GERMLINE TRANSFORMATION AND ISOLATION OF MIDGUT RELATED GENES FROM THE POTATO TUBER MOTH, *PHTHORAMIAEA OPERCULELLA*, (LEPIDOPTERA: GELECHIIDAE)

A Dissertation

by

AHMED MOHAMMED AHMED MOHAMMED

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Entomology

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Approved as to style and content by:

Craig J. Coates (Chair of Committee)	Pete Teel (Member)
Patricia Pietrantonio (Member)	Joseph Nagyvary (Member)

Kevin Heinz
(Interim Department Head)

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ABSTRACT

Germline Transformation and Isolation of Midgut Related Genes from the Potato Tuber Moth, *Phthoramiaea operculella*, (Lepidoptera: Gelechiidae). (August 2003)

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Potato production in tropical and subtropical countries suffers from damage caused by the potato tuber moth (PTM), *Phthoramiaea operculella*. Development of a germline transformation system and the identification of genes that are differentially expressed within the PTM midgut are the main goals of this research. We tested three components that are critical to genetic transformation systems for insects; promoter activity, marker gene expression, and transposable element function. We compared the transcriptional activities of five different promoters, *hsp70*, *hsp82*, *actin5C*, *polyubiquitin* and *ie1*, within PTM embryos. The *ie1* promoter flanked with the enhancer element, *hr5*, showed a very high level of transcriptional activity compared with the other promoters. The expression of the enhanced green fluorescent protein (EGFP) was detected under UV-illumination within the embryonic soma demonstrating that it can be used as an effective marker gene for PTM. The transpositional activities of the *Hermes*, *mariner* and *piggyBac* transposable elements were tested in interplasmid transposition assays. The *piggyBac* element was shown mobile within the embryonic soma with a transposition frequency of 4.2×10^{-5} transposition/donor plasmid. The *piggyBac* mobility has been enhanced by incorporating a transactivator plasmid expressing the IE1 protein from the bacoluvirus *Autographa californica* nuclear polyhedrosis virus. Seven transformation experiments were performed. The experiments failed to produce a transgenic PTM.

The insect midgut is a rich region of molecular targets involved in food processing that could be potentially used to design a new control strategy. The suppression subtractive hybridization (SSH) method was used to identify differentially expressed genes from the PTM midgