; Panchin *et al.*, 2010). Although it has been suggested that gene duplication occurs at a high rate of 0.01/gene /million years, the most common fate of duplicated genes is silencing and loss (Lynch and Conery, 2000). In some cases however, the duplicate is retained as functional gene and either a new function is acquired by one of the copies or the ancestral function is maintained or subdivided between the two copies (Hughes, 1994). The fact that the cleavage site differ between the two subunits (TETD for Caspase-1 and xETD for Caspase-2), and that Caspase-2 may lack a prodomain, suggests that they might be activated by different signals. This could indicate a subfunctionalization of Caspase-2 in Noctuidae.

Ms-Caspase-4 subfamily has evolved through duplication events

Several contigs corresponding to putative Caspase-4 transcriptswere identified from an EST dataset derived from *M. sexta* larval midgut cDNA library, sequenced by 454 pyrosequencing(Pauchet, *et al*., 2010). Analyses of complete sequences obtained by RACE PCR resulted in 3 distinct cDNAs.

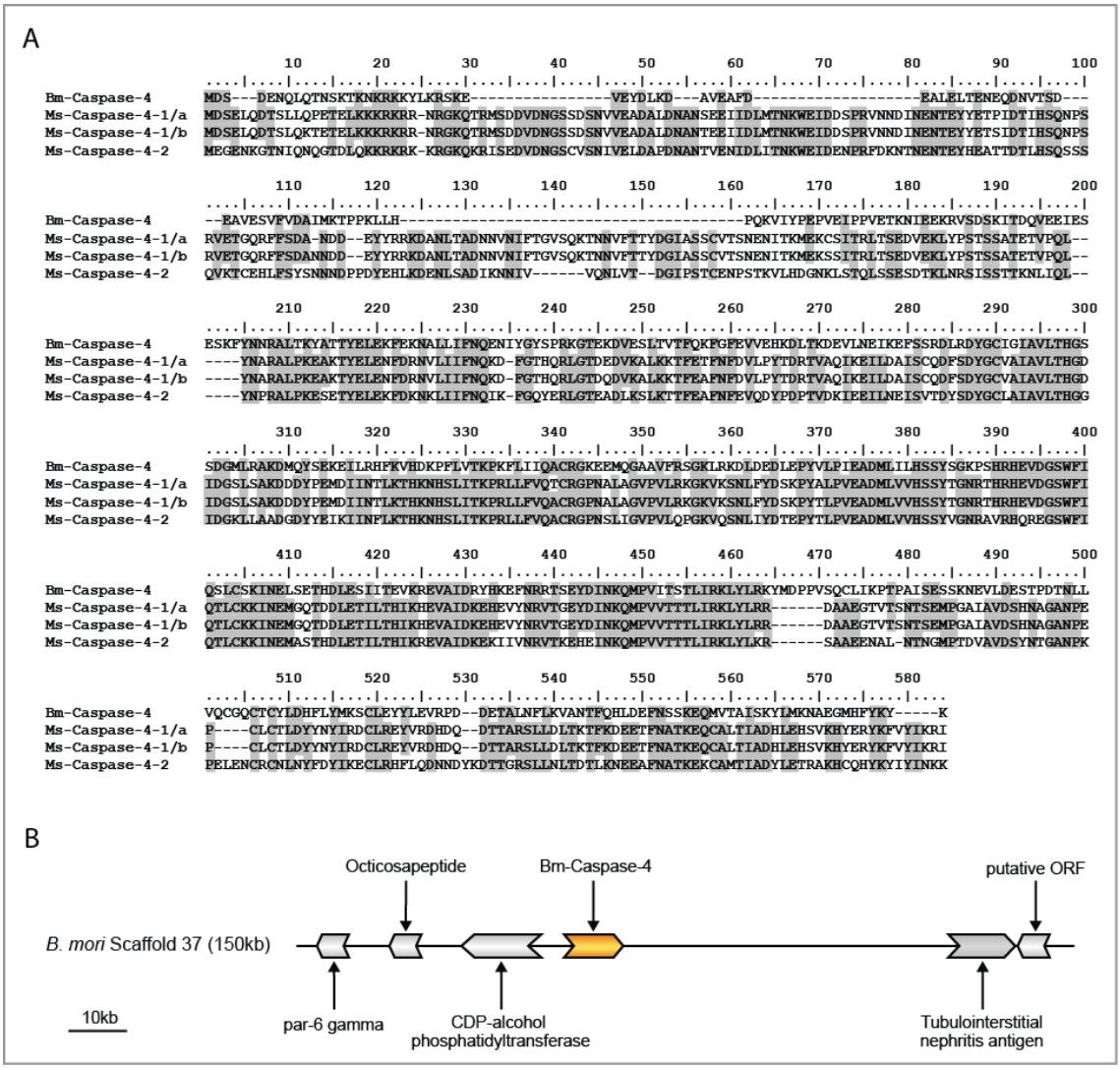


Figure 8. Recent gene duplications of the Caspase-4 gene in *M. sexta*.
(A) Amino acid alignment of the Caspase-4 sequences from *B. mori* and *M. sexta* Identical amino acids are shaded in grey.
(B) Schematic representation of the genomic region surrounding the Caspase-4 gene deduced from a *B. mori* scaffold. Gene annotation was performed using Kaikogaas.

Two of them, MsCaspase-4-1/a and -4-1/b, differ only by 20 single nucleotide polymorphisms (SNPs), 3 of which are nonsynonymous, as well as an extra codon encoding ASN^{113} in Ms-Caspase-4-1/b (Fig.9A). The third sequence, Ms-Caspase-4-2, encodes a protein sharing ~63% amino acid identity with both MsCaspase-4-1/a and-4-1/b (Fig.9A). The

occurrence of several unique transcripts for Lep-Caspase-4 has only been found in that species so far. A closer look at the region where Bm-Caspase-4 is located on the silkworm genome did not reveal any extra Lep-Caspase-4 genes or even pseudogenes nearby Bm-Caspase-4 (Fig. 9B). These results suggest that Caspase-4 has undergone at least two duplication events in *M. sexta*. A phylogenetic study of dipteran caspases suggest that Damm and Strica, the homologs of Lep Caspase-4, have arisen by duplication in the *melanogaster* and *obscura* clades, while only Strica is present in the other drosophilid clades (Bryant, *et al*., 2010). In mosquitoes, the ancestor of Damm/Strica has undergone many duplication events after the divergence of the *Anopheles gambiae, Culex quinquefasciatus* and *Aedes aegypti* lineages. Two homologs of Damm/Strica have been described in *An. gambiae*, as well as two in *C. quinquefasciatus* and four in *A. aegypti* (Bryant, *et al*., 2008; Bryant, *et al*., 2010). All of the duplicates are potentially active since they all harbor the critical amino acids involved in binding and active site. However, no biochemical characterization has been carried out on these caspases so far. The paucity of EST data for members of the family Sphingidae does not allow us to conclude whether the duplication of Lep-Caspase-4 is specific only to *M. sexta* or if it has also happened in sister species.

Future directions and conclusions

Despite obvious limitations of EST datasets in term of available species and transcriptome coverage, we were able to identify 63 caspase genes coming from 27 different lepidopteran species. Based on the phylogenetic analyses, we showed that the lepidopteran caspase family is represented by at least 5 members. Nevertheless, the biochemical characterization of these caspases has to be performed to clarify the exact function and their potential interactions. Our data also suggest that gene duplication is one of the shaping forces of this gene family in Lepidoptera, as it has been shown in Diptera and mammals. Availability in the near future of whole genome sequences and transcriptomes from other species of the order Lepidoptera as well as from other insect orders, will improve our understanding of the complexity of this gene family and its evolutionary history in insects in general.

Supplementary materials

Table S1. Details of the caspase sequences characterized in this study. Sequences marked with an asterisk are new to this study.

Species	Common name	Family	Gene	Database	accession
Caspase 1					
Antheraea assama	Muga silkworm	Saturniidae	AaCASP-1	Genbank	FG217522
Bicyclus anynana	Squinting bush	Nymphalidae	BaCASP-1	Genbank	GE695124
Bombyx mori	Silkworm	Bombycidae	BmCASP-1	Genbank	AF448494
Epiphyas postvittana	light brown apple	Tortricidae	EpCASP-1	Genbank	EV811051
Euphydryas aurinia	Marsh fritillary	Nymphalidae	EaCASP-1	Genbank	HM234680*
Galleria mellonella	Greater Wax Moth	Pyralidae	GmCASP-1	in house	HQ328948*
Heliconius erato	Red Postman	Nymphalidae	HeCASP-1	Genbank	EF207976
Heliconius numata	Numata longwing	Nymphalidae	Hn-CASP-1	Insecta central	IC33419AbAorf6448
Heliconius melpomene		Nymphalidae	HmCASP-1	Genbank	EF211965
Helicoverpa armigera	Cotton Bollworm	Noctuidae	HaCASP-1	Genbank	EF688063
Heliothis subflexa		Noctuidae	HsCASP-1	PCR	HQ328949*
Heliothis virescens	Tobacco Budworm	Noctuidae	HvCASP-1	PCR	HQ328950*
Lymantria monacha	Black Arches	Lymantriidae	LmCASP-1	in house	HQ328951*
Manduca sexta	Tobacco hornworm	Sphingidae	MsCASP-1	Genbank	HM234675*
Mamestra brassicae	Cabbage Moth	Noctuidae	MbCASP-1	in house	HQ328952*
Spodoptera exigua	Beet Armyworm	Noctuidae	SeCASP-1	in house	HQ328953*
Spodoptera frugiperda	Fall Armyworm	Noctuidae	SfCASP-1	Genbank	U81510
Spodoptera littoralis	African Cotton	Noctuidae	SlCASP-1	Genbank	AF548387
Trichoplusia ni	Cabbage Looper	Noctuidae	TnCASP-1	Genbank	AY159381
Caspase 2					
Helicoverpa armigera	Cotton Bollworm	Noctuidae	HaCASP-2	in house	HQ328954*
Heliothis subflexa		Noctuidae	HsCASP-2	PCR	HQ328955*
Heliothis virescens	Tobacco Budworm	Noctuidae	HvCASP-2	PCR	HQ328956*
Mamestra brassicae	Cabbage Moth	Noctuidae	MbCASP-2	in house	HQ328957*
Spodoptera exigua	Beet Armyworm	Noctuidae	SeCASP-2	in house	HQ328958*
Caspase 3					
Anthocharis cardamines	Orange Tip	Pieridae	AcCASP-3	in house	HQ328959*
Bombyx mori	Silkworm	Bombycidae	BmCASP-3	Genbank	DQ360829 (variant a) AY885228 (variant b) DQ360830 (variant c)
Eucheira socialis	Social White	Pieridae	EsCASP-3	in house	HQ328960*
Euphydryas aurinia	Marsh fritillary	Nymphalidae	EaCASP-3	Genbank	HM234681*
Galleria mellonella	Greater Wax Moth	Pyralidae	GmCASP-3	in house	HQ328961*
Helicoverpa armigera	Cotton Bollworm	Noctuidae	HaCASP-3	in house	HQ328962*
Heliothis virescens	Tobacco Budworm	Noctuidae	HvCASP-3	in house	HQ328963*
Lymantria monacha	Black Arches	Lymantriidae	LmCASP-3	in house	HQ328964*
Mamestra brassicae	Cabbage Moth	Noctuidae	MbCASP-3	in house	HQ328965*
Ostrinia nubilalis	European Corn Borer	Crambidae	OnCASP-3	Genbank	GH987442
Spodoptera exigua	Beet Armyworm	Noctuidae	SeCASP-3	in house	HQ328966*
Spodoptera littoralis	African Cotton	Noctuidae	SlCASP-3	in house	HQ328967*

Table S1 (continued)

Caspase 4					
Bombyx mori	Silkworm	Bombycidae	BmCASP-4	Kaikobase	HQ456874*
Colias eurytheme	Orange Sulphur	Pieridae	CeCASP-4	in house	HQ328968*
Galleria mellonella	Greater Wax Moth	Pyralidae	GmCASP-4	in house	HQ456875*
Helicoverpa armigera	Cotton Bollworm	Noctuidae	HaCASP-4	in house	HQ328969*
Heliothis virescens	Tobacco Budworm	Noctuidae	HvCASP-4	in house	HQ328970*
Lymantria dispar	Gypsy moth	Lymantriidae	LdCASP-4	in house	HQ328971*
Lymantria monacha	Black Arches	Lymantriidae	LmCASP-4	in house	HQ328972*
Mamestra brassicae	Cabbage Moth	Noctuidae	MbCASP-4	in house	HQ328973*
Manduca sexta	Tobacco hornworm	Sphingidae	MsCASP-4-1a	Genbank	HM234676* (variant a)
Manduca sexta	Tobacco hornworm	Sphingidae	MsCASP-4-1b	Genbank	HM234677* (variant b)
Manduca sexta	Tobacco hornworm	Sphingidae	MsCASP-4-2	Genbank	HM234678*
Ostrinia nubilalis	European Corn Borer	Crambidae	OnCASP-4	Genbank	GH998299 / GH991688
Plutella xylostella	Diamondback moth	Plutellidae	PxCASP-4	Genbank	BP937230
Pontia daplidice	Bath White	Pieridae	PoCASP-4	in house	HQ328974*
Spodoptera exigua	Beet Armyworm	Noctuidae	SeCASP-4	in house	HQ328975*
Caspase 5					
Bombyx mori	Silkworm	Bombycidae	BmCASP-5	Kaikobase	HQ328976* (variant a) HQ328977* (variant b)
Danaus plexippus	Monarch butterfly	Nymphalidae	DpCASP-5	Genbank	EY269609
Helicoverpa armigera	Cotton Bollworm	Noctuidae	HaCASP-5	Ha Genome assembly	HQ328978*
Pieris rapae	Small Cabbage	Pieridae	PrCASP-5	in house	HQ328979*
Caspase 6					
Bombyx mori	Silkworm	Bombycidae	BmCASP-6	Genbank	AB292816
Galleria mellonella	Greater Wax Moth	Pyralidae	GmCASP-6	in house	HQ328980*
Heliconius erato	Red Postman	Nymphalidae	HeCASP-6	Genbank	DT664351
Helicoverpa armigera	Cotton Bollworm	Noctuidae	HaCASP-6	PCR	HQ328981*
Heliothis virescens	Tobacco Budworm	Noctuidae	HvCASP-6	in house	HQ328982*
Manduca sexta	Tobacco hornworm	Sphingidae	MsCASP-6	Genbank	HM234679*
Spodoptera exigua	Beet Armyworm	Noctuidae	SeCASP-6	in house	HQ328983*

Figure S1. Amino acid alignment of Lep-Caspase1 sequences Identical residues are boxed in grey. Critical amino acids involved in substrate binding are boxed in purple. Critical amino acids involved in the active site, including the catalytic cysteine residue, are boxed in red. Blue frames indicate cleavage sites.

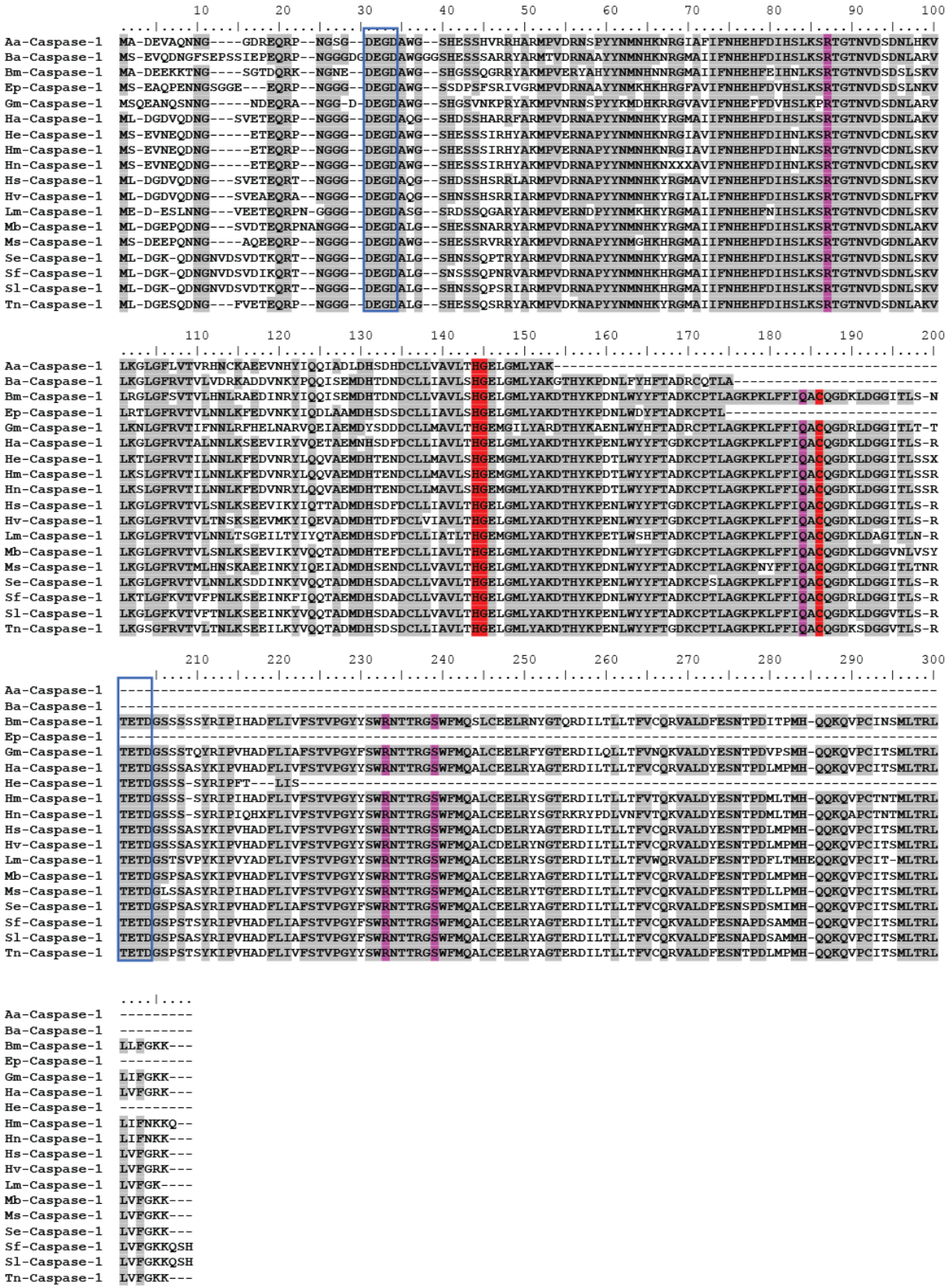


Figure S2. Amino acid alignment of Lep-Caspase-2 sequences Identical residues are boxed in grey. Critical amino acids involved in substrate binding are boxed in purple. Critical amino acids involved in the active site, including the catalytic cysteine residue, are boxed in red. Blue frames indicate putative cleavage sites.

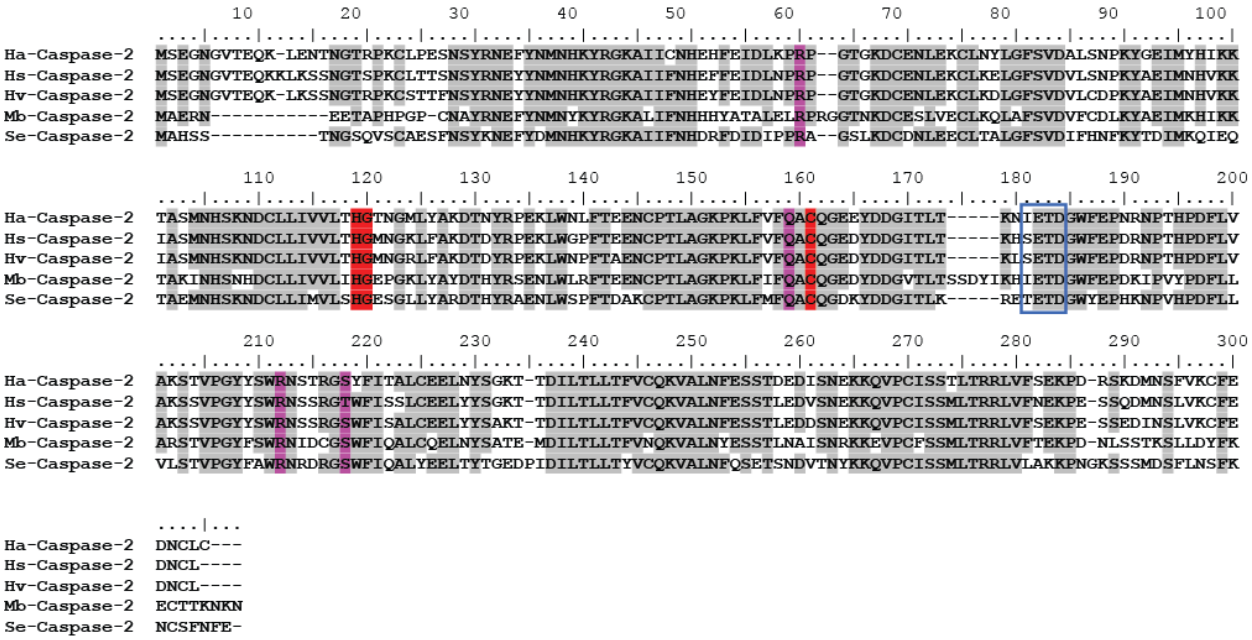


Figure S3. Amino acid alignment of Lep-Caspase-3 sequences. Identical residues are boxed in grey. Critical amino acids involved in substrate binding are boxed in purple. Critical amino acids involved in the active site, including the catalytic cysteine residue, are boxed in blue. Blue frames indicate putative cleavage sites.

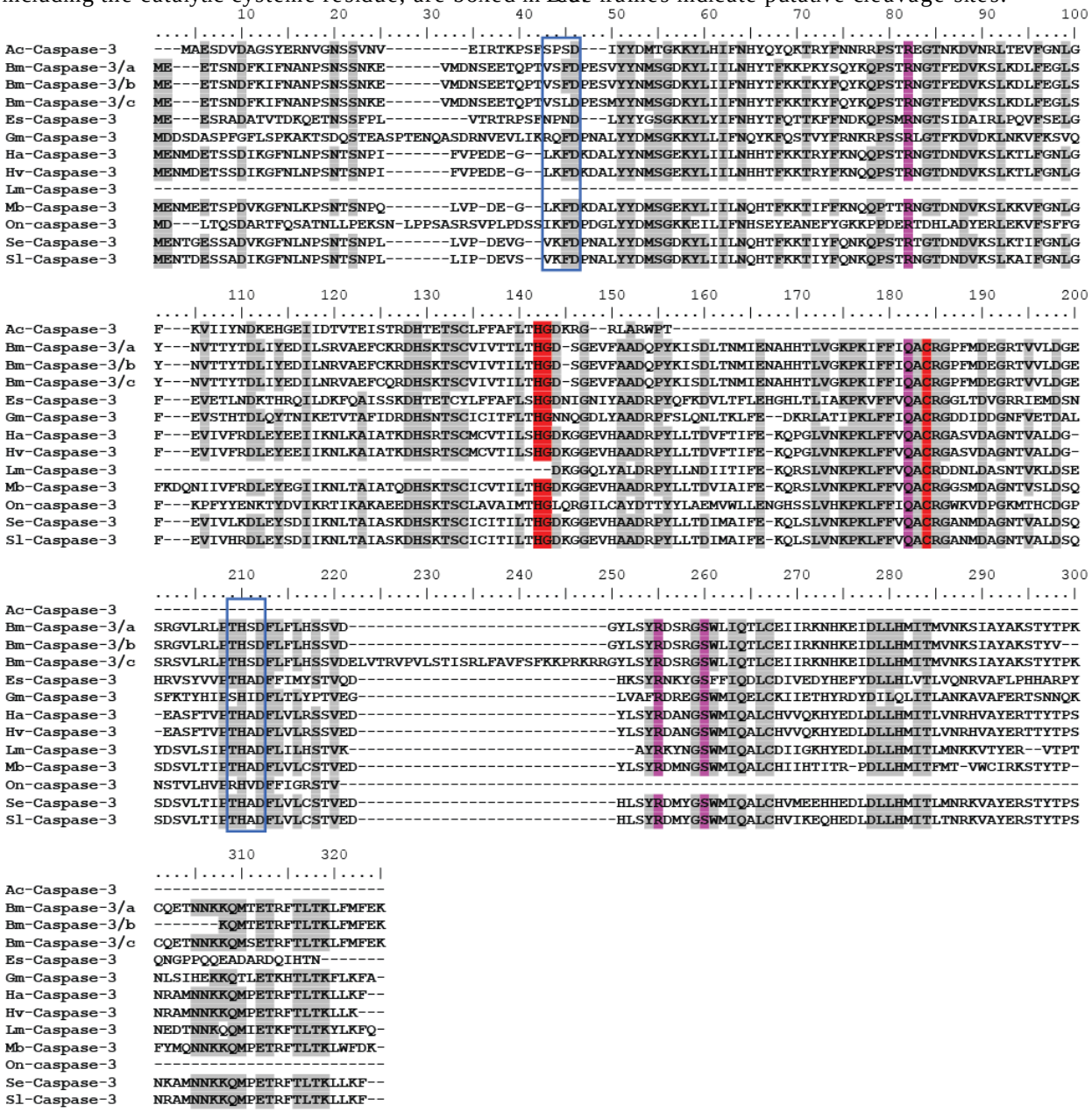


Figure S4. Amino acid alignment of Lep-Caspase4 sequences Identical residues are boxed in grey. Critical amino acids involved in substrate binding are boxed in purple. Critical amino acids involved in the active site, including the catalytic cysteine residue, are boxed in red. Blue frames indicate putative cleavage sites.

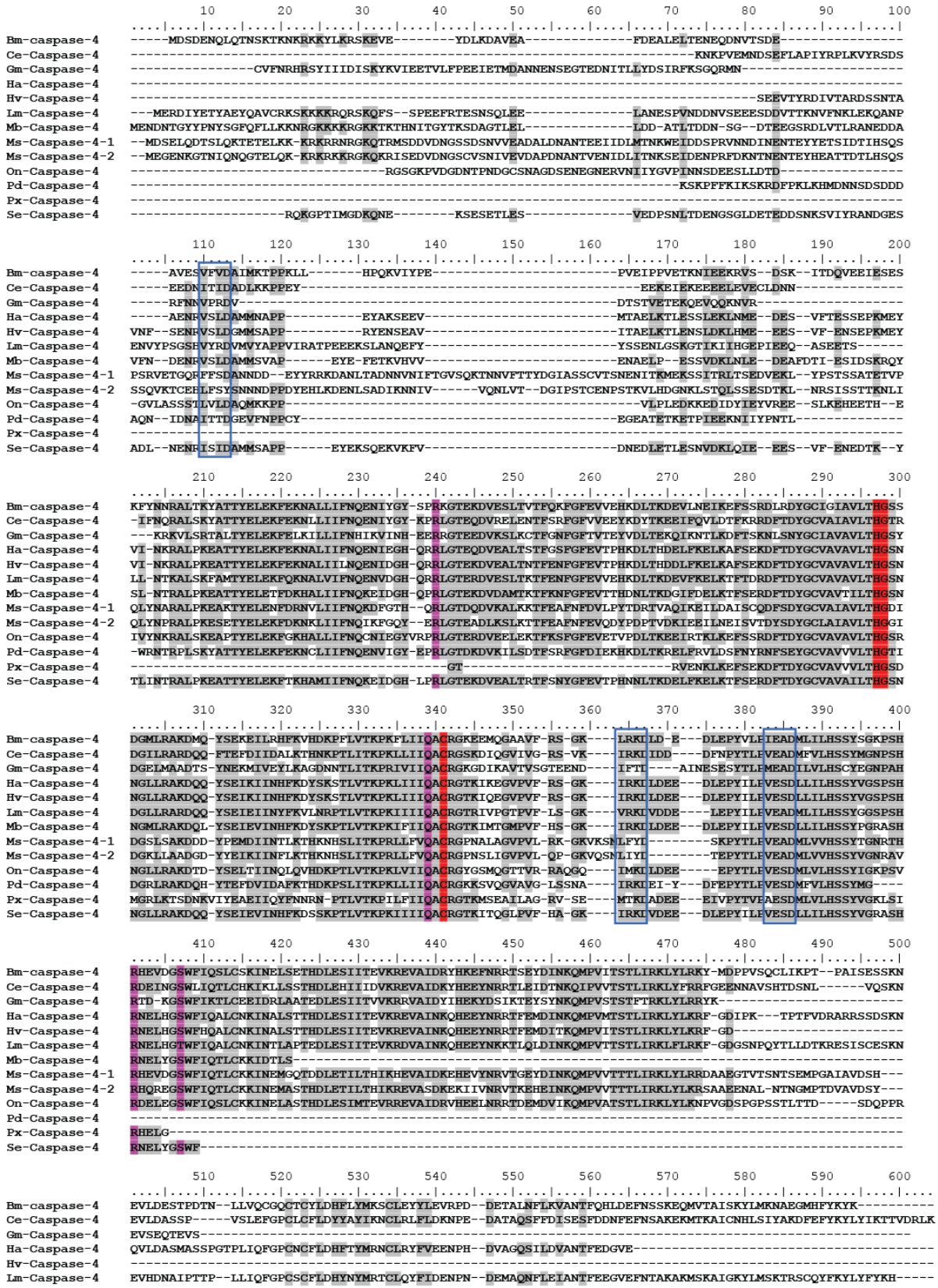


Figure S5. Amino acid alignment of Lep-Caspase-5 sequences. Identical residues are boxed in grey. Critical amino acids involved in substrate binding are boxed in purple. Critical amino acids involved in the active site, including the catalytic cysteine residue, are boxed in red. Blue frames indicate putative cleavage sites.

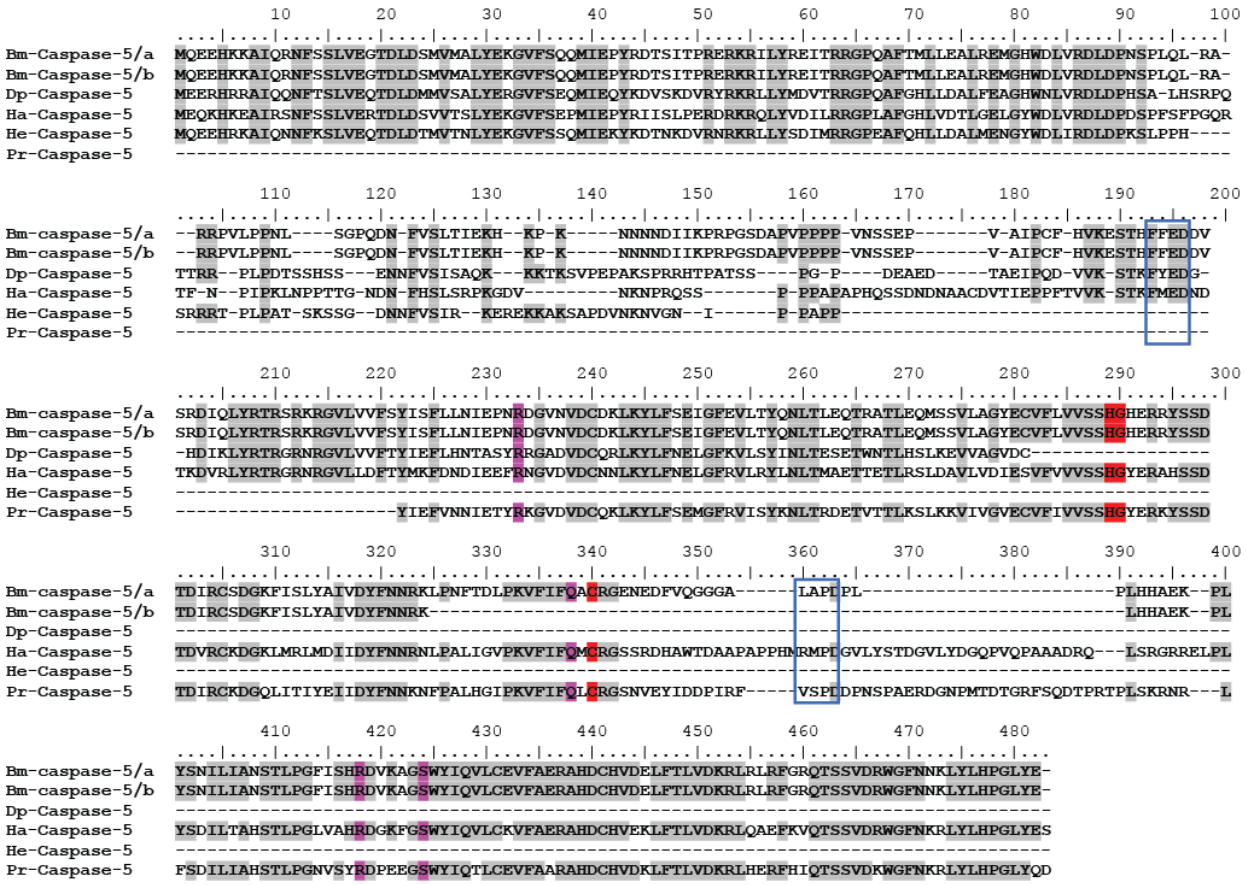
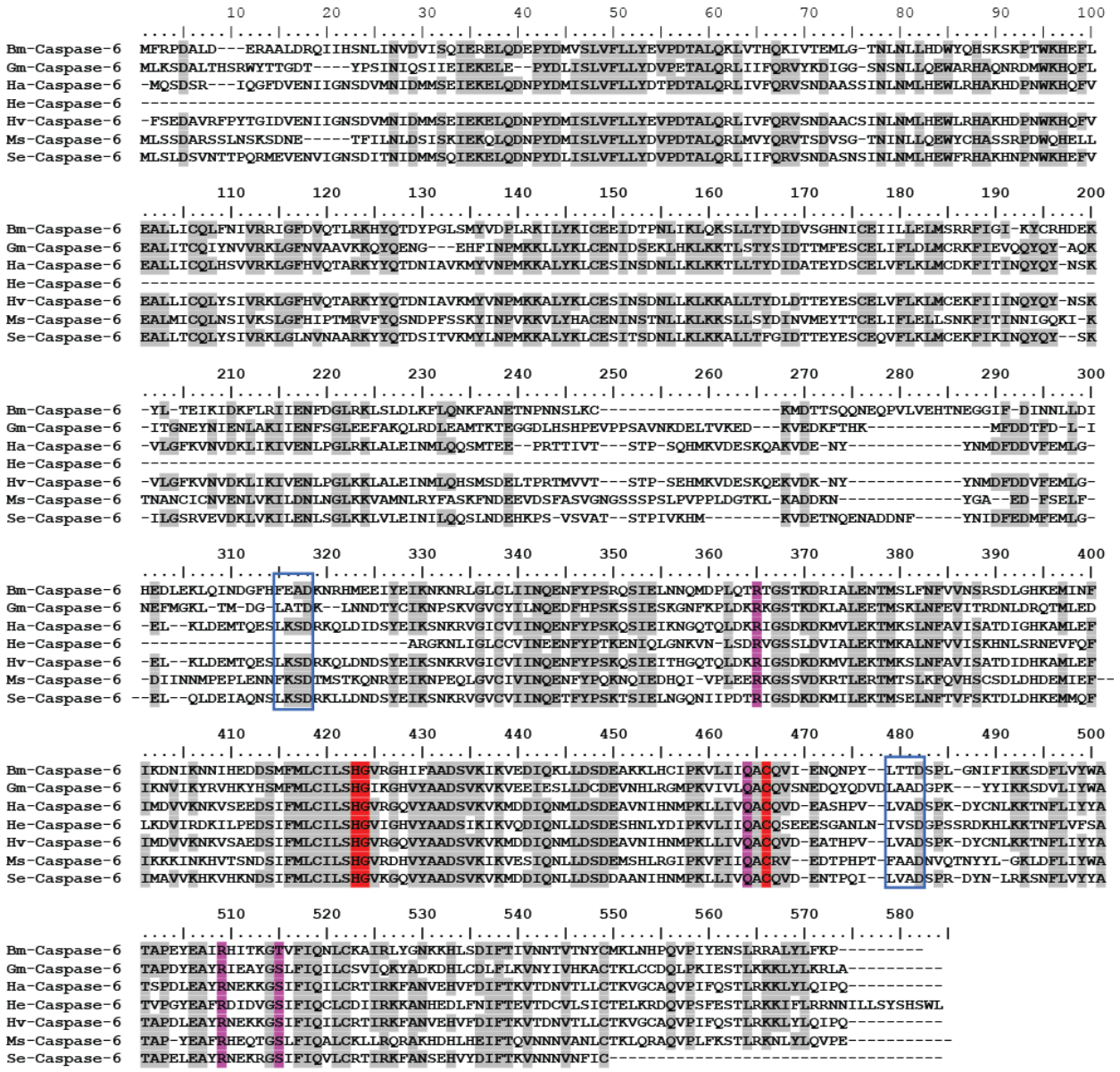


Figure S6. Amino acid alignment of Lep-Caspase-6 sequences. Identical residues are boxed in grey. Critical amino acids involved in substrate binding are boxed in purple. Critical amino acids involved in the active site, including the catalytic cysteine residue, are boxed in red. Blue frames indicate putative cleavage sites.



Chapter 2.
Functional analyses of Caspases in *Helicoverpa armigera*

Introduction

Apoptosis is a crucial event in the life of multicellular eukaryotes. This form of cell suicide is essential for proper development, maintenance of organism integrity and defense against pathogens. Cells undergoing apoptosis exhibit drastic morphological changes including cell shrinkage, chromatin condensation and membrane blebbing. These morphological transformations come with modifications of various cellular processes. It has been shown that the overall levels of mRNA and proteins are reduced. Early stages of apoptosis have been correlated with a decrease in mRNA synthesis as well as an increase of mRNA degradation (Degen, *et al*, 2000). In addition, protein synthesis is also decreased due to the cleavage of several components of the translation machinery by effector caspases (Bushell, *et al*, 2004; Martin, *et al.*, 1988; Mondino and Jenkins, 1995).

However, despite an overall decrease in mRNA and protein synthesis, apoptosis requires transcription and translation of a selection of critical genes. Among these, numerous apoptosis regulators are found, such as members of the Bax family or oncogenes *c-jun* and *Fos* (Estus, *et al.*, 1994; Rong *et al*, 1999), but also apoptotic effectors such as caspase genes (Bowen, *et al*, 1999; Eldadah, *et al*, 1997). In *Drosophila*, the transcription of the caspase Dronc, together with the pro-apoptotic factors Ark, Reaper and Grim, is upregulated by steroid hormones (Baehrecke, 2000; Cakouros, *et al*, 2002; Lee, *et al*, 2002). In *H. virescens*, it has been shown that Caspase-1 is upregulated during the early stages of the gut remodeling whereas Caspase-3 is upregulated later during pupation (Parthasarathy and Palli, 2007).

In an attempt to get a picture of the expression profiles of caspases in *H. armigera*, we used a real time quantitative RT-PCR approach to determine the resting mRNA expression levels of each of the 6 caspase genes in the different larval tissues and during development. Then the expression levels were also investigated upon induction of apoptosis induction by actinomycin D or 20-hydroxyecdysone and upon immune challenge with pathogenic microbes or with bacterial cell wall extracts. We observed that the expression of Caspase-5 is upregulated after the 20-hydroxyecdysone peak, similarly to what has been observed for Dronc, the ortholog of

Lep-Caspase-5 in *Drosophila*, this shows that Caspase-5 is involved in developmental apoptosis. Our data also show that Lep-Caspase-6, the ortholog of *Drosophila* Dredd, is upregulated upon immune challenge by Gram positive and Gram negative bacteria, suggesting that it shares a similar function in the *imd* pathway. In addition, immune challenge also triggers a strong upregulation of the expression of the effector Caspase-3, suggesting that this caspase may also be involved in the *imd* pathway.

Materials and methods

Apoptosis induction

A cell line derived from *H. armigera* pupal ovaries (BCIRL-HaAM1) (McIntosh, *et al*, 1983) was maintained in EX-CELL 420 medium (Sigma-Aldrich, USA) supplemented with 10% heat inactivated fetal bovine serum and 50 µg/mL gentamycin, at 27°C in 75 cm² flasks. When the cells reached 90% confluency, culture medium was replaced and actinomycin D was added to a final concentration of 0.5 µg/mL. Apoptosis inductions were performed in 6-well plates using 0.5 µg/mL actinomycin D or 100 µM 20-hydroxyecdysone. A non-induced culture was also prepared (referred as "control" below). Cells were harvested by centrifugation (1,000 x g, 15 min, 20 °C) after 4, 8 and 24 h post induction, and were subsequently washed twice with cold phosphate buffered saline (PBS). Cell pellets were then resuspended in 500 µL Trizol and stored at -80 °C until further extraction.

Feeding experiments, tissue and developmental stages expression

Fifteen early fifth-instar *H. armigera* larvae were fed for 24 h on an artificial pinto bean-based diet soaked with liquid culture of *Saccharomyces cerevisiae*, *Micrococcus luteus* (Gram positive), or *Escherichia coli* (Gram negative). Alternatively, larvae were fed on artificial diet soaked with bacterial cell wall polysaccharide (0.05% (w/v)). Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) were used as representative of Gram positive and Gram negative bacterial cell wall, respectively. Larvae fed on sterile artificial diet were used as a control group (Pauchet, *et al*, 2009). Three groups of five larvae per diet were used for the real-time quantitative RT-PCR experiments.

H. armigera larvae were reared on artificial diet. Hemolymph was sampled from 15 third-instar larvae, which were then dissected for tegument, fat body and midgut. For each developmental stage, between 10 and 20 insects were pooled for further RNA extraction.

Real time quantitative RT-PCR

TRIzol reagent (Invitrogen) was used to isolate total RNA from the different samples described above. Total RNA was treated with 2 units TurboDNase (Ambion) followed by a clean-up step (RNeasy mini elute cleanup kit, Qiagen). Five hundred nanograms of DNA free total RNA were converted into single stranded cDNA using the RevertAid H Minus first strand synthesis kit and a mixture of random and oligo(dT) primers (Thermo Scientific). Real time quantitative RT-PCR reactions were performed in optical 96-well plates on a MX3000P Real time PCR detection system (Stratagene), using the ABsolute QPCR SYBR Green Mix (Thermo Scientific) to monitor double-stranded DNA synthesis, in combination with Rox as a passive reference dye included in the PCR master mix. As an endogenous control, ribosomal protein L1 (RpL1) was used. Specific primers were designed for each gene (Table 1).

Table 1. Quantitative RT-PCR primer sequences

Gene	Primer name	Primer sequence
Ha-Caspase-1	qHaC1F1	ATGTTCAATGGCTGTTGGAAG
	qHaC1R1	TGTGACATGGCATTGTTGTCT
Ha-Caspase-2	qHaC2F2	GGTGTTAACTCACGGAACGAA
	qHaC2R2	CCATCCATCTGTCTCGATGTT
Ha-Caspase-3	qHaC3F1	GGAATGGTACGGACAATGATG
	qHaC3R1	TCCATGGCTCAGGATAGTGAC
Ha-Caspase-4	qHaC4F1	TCGCAAGGACCTAGATGAAGA
	qHaC4R2	GCTCTCCATCGCTAGTTGTTC
Ha-Caspase-5	qHaC5F1	AGCATTCGGCCATCTTGTAG
	qHaC5R1	GCGTTGACCTGGAAATGAGA
Ha-Caspase-6	qHaC6F1	GAATTTGCCTGGACTGAGGA
	qHaC6R1	TGCTGAGAGGGAGTACTTGTGA

For the resting expression pattern, results are expressed as fold differences compared to the expression level of the control gene RpL1. For the immune challenge and induction of apoptosis, caspase expression levels were normalized with the expression of RpL1 and then compared to the expression of the respective caspases in control samples. One-Way ANOVA, followed by a Bonferroni post-hoc test, was performed to determine if the relative expression of each caspase for the different treatment was significantly different than in the control. Expression differences between 0.5- and 2-fold (which correspond to a difference of +/- 1 Ct) were regarded as non significant.

Results

Caspase expression patterns in H. armigera

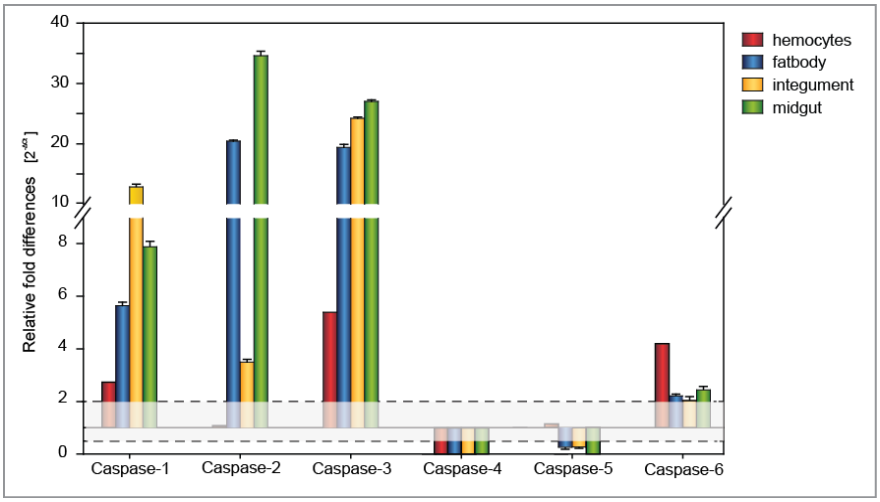


Figure 1. Caspase mRNA expression in the different tissues of third instar larvae
Expressed as relative fold differences compared to the expression of the control gene RpL1 ($2^{-\Delta Ct}$).

To test whether caspase genes have a tissue specific pattern of expression in *H. armigera*, we determined their resting mRNA expression level in hemocytes, midgut, fatbody and integument using real time quantitative RT-PCR (Fig. 1). The 6 caspases seem to be ubiquitously expressed in the different tissues, however at different levels. Caspase-1 is strongly expressed in integument, midgut and fatbody, but is also expressed to a lesser extent in hemocytes. The expression of Caspase-2 in hemocytes is comparable to the expression of the control gene RpL1, whereas it is ~20 fold higher in fatbody and ~35 fold higher in midgut. In integument, expression of Caspase-2 is ~3-fold lower than Caspase-1. Caspase-3 is highly expressed in all tissues. On the other hand Caspase-4 is expressed at very low level in all tissues. The expression of Caspase-5 is comparable to the expression of the control gene RpL1 in all tissues. The expression of Caspase-6 is ~2-fold higher than RpL1 in integument, fatbody and midgut and ~4-fold higher in hemocytes.

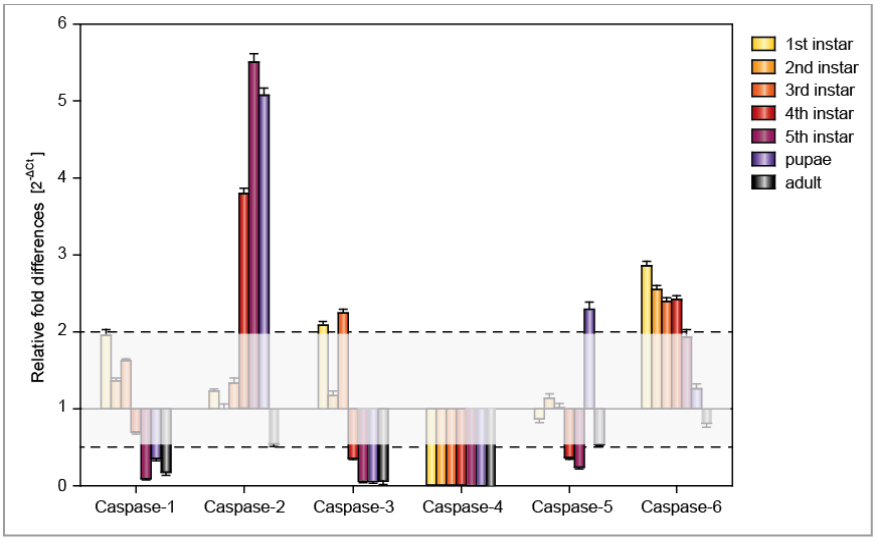


Figure 2. Caspase mRNA expression in the different developmental stages.
Expressed as relative fold differences compared to the expression of the control gene RpL1 ($2^{-\Delta Ct}$).

To determine whether the expression of caspases varies in function of the developmental stage of the insect, we measured the transcript levels of each of the 6 caspases by quantitative RT-PCR in the 5 larval instars, as well as in pupae and in adults (Fig. 2). Caspase-1 and -3 transcripts are more abundant in the first three larval instars than in any other developmental stages. The opposite is observed for Caspase-2 transcripts. Similarly to what is observed in the different larval tissues (Fig. 1), Caspase-4 mRNA expression is very low in all developmental stages. Caspase-5 mRNA expression remains low during larval development but is 2 to 3 fold higher at pupation, before dropping again in adults. Finally, the level of Caspase-6 transcripts decreases gradually from the 1st larval instar to the adult stage.

Caspase expression after immune challenge

Helicoverpa armigera larvae were fed for 24 h on artificial diet containing non-pathogenic microbes: Gram negative (*E. coli*), Gram-positive (*M. luteus*) bacteria or yeast (*S. cerevisiae*). The expression of each caspase was investigated by real-time quantitative RT-PCR (Fig. 3). Transcript levels are expressed as relative fold differences compared to the control treatment (larvae fed on a microbe free diet). In the midgut (Fig. 3A), the different treatments induce a significant upregulation of the expression of Caspase-3 and -6. In addition, the 2 bacterial cell wall polysaccharides, LTA and LPS also induce the upregulation of Caspase-1, -2 and -4. In the rest body (Fig. 3B), Caspase-3 is upregulated by the ingestion of LTA and LPS, and in a lesser extent by *E. coli*. The expression of Caspase-1 and -4 remains constant in response to LTA and LPS, but decreases significantly when larvae were fed on the different

microorganisms. In hemocytes, Caspase-3 is strongly upregulated, up to 40-fold, in response to the ingestion of microorganisms (Fig. 3C).

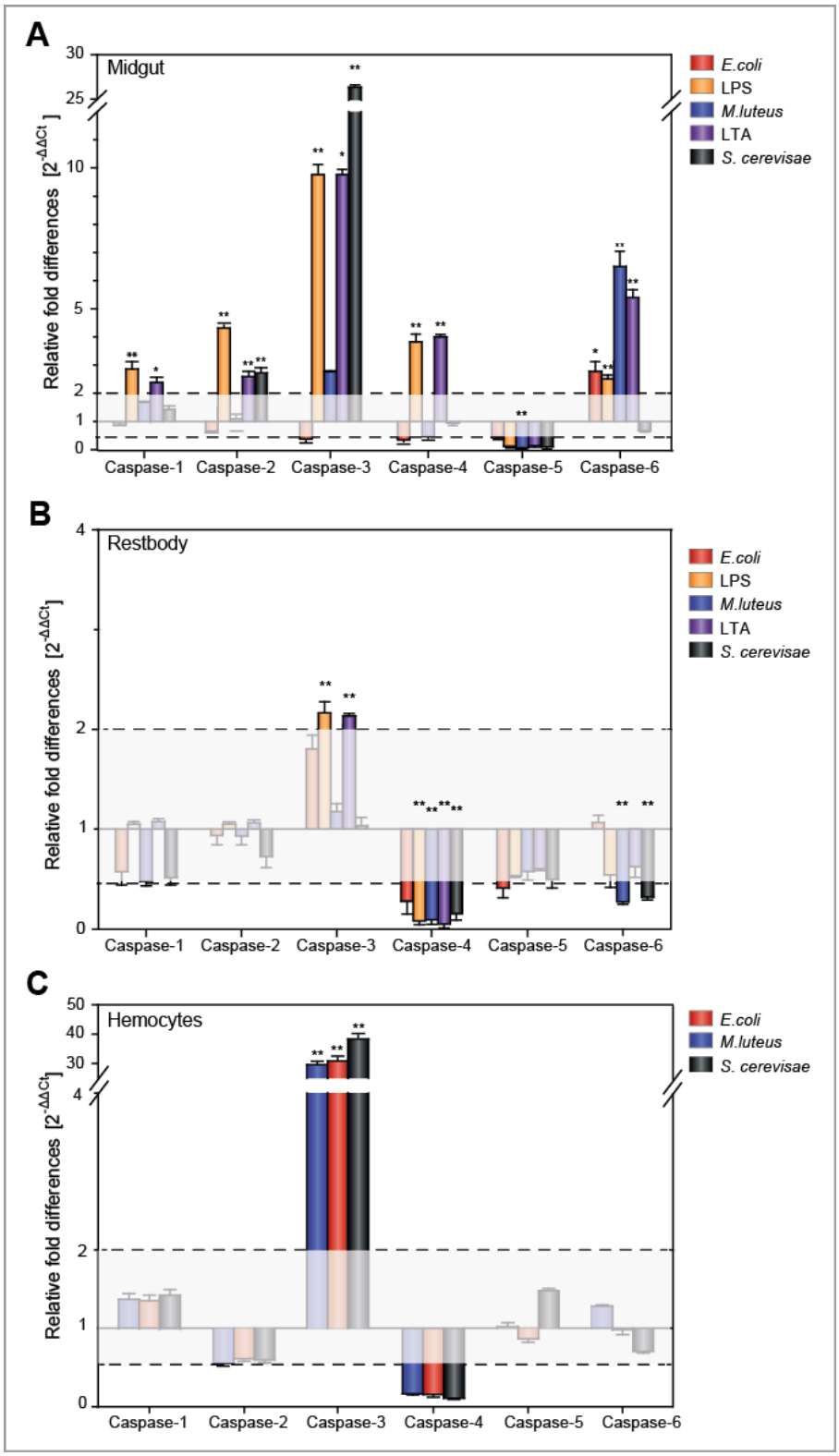


Figure 3. Caspase mRNA expression after immune challenge with 3 microorganisms, *E. coli*, *M. luteus* or *S. cerevisae*, or with bacterial cell wall polysaccharide LPS or LTA, in the midgut (A), in the restbody (B) or in hemocytes (C), expressed as relative fold differences ($2^{-\Delta\Delta Ct}$) compared to caspase mRNA expression in larvae fed on sterile diet. ** $p<0.001$, * $p<0.01$.

Caspase expression upon induction of apoptosis

To investigate whether the expression of caspases is altered upon induction of apoptosis, we treated *H. armigera* derived cells (HaAM1) with both actinomycin D and 20-hydroxyecdysone which are typical apoptotic inducers.

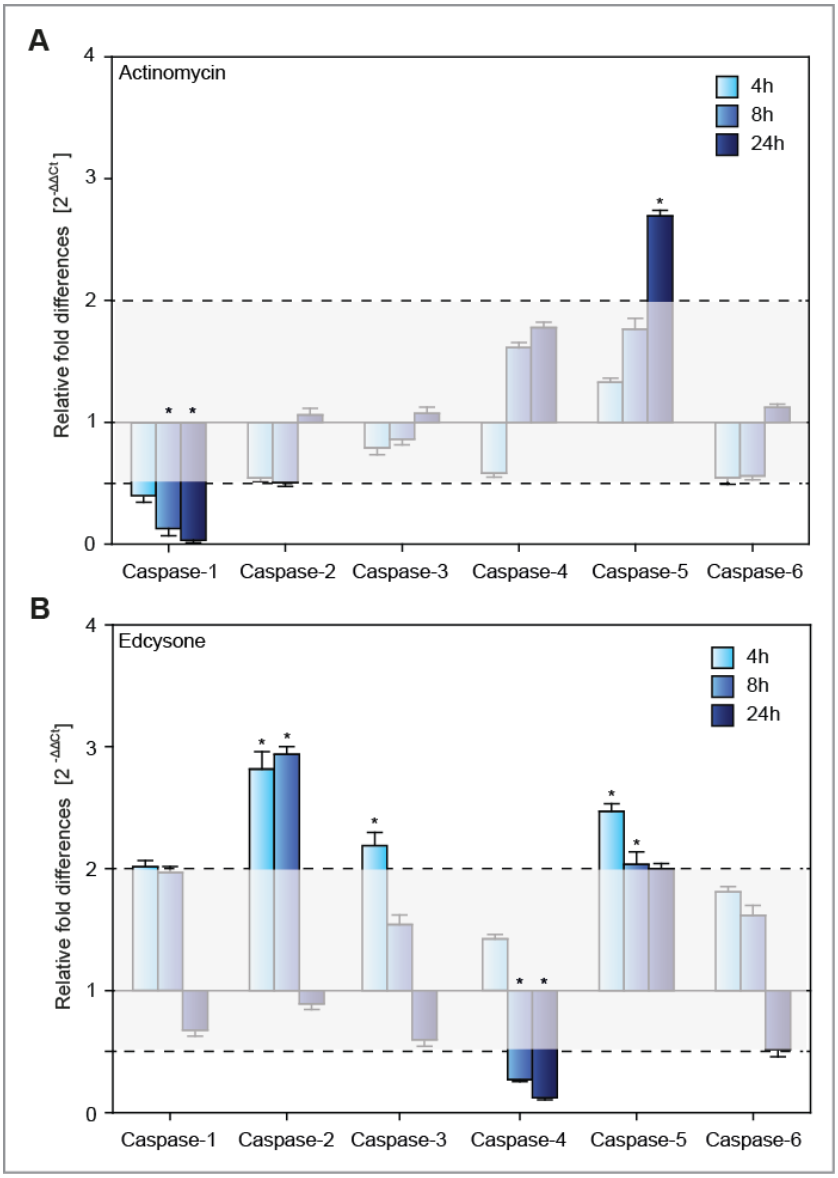


Figure 4. Caspase mRNA expression in HaAM1 cells 4, 8 and 24 hours after induction of apoptosis (A) with 0.5 µg/mL actinomycin D or (B) with 100 µM 20-hydroxy ecdysone Expressed as relative fold differences ($2^{-\Delta\Delta Ct}$) compared to the caspase mRNA expression in non-induced cells * $p<0.001$

Actinomycin D is a well known antibiotic which is widely used as an apoptotic inducer. When HaAM1 cells are treated with 0.5 µg/mL actinomycin D, the expression level of Caspase 1 decreases over time whereas the expression of Caspase-2, -3, -4 and -6 remains constant (Fig. 4A). On the other hand, the expression of Caspase-5 increases significantly during the

same period of time, until reaching a level 2.5-fold higher than in the control 24 hours after induction (Fig. 4A). After 4 hours treatment with 20-hydroxyecdysone (Fig. 4B), the expression level of all caspases is higher than in the control. These higher expression levels are maintained during the first 8 hours post-induction, before dropping completely after 24 hours, at the exception of Caspase-5 for which mRNA expression level is still 2-fold higher than in the control.

Discussion

Caspase-1 and -2, shared functions?

As described in Chapter 1, Caspase-1 and -2 are effector caspases and Caspase-2 is a noctuid specific caspase, arisen from a duplication of Caspase-1. We hypothesized that these two caspases might be activated by different signals. Caspase-1 mRNA is more abundant in hemocytes and integument, whereas Caspase-2 transcripts are more abundant in midgut and fatbody. During development, Caspase-1 transcripts are more abundant than those from Caspase-2 during the first 3 larval instars, and the opposite is observed during the last two larval instars and in pupae. These results suggest that Caspase-1 and -2 mRNA expression is differentially regulated, which supports the hypothesis of a subdivision of the ancestral function of Caspase-1.

Caspase-5, the development manager?

Lep-Caspase-5 is orthologous to Dronc, which is a known *Drosophila* caspase involved in developmental apoptosis. It has been shown that Dronc is ubiquitously expressed during the development of *Drosophila* and that its expression is stimulated by ecdysone during metamorphosis (Dorstyn, *et al*., 1999a). The real time quantitative RT-PCR analyses show an upregulation of Ha-Caspase-5 transcription during pupation, which can be correlated with the ecdysone peak triggering metamorphosis (Fig. 5).

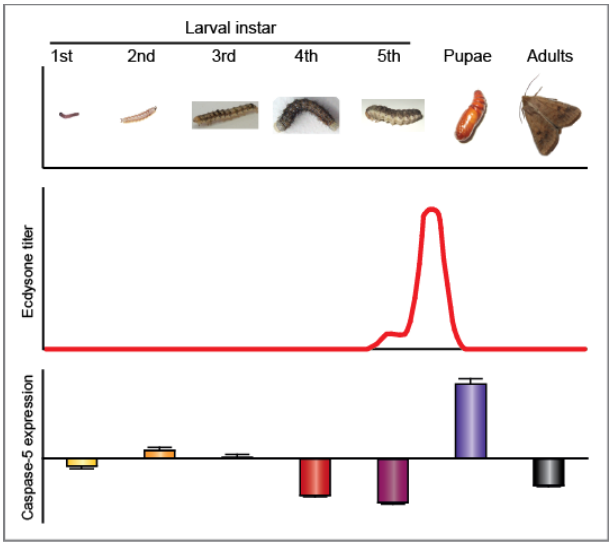


Figure 5. Schematic representation of the relationship between developmental stages, ecdysone titer and Ha-Caspase-5 expression in the cotton bollworm *H. armigera*.

When HaAM1 cells were treated with 20-hydroxyecdysone, Ha-Caspase-5 was the only caspase for which mRNA expression was still upregulated 24 hrs after induction. A similar study was performed on *Drosophila* l(2)mbn cells which showed that expression of the *Dronc* gene is upregulated after 12 hours exposure to 10 µM 20-hydroxyecdysone, and that the upregulation is maintained for 48 hours (Cakouros, *et al*., 2002). These results suggest that Lep Caspase-5 and Dronc not only share high sequence similarities but also share the same function during developmental apoptosis.

Caspase-3 and -6, to serve and protect?

As described in Chapter 1, Lep-Caspase-6 is closely related to *Drosophila* Dredd. Despite the first description of Dredd as a potential initiator caspase (Chen, *et al*., 1998), it now appears to be more important in activating the innate immune response upon infection by Gram negative bacteria (Leulier, *et al*., 2000). The insect midgut is the first barrier against potential bacterial infection (Ferrandon, *et al*., 1998). It has been shown that feeding nonpathogenic bacteria to lepidopteran larvae induces the expression of several antimicrobial peptides as well as an increase in phenoloxidase activity (Freitak, *et al*., 2007; Pauchet, *et al*., 2009).

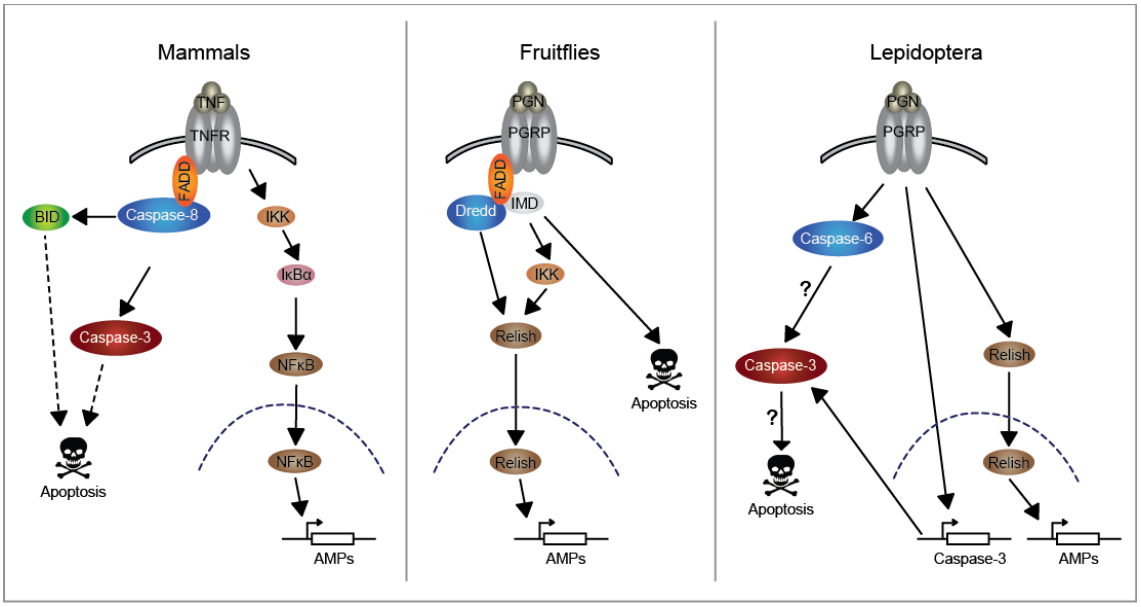


Figure 6. Comparison of the TNF / PGRP pathways in mammals, fruitflies and lepidopteran insects.
Proteins with a similar function are represented with the same color in the 3 systems

In *Drosophila*, the production of antimicrobial peptides is controlled either by the Toll pathway in the case of infection by Gram positive bacteria or fungi or by the Immune deficiency (*imd*) pathway in the case of infection by Gram negative bacteria (Lemaitre, *et al*., 2000; Michel, *et al*., 2001). In the *imd* pathway (Fig. 6), presence of bacteria is detected by peptidoglycan recognition proteins (PGRPs), and the signal is propagated via kinases, which phosphorylate the transcription factor Relish. The initiator caspase Dredd is also activated and is required for the catalytic activation of Relish (De Gregorio, *et al*., 2002; Steiner, 2004). Relish, a member of the NF-κB transcription factor family, then promotes the transcription of antimicrobial peptides (AMPs). The *imd* pathway is highly similar to the Tumor necrosis factor α (TNF-α) pathway in mammals (Hoffmann and Reichhart, 2002). TNF-α can induce both an innate immune response via NF-κB, or apoptosis via Caspase-8 activation (Fig. 6). In *Drosophila*, overactivation of the *imd* pathway has been shown to promote apoptosis (Georgel, *et al*., 2001). Furthermore, the production of AMPs induced by the *imd* pathway can be inhibited by the pan-caspase inhibitor p35 (Hay, *et al*., 1994). The baculovirus protein p35 has been shown to inhibit effector, not initiator caspases (LaCount, *et al*., 2000) which suggests that Dredd is not the only caspase involved in the *imd* pathway.

Analyses of the levels of Caspase-6 transcript show that bacterial challenge induces a 3 to 6-fold increase of its expression in the midgut. A similar expression pattern is also observed when larvae were challenged with bacterial cell wall extracts. These results suggest that the

major components of the bacterial cell walls are responsible for triggering the upregulation of Caspase-6 in the midgut. However, our results also show that both Gram negative and Gram positive bacteria induce Caspase-6 upregulation and not only Gram negative as we would expect from what has been observed in *Drosophila* (Leulier, *et al.*, 2000). Surprisingly, Ha-Caspase-6 mRNA is not upregulated in hemocytes from immune challenged larvae and is even downregulated in the rest of the body. The expression of Ha-Caspase-3 is strongly upregulated in hemocytes from larvae fed with bacterial diet. In a lesser extent, Caspase-3 is also upregulated in the midgut and rest body of immune challenged larvae. These results suggest that Caspase-3, like Caspase-6, is involved in immune response.

Caspase-4, an outcast?

Lep-Caspase-4 is characterized by a long prodomain, lacking any known death domain, but also by a large small subunit, which is unusual among caspases. Analyses of the transcript levels of this caspase confirmed that Caspase-4 is indeed a peculiar caspase, as its resting level of expression is extremely low in the different tissues or during development. Furthermore, expression of Caspase-4 is strongly downregulated in the hemocytes and rest of the body of immune challenged larvae as well as in ecdysone-induced cells, suggesting that Caspase-4 plays no role either in immune response or in developmental apoptosis.

Conclusion and further directions

Analyses of the expression patterns of the different caspases support the hypothesis that Lep-Caspase-5 and -6 play the same role as their *Drosophila* counterparts Dronc and Dredd. The upregulation of Lep-Caspase-3, an effector caspase, suggests that *imd* pathway in Lepidoptera may trigger the innate immune response and apoptosis, similar to the TNF signaling pathway in human. However, biochemical analyses need to be performed to validate these hypotheses.

Chapter 3. Comparative proteomic analysis of *Helicoverpa armigera* cells undergoing apoptosis

Introduction

Apoptosis is a tightly controlled cell suicide pathway which has a fundamental role in the life of multicellular organisms. It is involved in numerous biological processes ranging from development to homeostasis and immune response (Ameisen, *et al*., 1998; Creagh, *et al*., 2003; Penaloza, *et al*., 2008). Apoptosis is characterized by drastic morphological changes, such as cell shrinkage, chromatin condensation, blebbing of the plasma membrane and the formation of so-called apoptotic bodies, which ultimately result in the engulfment of the dying cell by the surrounding cells or by specialized cells, such as macrophages or phagocytes. In order to achieve this characteristic morphological transformation, a number of different proteins have to be altered. The apoptosis pathway is a very complex and tightly regulated network of signal transduction and regulation in which caspases, an evolutionarily conserved family of proteases, play an essential role. To date, up to 280 proteins have been shown to be potential targets of caspases (Fischer, *et al*., 2003). These proteins are involved in cell structure, cell cycle, translation, or DNA synthesis and repair. The cleavage of target proteins by caspases does not necessarily imply their inactivation; but can also result in their activation or the translocation from one sub-cellular compartment to another. Classical proteomic approaches, such as the combination of protein separation by two-dimensional gel electrophoresis and protein identification by mass spectrometry (MS), has enabled, mostly in mammalian systems, the discovery of more than a hundred proteins altered during apoptosis. Insights about protein degradation, modification, translocation, and synthesis have also been obtained by these methods (Thiede and Rudel, 2004).

Development of holometabolous insects, such as butterflies and moths (Lepidoptera), is characterized by a complete metamorphosis between the wingless larval stage, mostly dedicated to nutrient acquisition and growth, and the winged adult, fully dedicated to reproduction. These drastic modifications in appearance and physiology require massive histolysis and histogenesis. However, despite the description of the morphological features of apoptosis in Lepidopteran

insects as early as in the 1996, the molecular pathways involved are still poorly understood. The first Lepidopteran caspase was characterized in 1997 (Ahmad, *et al*., 1997) and four others were described almost 20 years later (Chapter 1.). A recent study showed that in Lepidoptera, apoptosis induces a transient mitochondrial membrane permeability, allowing the translocation of cytochrome c from mitochondria to the cytosol (Liu, *et al*., 2007), similar to the pathway extensively described in mammalian systems. Therefore, this suggests that apoptosis pathways in Lepidoptera may share similarities with those described in mammals. Nonetheless, a better understanding of the different partners involved in apoptosis and how they interact with each other is necessary to improve our knowledge of developmental mechanisms and the immune response in Lepidopteran species.

Here, we report on the two-dimensional gel electrophoretic profiles of fluorescently labeled proteins (2DE-DIGE), isolated from a *Helicoverpa armigera*-derived cell line (HaAM1) treated with actinomycin D, a typical inducer of apoptosis in eukaryotic cells. This unbiased, non candidate gene-driven, approach allowed us to identify a total of 13 proteins for which the relative abundance was significantly altered in cells induced with actinomycin D versus untreated cells. Among these, the relative abundance of procaspase-1, the main effector caspase in Lepidoptera, decreased in cells undergoing apoptosis, reflecting its processing into the active form. We characterized the properties of this caspase by heterologous expression and correlated the observed substrate specificity patterns with increases in caspase activity after induction. We also observed changes in the relative abundance of chaperones and other proteins previously characterized as pro- or anti-apoptotic, and compared these with changes in mRNA levels determined by quantitative RT-PCR.

Material and Methods

Cell culture, apoptosis induction and extraction of protein samples.

A cell line derived from *H. armigera* pupal ovaries (BCIRL-HaAM1) (McIntosh, *et al*., 1983) was maintained in EX-CELL 420 medium (Sigma-Aldrich, USA) supplemented with 10% heat inactivated fetal bovine serum and 50 µg/mL gentamycin, at 27°C in 75 cm² flask. When the cells reached 90% confluency, culture medium was replaced and actinomycin D was added to a final concentration of 0.5 µg/mL. A non-induced culture was also prepared (referred as "control" below). Cells were harvested by centrifugation (1,000 x g, 15 min, 20 °C) after 4, 8 and 24 h post induction, and were subsequently washed twice with cold phosphate buffered

saline (PBS). Cell pellets were then resuspended in 500 µL lysis buffer (25 mM Hepes, 5 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 0,2% CHAPS and a cocktail of protease inhibitors (Complete, Roche)), and incubated on ice with a 10 s vortexing pulse every 5 min for a total of 15 min. Cytosoluble proteins were then collected by centrifugation (16,000 *g*, 20 min, 4 °C). Protein concentration was determined using the Bio-Rad protein assay kit (BioRad) according to the manufacturer's instructions, and using bovine serum albumin as a standard. Three biological replicates were collected for each time point (control, 8 h, and 24 h).

Two-dimensional Differential Fluorescence Gel Electrophoresis (DIGE).

Samples were precipitated by 6% TCA using 0.02% sodium deoxycholate as a carrier. After 2 washes with cold acetone, samples were solubilized in 500 µL IEF sample buffer (7 M urea, 2 M thiourea, 30 mM Tris pH 8.5, 4% CHAPS). After resolubilization, the pH of a 100 µL aliquot was checked and adjusted to pH 8.5 by adding an adequate volume of the same buffer at pH 9.5. Protein concentration was then determined using the 2-D Quant kit (GE Healthcare), according to the manufacturer's protocol. Fifty micrograms of proteins were labeled with 300 pmol of Cy3 or Cy5 dyes (GE Healthcare). An internal standard (IS) was prepared by mixing an equal amount of each protein sample, and then 50 µg of this IS was labeled with 300 pmol of Cy2 dye (GE Healthcare). Labeling was performed for 30 min in the dark; the reaction was then stopped by adding 10 mM lysine followed by additional 10 min incubation in the dark.

Table 1. DIGE experimental setup

Comparison	Cy3 Dye	Cy5 Dye	Cy2 Dye
1	Control 1	Treatment 1	(C1 + T1 + C2 +T2 +C3 + T3)
2	Treatment 1	Control 1	
3	Control 2	Treatment 2	
4	Treatment 2	Control 2	
5	Control 3	Treatment 3	
6	Treatment 3	Control 3	

Cy3 and Cy5 labeled proteins to be separated on the same gel (Table 1) were then combined with an aliquot of Cy2 labeled IS and an equal volume of 2x sample buffer (7 M urea, 2 M Thiourea, 2% CHAPS) was added to the mixture. The mixture was then brought to 450 µL with rehydration buffer (Sample buffer + 2% DTT and 2% carrier ampholytes). Isoelectric focusing was performed on a PROTEAN IEF Cell (BioRad) using 24 cm IPG strips, pH 3-11 NL (GE Healthcare). IPG strips were loaded with the 450 µL labeled protein samples and

passively rehydrated for 16 h at 20°C. Isoelectric focusing run followed a program of 8 h at maximum 50 mA per strip, at 500 V for 1 h; 500-1,000 V in 1 h; 1,000-8,000 V in 3 h; and 8,000 V for 3 h. Prior to running in the second dimension, strips were equilibrated for 30 min with 10 mL equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) containing 1% (w/v) DTT followed by 30 min with 10 mL equilibration buffer containing 2.5% (w/v) iodo-acetamide. The IPG strips were then sealed with a solution containing 0.3% agarose in SDS-PAGE buffer on top of a 12% SDS-PAGE gel used for the second dimension. Electrophoresis was performed at a maximum power of 20 W per gel with a voltage limit of 500 V at 20°C using an Ettan DALT 6 apparatus (GE Healthcare). All the electrophoresis procedures were conducted in the dark. Protein spots were visualized by scanning the gels with a Typhoon 9410 scanner (GE Healthcare) at a resolution of 100 µm and at wavelengths of 488 nm (Cy2), 532 nm (Cy3), and 633 nm (Cy5). Spot detection of cropped images was performed with the Decyder 6.5 software package (GE Healthcare) using the following parameters: detection sensitivity, 2,500 spots; slope >1.6; area not filtered; peak height not filtered; volume <10,000. Changes in protein abundance were regarded as being significant with a threshold of twice the model standard deviation (2SD). Twelve images per comparison were analyzed and average ratio and Student's *t*-test values were calculated with the Decyder 6.5 software. Spots with a *t*-test *p* value <0.05 were considered as being significant.

Protein identification by mass spectrometry.

For the tentative identification of the differentially expressed proteins, two separate preparative gels were run for each time point under the same electrophoresis conditions as for the DIGE gels. For each time point, 250 µg of a protein sample composed of an equal amount of each biological replicate were used. After the run, gels were fixed overnight in 40% ethanol / 10% acetic acid, then stained for 2 h using colloidal Coomassie (2% orthophosphoric acid, 10% ammonium sulfate, 0.1% Coomassie blue G250) (Neuhoff, *et al*, 1988). After destaining the gels with 1% acetic acid, proteins of interest were manually picked, destained, digested with porcine trypsin and extracted as previously described in Paris *et al* (2008). Tryptic peptides were analyzed by nanoLC MS/MS and their tandem mass spectra were interpreted *de novo*. The obtained peptide sequences were used in a homology-based search strategy using the MS BLAST program (Shevchenko, *et al*, 2001). We used an 'in-house' MS BLAST server for searching the NCBI_insecta database and a locally generated EST database from *H. armigera* cDNA libraries (179,766 predicted protein sequences deduced from gut, fat body, rest of body,

hemocytes and salivary glands cDNA libraries. We also used an MS BLAST server installed on the ButterflyBase web page (http://butterflybase.org/) for searching the ButterflyBase EST database from Lepidoptera, exclusive of *Bombyx mori* (34,882 protein sequences) (Papanicolaou *et al*., 2008).

Heterologous expression of Ha-Caspase-1.

The complete open reading frame encoding Ha-Caspase-1 was amplified from cDNAs by RT-PCR using gene-specific primers and subcloned into the pCR-T7/CT-TOPO/TA expression vector (Invitrogen) in frame with the Carboxy-terminus V5-$(His)_6$ tag. Correct insertion and orientation of the construct were verified by sequencing. Cultures of *Escherichia coli* BL21(DE3) (Invitrogen) harboring the expression construct were grown overnight in 3 mL 2x YTA medium supplemented with 50 µg/mL ampicillin at 37°C and 250 rpm. Then, 500 µL of the preculture were used to inoculate 50 mL 2x YTA medium supplemented with 50 µg/mL ampicillin. The cultures were grown at 37°C and 250 rpm, until reaching an optical density at 600 nm of 0.6-0.8 before isopropyl-1-thio-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 3 h at 30°C (250 rpm), soluble proteins were extracted in 3 mL BugBuster Protein Extraction Reagent (Novagen). Proteins from non-induced cultures (-IPTG) were also extracted as a control. Successful expression of Ha-Caspase-1 was estimated by western blot using an anti-V5-HRP antibody (Invitrogen).

Caspase activity assays.

Seven characterized caspase tetra- or pentapeptide substrates (Caspase substrates set III, Calbiochem) as well as 4 known caspase inhibitors (LEHD-CHO, IETD-CHO, VDVAD-CHO and DEVD-CHO) were tested. Cytosoluble proteins extracted from HaAM1 cells after induction with 0.5 µg/mL actinomycin D, as well as soluble proteins extracted from *E. coli* BL21 expressing Ha-Caspase-1 were tested. In a 96-well plate, 20 µL of proteins were mixed with 20 µL of caspase lysis buffer (25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA and Complete protease inhibitor cocktail) and were incubated with 55 µL caspase activity buffer (0.1% CHAPS, 10 mM DTT, 1% DMSO, 100 mM HEPES pH 7.5 and 10% sucrose) for 30 min at 37°C before adding 5 µL of substrate, using 100 µM of *p*-nitroanilide (*p*NA) as a standard. Absorbance readings were performed every 3 min during 90 min at a wavelength of 405 nm with an Infinite M200 microplate reader (TECAN). Three biological replicates were prepared and each sample was measured in triplicate. Average absorbance values obtained during the linear

phase were used to determine the tetrapeptide cleavage activity, expressed in pmol of pNA cleaved/minute/mg of protein. Inhibition assays were performed using concentrations ranging from 10nM to 10µM of the different inhibitors detailed above.

RNA isolation and quantitative real-time PCR.

TRIzol reagent (Invitrogen) was used to isolate total RNA from HaAM1 cells after induction of apoptosis by actinomycin D. Total RNA was treated with 2 units TurboDNase (Ambion) following by a clean-up step (RNeasy mini elute cleanup kit, Qiagen). Five hundred nanograms of DNA-free total RNA were converted into single stranded cDNA using the RevertAid H- MAX first strand synthesis kit and a mixture of random and oligo(dT)20 primers (Thermo Scientific). Quantitative RT-PCR reactions were performed in optical-well 96 plates on a MX3000P Real-time PCR detection system (Stratagene), using the ABsolute QPCR SYBR Green Mix (Thermo Scientific) to monitor double-stranded DNA synthesis in combination with Rox as a passive reference dye included in the PCR master mix. As an endogenous control gene, ribosomal protein L1 (RpL1) was used. Specific primers were designed for each gene (Table 2).

Table 2. Primer sequences used for qPCR

Gene	Primer name	Primer sequence
VDAC	Spot3F1	GGTTTTCGGTAGCCTTTCATC
	Spot3R1	GCAATCTTGTCCTGGATCGTA
GAPDH	Spot5F1	AGGCCTCTGCTCATTTAGAGG
	Spot5R1	TTGTCATGGATGACCTTAGCC
Annexin IX	Spot8F1	TCCTCTGCACACTCTCCAACT
	Spot8R1	CCATGCAGAGAGACACACAGA
HSP105-110	Spot15F1	GATTTGGGTGGTGGTACCTTT
	Spot15R1	CACGGTTGTCTTTCCTGATGT
Caspase-1	qHaC1F1	ATGTTCAATGGCTGTTGGAAG
	qHaC1R1	TGTGACATGGCATTGTTGTCT
RpL1	qRPL1F1	CACATCAGCAAACACATCACC
	qRPL1R1	AGAAGTGAGAGCCGTGTGAAA

Results and Discussion

Comparative analysis and protein identification.

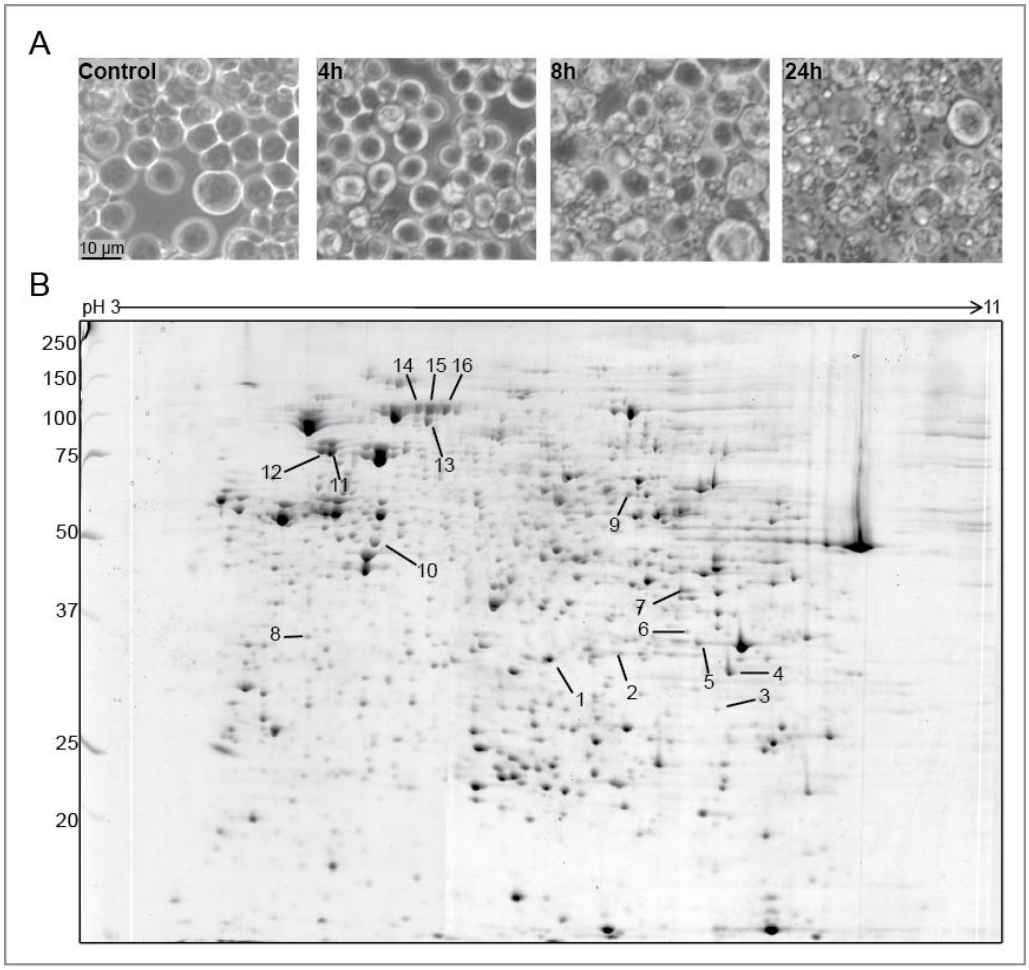


Figure 1. Induction of apoptosis by actinomycin D drastically alters the morphology of HaAM1 cells as well as their cytosoluble proteome.
(A) Morphological changes observed by phase contrast microscopy on HaAM1 cells treated with actinomycin D for 0, 4, 8 and 24 h.
(B) Separation of cytosoluble proteins extracted from HaAM1 cells by 2D-gel electrophoresis followed by staining with colloidal Coomassie. Numbers designate the protein spots for which the relative abundance was found to be significantly different between control cells and cells treated with actinomycin D for 8 h, and which were analyzed by mass spectrometry.

Several "classical" inducers of apoptosis such as UV, 20-hydroxyecdysone, actinomycin D, hydrogen peroxide, staurosporine and etoposide were tested on HaAM1 cells by both morphological observation and caspase activity assays (data not shown). Actinomycin D was eventually chosen because it gave us the best reproducible response with this cell line. Typical morphological changes could be observed on HaAM1 cells treated with 0.5 µg/mL actinomycin D (Fig. 1A). After 4h treatment, blebbing of the plasma membrane started to

appear on some cells, followed by the appearance, at 8 h post induction, of small spherical vesicles known as apoptotic bodies. After 24 h treatment, most of the cells had entered apoptosis and the number of apoptotic bodies increased dramatically (Fig. 1A).

We used 2DE DIGE analysis to examine the physiological changes occurring in HaAM1 cells upon induction of apoptosis by actinomycin D. Differential analysis showed no statistically significant differences in the relative abundance of proteins between samples extracted 14 h after induction and the control (data not shown). Also, the amount of cytosoluble proteins recovered from cells induced for 24 h was too low to be able to perform the differential analysis, indicating that extensive cell damage and protein degradation had occurred by this time. We therefore focused our effort in comparing control cells with cells induced for 8 h. A total of 1,576 protein spots corresponding to proteins with molecular sizes ranging from 15 to 200 kDa and with isoelectric points between pI 4 and pI 10 were resolved by 2DE (Fig. 1B). Among them, the relative abundance of 16 protein spots was significantly different between the 2 samples (Fig. 1B and Fig. 2). Half of them were more abundant in the 8 h induction treatment compared to the control, whereas the others were less abundant (Table 3). From these 16 spots, 13 were successfully identified (Table 3 and supplementary Table S12).

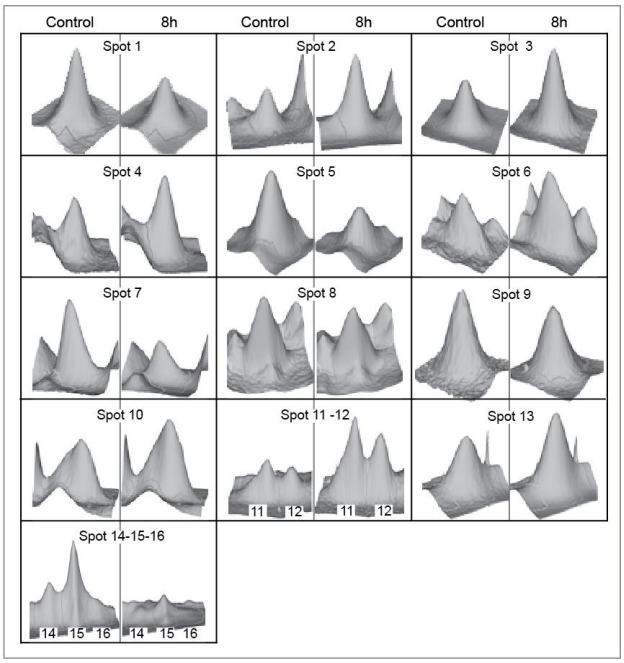


Figure 2. Three dimensional representation of the densitometric spot volume for the 16 protein spots for which the relative abundance was significantly different between the "control" and "8 h post induction" samples, as pictured in Decyder 6.5

Cytosolic proteins from "8 h post induction" and "control" cells were labeled with Cy dyes and separated on 24 cm 2D-gel electrophoresis. After scanning the gels at 488 nm (Cy2), 532 nm (Cy3), and 633 nm (Cy5) with a Typhoon 9410 scanner, protein spot detection and quantitation was performed with the Decyder 6.5 software. Average ratio and Student's *t*-test values were calculated for each spot, and spots with a *t*-test *p* value <0.05 were considered significant.

Searching against the NCBI_insecta database, four protein spots were identified by MALDI-TOF/MS, protein spot 1 as the *H. armigera* Caspase-1, protein spot 4 as a receptor for activated protein kinase C, and protein spot 11 and 12 as HSP70 (supplementary Table 1). The other protein spots were further processed by nanoLC-MS/MS analysis and the peptides obtained were then interpreted *de novo*. Protein spots 6 and 14 returned no peptides at all and were excluded from the rest of the analysis. The peptide sequences obtained from the other spots were then submitted to a homology based search using the MS BLAST program (Shevchenko, *et al.*, 2001). The first round of MS BLAST searches was performed against the NCBI_insecta database, allowing the identification of 9 more protein spots (supplementary Table 1). The second round was performed against the ButterflyBase database (Papanicolaou, *et al.*, 2008). The unannotated clusters identified from ButterflyBase were subsequently submitted to a BLASTp search against the Uniref database to identify the most similar proteins present in this database (supplementary Table 2). A similar search was performed against our "in house" *H. armigera* database in order to get the corresponding cDNA sequences, which were subsequently used to design primers for the quantitative real time RT-PCR analyses (see below). The deduced *H. armigera* protein sequences were submitted to an InterProScan search, to link them to conserved domains and confirm their accurate identification and annotation (Table 3). Protein spot 13 did not return any hits during these MS-BLAST searches and could not be identified.

Table 3. Differentially regulated proteins in HaAM1 cells 8h after induction of apoptosis with 0.5µg/mL actinomycin D.

Spot	Relative abundance[a]	*t*-test[b]	Identification	InterProScan		Genbank Accession
1	-1,07	**	Caspase-1	IPR011600	Peptidase C14, caspase catalytic	EF688063
2	1,35	**	Aldo-Keto reductase	IPR001395	Aldo/keto reductase	JF417982
3	1,61	***	Voltage dependent anion channel	IPR001925	Porin, eukaryotic type	JF417989
4	1,14	*	Receptor for activated PKC	IPR001680	WD40 repeat	JF417987
5	1,05	*	GAPDH	IPR006424	Glyceraldehyde-3-phosphate dehydrogenase	JF417983
6	1,35	*	Not identified	-		-
7	-1,08	**	Actin	IPR004000	Actin-like	JF417981
8	-1,19	**	Annexin IX	IPR001464	Annexin	JF417990
9	-1,17	*	Seryl tRNA synthetase	IPR002317	Seryl-tRNA synthetase, class IIa	JF417988
10	1,08	*	Protein disulfide isomerase	IPR005788	Disulfide isomerase	JF417986
11	1,56	**	GRP78/BiP	IPR001023	HSP 70	JF417984
12	1,42	***	GRP78/BiP	IPR001023	HSP 70	JF417984
13	-1,18	***	Not identified	-		-
14	-1,06	*	Not identified	-		-
15	-1,11	***	HSP105/110	IPR001023	HSP 70	JF417985
16	-1,13	*	HSP105/110	IPR001023	HSP 70	JF417985

[a]degree of difference in log standardized abundance between the control and the 8 h samples ; *p<0.05 ; **p<0.01 ; ***p<0.001.

Functional characterization of Ha-Caspase-1.

Protein spot 1, for which the relative abundance significantly decreases post 8 h induction, was positively identified as *H. armigera* Caspase-1 (Fig. 1B and Fig. 2, Table 3). According to its apparent molecular size on the 2DE gel, ~33 kDa (Fig. 1B), spot 1 corresponds to the inactive form pro-caspase-1. Upon activation, pro-caspases are cleaved into a large and a small subunit of ~20 kDa and ~10 kDa respectively which then associate to form the active caspase. The decrease in pro-caspase-1 observed 8 h post induction thus reflects the transition to its active form. However, we could not detect the appearance of the resulting subunits in our 2DE DIGE experiments.

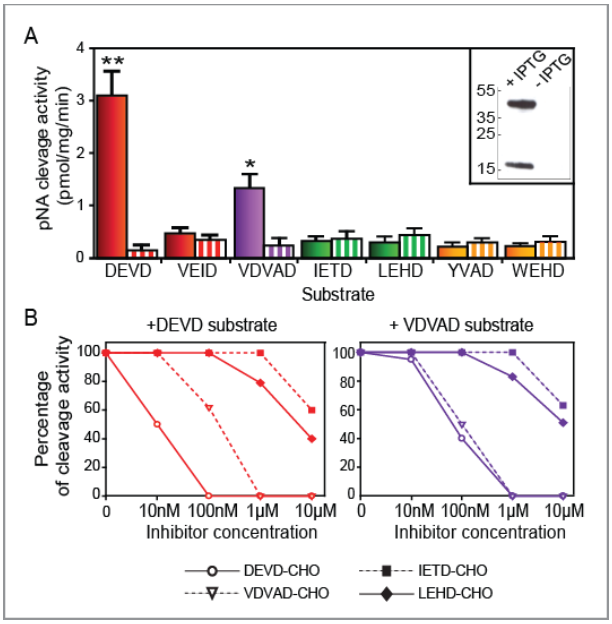


Figure 3. Characterization of the substrate specificity of Ha-Caspase1 expressed in *E. coli*.
(A) Successful expression of Ha-Caspase-1 was assessed by western blot using an antibody directed against the carboxy-terminal V5 epitope. Soluble proteins were extracted from bacteria harboring the Ha-Caspase1 construct after induction of expression by 1 mM IPTG (plain bars) or from non-induced cultures (striped bars). Data are expressed in pmol of pNA released per mg of protein per minute. Statistical significance of the data was assessed by student's *t* test (**p<0.01, *p<0.05).
(B) Ha-Caspase-1 inhibition assays were performed using DEVD-pNA or VDVAD-pNA as a substrate and concentrations ranging from 0 to 10 µM of the specific inhibitors DEVD-CHO and VDVAD-CHO, as well as the non-specific inhibitors IETD-CHO and LEHD-CHO, expressed in percentage of non-inhibited caspase activity.

We decided to test the functional properties of Ha-Caspase-1 by heterologous expression in *E. coli*. Western blotting with anti-V5 antibody revealed 2 bands in the induced culture (insert in Fig. 3A). The upper band corresponds to the unprocessed caspase-1, whereas the lower band most likely corresponds to the cleaved small subunit, showing the partial activation of Ha Caspase-1 in *E. coli*. Activity assays were performed on crude protein extracts of bacteria expressing Ha-Caspase-1. Ha-Caspase-1 showed substrate specificity towards the tetrapeptide DEVD and the pentapeptide VDVAD (Fig. 3A). These cleavage activities are inhibited by the specific inhibitors DEVD-CHO and VDVAD-CHO but not by non-specific inhibitors such as LEHD-CHO and IETD-CHO (IC50 ≥ 10 µM) (Fig. 3B). The substrate specificity observed for Ha-Caspase-1 is similar to that described for the ortholog in *Spodoptera littoralis*, Sl-Caspase-1 (Liu, *et al*, 2005)

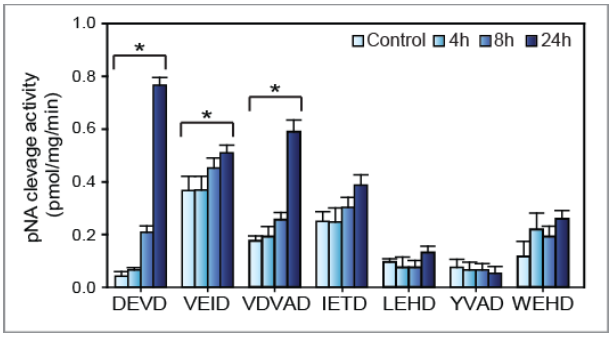


Figure 4. Actinomycin D induces an increase in caspase enzymatic activities in HaAM1 cells.
Cytosoluble protein extracts were harvested from HaAM1 cells treated with 0.5 µg/mL actinomycin D for 0, 4, 8 and 24 h. Caspase activity assays were performed using a set of well characterized synthetic substrates. Results are expressed in pmol of pNA released per mg of protein per minute. For each substrate, Spearman correlation was used to determine whether caspase activities significantly increased overtime (*p<0.001).

In order to confirm that the decrease in relative abundance observed for protein spot 1 is due to the activation of HaCaspase-1 upon apoptosis induction in HaAM1 cells, caspase activity assays were also performed on cytosoluble protein samples extracted 0, 4, 8 h and after 24 h apoptosis induction (Fig. 4). Spearman correlation indicates a significant increase of DEVD, VDVAD and VEID cleavage during the course of the induction by actinomycin D (r=0.930; 0.845 and 0.720 respectively, p<0.001). The increase in DEVD and VDVAD cleavage correlates well with the substrate specificity of HaCaspase-1 expressed in *E. coli*, indicating that the decrease in abundance of pro-caspase-1 observed 8 h post induction reflects the catalytic activation of HaCaspase-1. Interestingly, a decrease in mRNA level of HaCaspase-1 over the same period of time was also detected by qRT-PCR (Fig. 5). The increase in VEID cleavage activity is most probably due to another caspase which was not detected during the differential proteomic analysis.

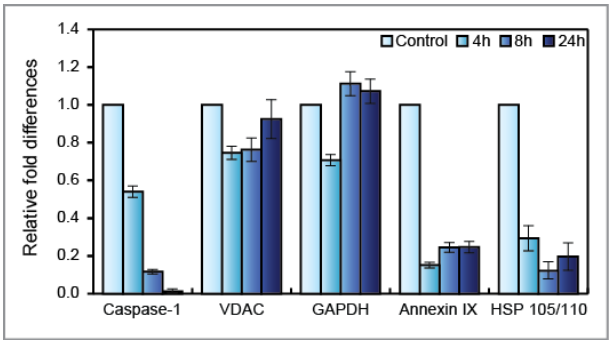


Figure 5. Quantitative real time RT-PCR analysis of transcripts corresponding to proteins for which the relative abundance was found to be significantly different between control cells and cells treated for 8 h by with actinomycin D.
Transcript levels of Caspase-1, VDAC, GAPDH, annexin IX and HSP 105/110 were measured after 0, 4, 8 and 24 h induction of apoptosis with 0.5 µg/mL actinomycin D. Results are expressed in relative fold differences compared to the non treated sample (control).

Actin.

The relative abundance of protein spot 7, identified as actin, was found to decrease after 8 h induction by actinomycin D (Fig. 1B and Fig. 2, Table 3). In mammals, actin has been implicated in apoptotic processes in several ways. It is involved in the regulation of sensitivity of transmembrane death receptor in response to apoptotic stimuli, in the formation of plasma membrane blebs, and in the stabilization of voltage dependent anion channels (Franklin-Tong and Gourlay, 2008). The decrease observed here could indicate the translocation or the degradation of actin. A similar decrease in actin abundance during apoptosis has been observed in several studies (Gerner, *et al*., 2000; Gerner, *et al*., 2002; Wan, *et al*., 2001), which can be explained by the degradation of the protein, most probably by caspases. Indeed, human cytoskeletal actin possesses a YELPD pentapeptide motif which is recognized as a cleavage site by the effector Caspase-3 (Mashima, *et al*., 1997). Further investigation of the 2 resulting actin fragments, called Fractin and l-actin, indicated that l-actin is partially responsible for the morphological changes occurring during apoptosis (Mashima, *et al*., 1999). We found a similar YELPD pentapeptide motif in the *H. armigera* sequence from our cDNA libraries (Genbank: JF417981) corresponding to actin identified by MS, indicating that in *H. armigera*, cytoskeletal actin can also be a target for an effector caspase, such as Caspase-1 (supplementary Fig. 1).

Voltage dependent anion channel.

The relative abundance of a voltage dependent anion channel protein (protein spot 3) increases 8 h post induction (Fig. 1B and Fig. 2, Table 3). Voltage dependent anion channels (VDACs) are integral membrane proteins forming pores in the mitochondrial outer membrane. There, they act as the main general diffusion pore for small molecules such as ATP, phosphocreatine, and small ions. They have also been shown to play a role during apoptotic events by regulating the release of pro-apoptotic factors into the cytosol (Franklin-Tong and Gourlay, 2008; Leadsham, *et al*., 2010). In human, VDAC 2 has been found to interact with Bak, one of the pro-apoptotic proteins of the Bcl2 family (Cheng, *et al*., 2003; Setoguchi, *et al*., 2006). However, the importance of VDAC in the release of cytochrome c from the mitochondria to the cytosol remains controversial, since cells can also carry out cytochrome c release and subsequent caspase activation in the absence of VDACs (Baines, *et al*., 2007). Despite this role of VDAC during apoptosis, it remains unclear why this protein is detected in the cytosol 8 h post induction, as its usual location is the mitochondrial outer membrane. The qRT-PCR experiments

show that the VDAC mRNA level remains stable during apoptosis (Fig. 5), one hypothesis could be that VDAC protein expression remains stable but the protein is unable to translocate to the mitochondria outer membrane because of apoptotic modification of this organelle, resulting in its accumulation in the cytosol.

Glyceraldehyde 3-phosphate dehydrogenase.

The relative abundance of protein spot 5, identified as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), decrease significantly 8 h post induction (Fig. 1B and Fig. 2, Table 3). GAPDH is a key enzyme of the glycolysis pathway, catalyzing the simultaneous phosphorylation and oxidation of glyceraldehyde-3-phosphate to 1,3-Biphosphoglycerate. Besides its role in glycolysis, GAPDH has been implicated in many other cellular processes, including apoptosis (Colell, *et al*., 2009; Sirover, 1999, 2005). For instance, the increase in the relative abundance of GAPDH we observe 8 h post induction is similar to what has been previously observed during apoptosis of cultured human cerebral granulocytes (Ishitani, *et al*., 1996). However, it would be premature to assume that GAPDH, in Lepidoptera, plays a similar role during apoptosis as in mammalian systems. Furthermore, the increase in protein abundance is not reflected by an increase in mRNA level (Fig. 5) suggesting that the increase in GAPDH 8 h post induction is most likely due to translational or post-translational regulation.

Annexin IX.

We observed a significant decrease of the relative abundance of protein spot number 8, identified as Annexin IX after 8 h induction with actinomycin D (Fig. 1B and Fig. 2, Table 3). Annexins constitute a family of structurally related calcium-dependent phospholipid binding proteins. The core region at the carboxy-terminus is responsible for the interaction with phospholipids whereas the highly variable amino-terminus interacts with cytosolic proteins, conferring unique functions to the different annexins. In mammalian systems, annexins are involved in many cellular processes such as endo- and exocytosis (Monastyrskaya, *et al*., 2009; Rescher and Gerke, 2004), phospholipase inhibition and apoptosis inhibition via regulation of calcium flux (Chasserot-Golaz, *et al*., 2005; Futter and White, 2007; Gidon-Jeangirard, *et al*., 1999; Kim, *et al*., 2001; Mira, *et al*., 1997). In the silkworm *Bombyx mori*, during metamorphosis, the expression of annexin IX remains stable in tissues that survive through metamorphosis such as testes and Malpighian tubules, whereas its expression decreases in the silk glands which undergo apoptosis (Kaneko, *et al*., 2006). We observed similar results with a

decrease of annexin IX mRNA expression 4 h after induction (Fig. 5) correlated with a significant decrease of the protein abundance post induction (Table 3). However, the mechanism responsible for the regulation of annexin IX expression might be different from the one observed during metamorphosis in *B. mori* since we used actinomycin D as an apoptosis inducer and not ecdysone.

Aldo-keto reductase.

A significant increase in the relative abundance of protein spot 2 was observed after induction by actinomycin D, and was identified as a member of the aldo-keto reductase (AKR) superfamily (Fig. 1B and Fig. 2, Table 3). This superfamily is composed of over 150 members organized in 15 families. They catalyze the oxido-reduction of a wide range of natural and synthetic substrates (Hyndman, *et al*, 2003). They are involved in biosynthesis, metabolism as well as detoxification pathways. According to the AKR nomenclature (Hyndman, *et al*, 2003; Jez, *et al*, 1997) the one identified in this study is a member of the AKR1 family (between 50 and 58% identity with human AKR1 family members). Several members of this family have been shown to act as pro-survival factors modulating stress responses, regulating lipid biosynthesis and lipid peroxidation detoxification (Chang, *et al*, 2003; Marín, *et al*, 2009; Wang, *et al*, 2009). The increase in AKR abundance observed post induction could indicate a pro-survival action in order to counteract the apoptotic effect of actinomycin D.

Receptor for activated protein kinase C 1.

Protein spot 4 was identified as a receptor for activated protein kinase-C 1 (RACK1). Its relative abundance significantly increases after induction (Fig. 1B and Fig. 2, Table 3). RACKs are adaptor proteins involved in signal transduction. They are composed of seven WD40 repeats allowing the binding of several different proteins. The first interacting proteins identified were the various protein kinase C isozymes, but several other enzymes were later shown to interact with RACKs (Schechtman and Mochly-Rosen, 2001). Several studies showed that the up-regulation of RACK1 has a protective effect against apoptosis (Ishii, *et al*, 2003; Inayat-Hussain, *et al*, 2009; Mourtada-Maarabouni, *et al*, 2005). It has also been shown that RACK1 interacts physically with p73, a p53-related protein (Ozaki, *et al*, 2003). p73 is a nuclear transcription factor activating the transcription of various p53-responsive promoters, thus promoting apoptosis (Jost, *et al*, 1997). The interaction between RACK1 and p73 leads to the inhibition of p73 and to inhibition of apoptosis (Ozaki, *et al*, 2003). Similar to AKR, the

increase of RACK1 protein abundance 8 h post induction can be interpreted as an attempt to inhibit apoptosis.

Chaperone proteins.

The relative abundance of three different chaperone proteins was altered after 8 h induction by actinomycin D. A decrease in the relative abundance of HSP105/110 (protein spots 15-16) was observed, whereas the relative abundance of Grp78/BiP (protein spots 11-12) and a protein disulfide isomerase (protein spot 10) increased (Fig. 1B and Fig. 2, Table 3).

Members of the protein disulfide isomerase (PDI) family catalyze the formation of disulfide bonds and the reduction or isomerization of newly synthesized proteins in the endoplasmic reticulum (ER) lumen, therefore acting as chaperones. Although PDIs are mainly located in the ER, some of them have been detected in the nucleus, the cytosol and at the cell surface (Coppari, *et al*., 2002; Ndubuisi, *et al*., 1999; Pauchet, *et al*., 2010; Yoshimori, *et al*., 1990). However, the mechanism by which these proteins, harboring an ER retention signal (tetrapeptide KDEL), can escape from the ER remains unknown (Turano, *et al*., 2002). Nevertheless, a similar increase in PDI protein abundance in the cytosol of cells undergoing apoptosis or hypoxic stress has been previously observed in mammalian systems (Shen, *et al*., 2007; Tanaka, *et al*., 2000). The up-regulation of PDI during hypoxia has also been shown to have a protective effect against apoptosis (Tanaka, *et al*., 2000).

Protein spots 11 and 12 were first identified as HSP70 (supplementary Table 1), but the orthologous sequence from *Spodoptera frugiperda*, identified searching the ButterflyBase database, corresponds to an HSC70Cb (supplementary Table 2). The InterProScan analysis confirmed that the protein identified corresponding to protein spots 11 and 12 is indeed a member of the HSP70 family (Table 3). Intriguingly, the corresponding sequence in *H. armigera* harbors an ER-retention signal at its carboxy-terminus, similar to what we observed for PDI. In addition the sequence from *H. armigera* shares 85% amino acid identity with human Grp78/BiP and only 65% with HSP70. Therefore, we decided to name this protein Grp78/BiP and not HSP70. Protein spots 15 and 16 were first identified as heat shock cognate protein 70 (HSC70), however the observed size on the gel (Fig. 1) as well as the deduced molecular weight of the corresponding *H. armigera* protein suggest that this protein is most likely an HSP105/110. Furthermore, this protein shares 50% amino acid identity with the human HSP105/110 and only 34% with HSP70.

Heat shock proteins (HSPs) are molecular chaperones which mediate the proper folding of proteins. The HSP70 family is composed of several members such as the constitutively expressed HSC70, the stress inducible HSP70 and HSP105/110 as well as the ER-localized Grp78/BiP. Because of the presence of an ER-retention signal, Grp78/BiP should normally be located in the ER, but upon ER-stress, Grp78/BiP is up regulated and can be translocated to the cytosol or to the mitochondria where it acts as a pro-survival factor (Sun, *et al*., 2006; Zhang and Zhang, 2010). The increase in abundance of the GRP78/BiP observed in this study suggests a similar role in HaAM1 cells. The role of HSP105/110 during apoptosis remains controversial, as in mammalian systems it can have a protective effect against staurosporine-induced apoptosis, or an enhancer effect on apoptosis induced by oxidative stress, when over-expressed (Yamagishi, *et al*., 2006; Yamagishi, *et al*., 2002). The significant decrease of HSP105/110 mRNA expression level (Fig. 5), and protein abundance during the apoptotic events observed in this study, suggests that HSP105/110 acts as an inhibitor of apoptosis in *H. armigera* cells, and its down-regulation would then promote apoptosis.

Seryl tRNA synthetase (SerRS).

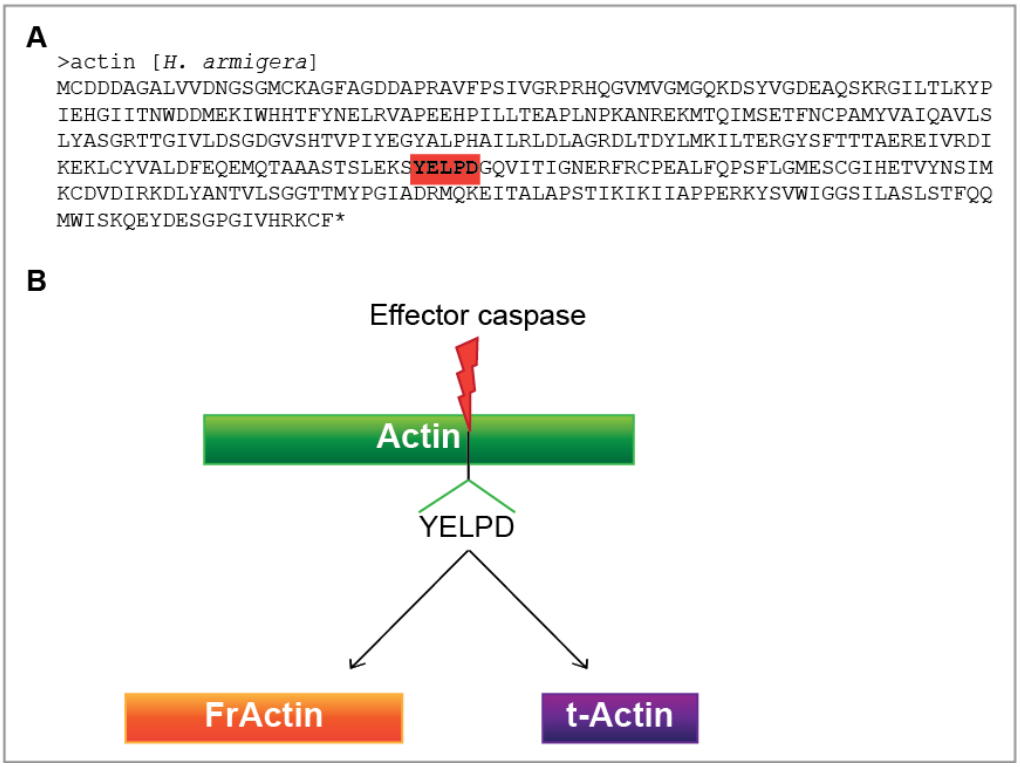
The relative abundance of Seryl-tRNA synthetase (protein spot 9) decreases after 8 h induction by actinomycin D (Fig. 1B and Fig. 2, Table 1). Amino-acyl tRNA synthetases catalyze the attachment of each amino acid to its respective tRNA. In addition to this essential role during protein synthesis, several alternative functions have been proposed for this protein family (Brown, *et al*., 2010; Guo, *et al*., 2010). These non-canonical functions have been associated with the acquisition of new domains during the course of evolution. Vertebrates acquired a unique sequence motif of 53 amino acids (SNE) located at the carboxy-terminus of their SerRS. This domain is absent in *Caenorhabditis elegans*, *Drosophila melanogaster* as well as in the SerRS identified in this study (Guo, *et al*., 2010). Furthermore, despite alternative SerRS functions in human vascular development for example (Herzog, *et al*., 2009), there is no previous evidence of SerRS involvement during apoptosis.

Conclusion

In the present study, we identified a set of candidate proteins involved in apoptotic events either as promoters (Caspase-1, VGAC, GAPDH), targets (actin) or inhibitors (AKR, RACK1, PDI, Grp78/BiP, HSP105/110), in a lepidopteran-derived cell system. In most cases, changes in protein abundance occur independently of changes in transcript levels, and in some cases they may reflect a shift of protein localization within the cell; thus illustrating the utility of the proteomic approach. With the exception of the Seryl tRNA synthetase, similar roles of all of these proteins in apoptosis have been previously described in mammalian systems. This suggests that several aspects of the apoptotic machinery are highly conserved along the Eukaryote lineage. Nevertheless, further investigations are needed to identify the exact function of these candidates in Lepidoptera.

Supplementary materials

Supplementary Figure 1 Potential cleavage site of actin by effector caspase
(A) Actin sequence from H. armigera, the caspase cleavage site is boxed in red.
(B) Representation of the cleavage of actin by effector caspase and the resulting fragments.



Supplementary table 1 : NCBI_insecta identification

Spot	Accession Number	Description	Organism	predicted MW / pI	Experimental MW	peptide hits PLGS 3	PLGS 3 score	peptide hits MS blast	MS BLAST score
1	A7L9Z3	Caspase 1	*H. armigera*	33.2 / 6.12	33.15	15	11,9666	-	-
2	NP_648485	aldo-keto-reductase	*D. Melanogaster*	36.24 / 6.17	33.95	-	-	4	190
3	XP_001993004	voltage-gated ion channel activity	*Drosophila grimshawi*	30.57 / 6.97	30.27	-	-	4	208
4	Q60FS2	receptor for activated C kinase 1	*S. Exigua*	35.9 / 7.64	32.49	15	11,7323	-	-
5	CAD33827	glyceraldehyde-3-phosphate dehydrogenase	*P. Xylostella*	35.47 / 6.54	36.39	-	-	4	288
6	**no peptides**	-	-	-	36.75	-	-	-	-
7	XP_001870756	conserved hypothetical protein	*Culex quinquefasciatus*	41.83 / 5.30	40.61	-	-	4	291
8	BAA92811	annexin IX-C	*Bombyx mori*	35.75 / 5	35.86	-	-	2	115
9	XP_968460	seryl-tRNA synthetase	*T. Castaneum*	56.8 / 6.33	63.38	-	-	2	166
10	XP_001660597	protein sulfide-isomerase A6 precursor	*A. Aegypti*	47.67 / 5.18	51.84	-	-	5	253
11	ABH09734	HSP 70	*T. Ni*	73.09 / 5.17	73.20	16	10,89	-	-
12	ABH09734	HSP 70	*T. Ni*	73.09/ 5.17	73.20	16	11,816	-	-
13	**no hits**	-	-	-	92.45	-	-	-	-
14	**no peptides**	-	-	-	96.94	-	-	-	-
15	XP_623199	HSC70cb	*A. Mellifera*	91.22 / 5.83	96.94	-	-	6	225
16	XP_623199	HSC70cb	*A. Mellifera*	91.22 / 5.83	96.94	-	-	4	221

Supplementary table 2 : Butterflybase identification

Spot	ButterflyBase Identification	Species	protein length (aa)	peptide hits	blastp Vs uniref100	Uniref100 identification	Species	E value
1	HAP00312	*H. armigera*	*294*	*10*	Caspase-1	A7L9Z3	*H.armigera*	*e-173*
2	SFP01005_2	*Spodoptera frugiperda*	*316*	*5*	Aldo-keto reductase	Q17DM5	*Aedes aegypti*	*e-119*
3	SFP00356_3	*S. frugiperda*	*285*	*4*	Voltage-dependent anion-selective channel	UPI0000D56CA4	*T.ni*	*e-101*
4	PXP00409	*Plutella xylostella*	*319*	*4*	Receptor for activated protein kinase C RACK1	Q95PD5	*H.virescens*	*0*
5	HAP00799_1	*H. armigera*	*297*	*4*	Glyceraldehyde 3-phosphate dehydrogenase	Q1EPM0	*Bombyx mori*	*e-156*
6	**no peptides**	-	-	-	-	-	-	-
7	HVP00018_1	*Heliothis Virescens*	*375*	*4*	Actin	Q95PD6	*H. virescens*	*0*
8	SFP14198_1	*S. frugiperda*	*194*	*2*	Putative Annexin IX	Q5F321	*Manduca sexta*	*5,00E-87*
9	HAP00516_1	*H. armigera*	*105*	*1*	putative Sery -tRNA synthetase	UPI00015B5C9A	*Nasonia vitripennis*	*9,00E-79*
10	SFP01726_1	*S. frugiperda*	*247*	*4*	Protein disulfide isomerase-1	5LHW0	*Haemaphysalis longicornis (Bush tick)*	*4,00E-81*
11	SFP00059_2	*S. frugiperda*	*661*	*8*	Heat shock cognate 70 protein	8I866	*S. Frugiperda*	*0*
12	SFP00059_2	*S. frugiperda*	*661*	*10*	Heat shock cognate 70 protein	8I867	*S. Frugiperda*	*0*
13	**no hits**	-	-	-	-	-	-	-
14	**no peptides**	-	-	-	-	-	-	-
15	HEP03159_0	*Heliconius erato*	545	5	PREDICTED: similar to Hsc70Cb	UPI00015B551B	*Nasonia vitripennis*	0
16	HEP03159_1	*Heliconius erato*	545	3	PREDICTED: similar to Hsc70Cb	UPI00015B551B	*Nasonia vitripennis*	0

General discussion

My research has contributed to understanding of apoptotic pathway(s) in Lepidoptera in several ways. My primary focus was to characterize a major player in apoptosis, the caspase gene family. The phylogenetic analysis of Caspase sequences reveals that Lepidoptera possess at least 5 caspases. According to their short prodomain and their close relationship to the *Drosophila* effector caspases DrICE, Dcp-1 and Decay, Lep-Caspase-1, -2 and -3 are effector caspases. Caspase cleavage activity assays, performed on Lep-Caspase-1 expressed heterologously, show substrate specificity toward DEVD and VDVAD peptides, which are known substrates of the effector caspases-3 and -7 in human and DrICE and Dcp-1 in *Drosophila*. Caspase-2 was found only in Noctuid species and the analysis of the genomic region surrounding Caspase-1 in *B. mori* and *H. armigera* shows that Caspase-2 is absent from the genome of *B. mori* but is found together with Caspase-1 within the same locus in *H. armigera*. The high identities between Caspase-1 and -2 sequences, together with their localization, strongly support the hypothesis of a tandem duplication of the Caspase-1 gene in the Noctuidae lineage. Further analysis of their relative rates of sequence evolution reveals that Caspase-2, despite being under purifying selection, has evolved under much relaxed selective constraints than Caspase-1 after the duplication event. The presence of a different cleavage site between the 2 subunits, the absence of a suitable cleavage site between the prodomain and the large subunit in Caspase-2, combined with their complementary mRNA expression profiles suggest that Caspase-1 and -2 are differentially regulated and that they may have undergone a subfonctionalization of the original function of Caspase-1. The analyses of Caspase-3 expression patterns show that it is strongly upregulated after immune challenge of *H. armigera*. These results suggest that Lep-Caspase-3 plays a role in the immune response in Lepidoptera, probably in a similar way as human caspase-3 in TNF-induced apoptosis. Caspase-4 is related to *Drosophila* Damm and Strica, the exact functions of which are not fully understood. The primary structure analysis shows that Lep-Caspase-4 possesses a long prodomain, suggesting an initiator role. However, unlike many initiator caspases, this prodomain does not harbor any known Death-Domain-related structure. Furthermore, the small subunit is of the usual size with a high variability among Lepidoptera in the last 140 amino acids. The analyses of Caspase-4 transcript levels show that the resting levels of expression are extremely low in the different tissues and that they present no significant variation during development or upon immune challenge. These data indicate that Caspase-4 is a peculiar caspase, the function of which cannot

be assessed by sequence or expression pattern comparisons. The phylogenetic analysis shows that Lep-Caspase-5 is the ortholog of Dronc, the *Drosophila* initiator caspase involved in developmental apoptosis. *In silico* analyses demonstrate that Caspase-5, like Dronc, harbor a CARD death domain within its prodomain. In addition, my data show that Caspase-5 expression is upregulated during pupation and by 20-hydroxyecdysone, which suggests that Caspase-5 shares a similar function as Dronc in developmental apoptosis. Caspase-6 possesses a long prodomain harboring a Death Domain structure and is closely related to the *Drosophila* initiator caspase Dredd. Furthermore, its transcript levels increase in the midgut after immune challenge. These results suggest that Lep-Caspase-6 may act in a similar fashion as Dredd in the *imd* pathway, promoting the synthesis of antimicrobial peptides. The use of an unbiased comparative proteomic approach has allowed me to identify several other key players of apoptosis in Lepidoptera. The decrease in procaspase-1 abundance, together with the increase in Caspase-1 cleavage activity show that this effector caspase is involved in apoptosis induced by actinomycin D in HaAM1 cells. The relative abundance of Voltage gated anion channel and Glyceraldehyde-3-phosphate dehydrogenase, 2 proapoptotic factors were found to increase. Similarly, the abundance of Aldo-keto reductase, receptor for activated protein kinase C1, GRP78/Bid and protein sulfide isomerase, four pro-survival factors, were found to increase, most probably in an attempt to counteract apoptosis or to regulate it.

A major drawback of my research resides in the lack of functional characterization of the different caspases identified. With the exception of Caspase-1, which was successfully expressed heterologously, and for which substrate specificity could be determined, the other caspases are still biochemically uncharacterized. A major difficulty faced in any biochemical characterization of a caspase is its biosynthesis as a proenzyme, which requires 2 cleavages at specific sites and proper refolding before obtaining an active enzyme. During the heterologous expression of Caspase-1 in bacteria, I could detect the cleavage of the proenzyme by western blot and the subsequent enzymatic activity. However, my several different attempts to express the other caspases were unsuccessful. The expression in bacteria of the different caspases, with or without their prodomain, resulted in proteins which were mostly expressed in inclusion bodies, without activation of the enzyme, and the activity assays were therefore unsuccessful. My transfection of Caspase-2 and -3 constructs into *Trichoplusia ni*-derived High Five cells, followed by induction of apoptosis by 200µM H_2O_2, 125µM etoposide, 100µM 20-hydroxyecdysone, 100µM staurosporine or 1min under UV, was unsuccessful as no cleavage

of the two heterologously expressed enzymes could be detected by western blot. The expression of human Caspase-1 and -3 in bacterial systems has shown that, similarly to LepCaspase-1, they are autoactivated and expressed in the cytosoluble fraction (Malinowski, *et al*., 1995). However the most successful way to express human caspases consists in expressing each subunit in bacteria independently, resolubilizing them from the inclusion bodies and then refolding them *in vitro* (Garcia-Calvo, *et al*., 1999; Scheer, *et al*., 2005). But this approach has major drawbacks as well, as the yield of expression of each subunit varies greatly. The expressed subunits have often a poor solubility, the refolding buffer has to be determined empirically for each caspase and the renatured enzymes are instable because of autolysis at high protein concentration.

The use of reverse genetic techniques, such as RNAi, to silence caspase expression has also been shown to be a powerful tool to investigate the physiological function (Hebert, *et al*., 2009; Leulier, *et al*., 2006; Liu and Clem, 2011). However, gene silencing by RNAi in Lepidoptera is not trivial. A study, analyzing over 150 gene silencing experiments in Lepidoptera (Terenius, *et al*., 2011) reveals that *B. mori* seems to be the most susceptible species, whereas the noctuid *S. exigua* is resistant. Furthermore, the silencing efficiency greatly depends on the targeted gene. Indeed, immune-related genes seem to be more easily silenced than any other classes of genes. However, in average, less than 50% of the silencing attempts were found to be successful. Another way to analyze the physiological function of caspases would be the use of specific antibodies targeting the active enzyme. This approach has been successfully used to study the time line of caspase activation by western blot or by immunohistobiochemistry (Bressenot, *et al*., 2009; Kaiser, *et al*., 2008; Srinivasan, *et al*., 1998).

Apoptosis is a fascinating process and revealing its secrets in lepidopteran insects would be of great interest for several reasons. It would help retracing the evolutionary history of apoptosis in eukaryotes. It would also give us a better understanding of the interactions between Lepidoptera and their pathogens, as some baculoviruses produce potent caspase inhibitors (Best, 2008). It would also be an opportunity to develop potential 'environmentally friendly' insecticides, as many host plants of lepidopteran herbivorous insects produce secondary compounds which were shown to be inducers of apoptosis and which are currently studied as potential treatment against cancer (Antonious, *et al*., 2007; Yang, *et al*., 2009). No currently used insecticides involve apoptosis in their mode of action, and work described here is a first step in revealing the likely players in such a response.

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Selbständigkeitserklärung

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller-Universität ist mir bekannt. Die vorliegende Dissertation habe ich selbständig verfasst und keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel benutzt. Es wurden keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen. Alle Personen, die an der Gewinnung von Daten beteiligt oder bei der Erstellung des Manuskripts hilfreich waren oder sonstige Hilfestellungen gaben, sind benannt.

Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere Wissenschaftliche Prüfung eingereicht.

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Publications

- Sialome of a lepidopteran herbivore: identification of transcripts and proteins from *Helicoverpa armigera* labial salivary glands
  Celorio-Mancera, M. P., **Courtiade, J.**, Muck, A., Heckel, D. G., Musser, R. and Vogel, H.
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- Characterization of the Caspase gene family in insects from the order Lepidoptera.
  Courtiade J., Pauchet Y., Vogel H. and Heckel D.G.
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- DETERMINATE and LATE FLOWERING are two TERMINAL FLOWER 1 / CENTRORADIALIS homologs that control two distinct phases of flowering initiation and development in Pea.
  Foucher F., Morin J., **Courtiade J.**, Cadioux S., Ellis N., Banfield M.J., Rameau C.
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Presentations

Oral presentations

- " The Caspase gene family"
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  8th International Workshop on Molecular Biology and Genetics of Lepidoptera ; Orthodox Academy of Crete ; Kolympari, Crete, Greece, Aug 2009

- "Apoptotic events in a symbiotic sea anemone: response against oxidative stress"
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  ICE Seminar , 08 Nov 2005 ; MPI for Chemical Ecology, Jena

Poster presentations

- Apoptosis in the Cotton Bollworm *Helicoverpa armigera* (Lepidoptera : Noctuidae)
  Juliette Courtiade, Yannick Pauchet, Heiko Vogel, Susanne Preiss David G. Heckel
  ICE Symposium, September 2008 ; MPI for Chemical Ecology, Jena, Germany.

- Apoptosis in Lepidoptera: Study of the caspase gene family
  Juliette Courtiade, Yannick Pauchet, Heiko Vogel, Susanne Preiss David G. Heckel
  Apoptosis world 2008: from mechanism to applications, January 2008 ; Recherches scientifiques Luxembourg, Luxembourg,

- Apoptosis in the Cotton Bollworm Helicoverpa armigera (Lepidoptera : noctuidae)
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  SAB Meeting, January 2008 ; MPI for Chemical Ecology, Jena, Germany

- Biochemical characterization of Caspase-like proteins in the cotton Bollworm Helicoverpa armigera (Lepidoptera : Noctuidae)
  Juliette Courtiade, Yannick Pauchet, Heiko Vogel, Susanne Preiss David G. Heckel
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- Digestive physiology of generalist and specialist Lepidoptera
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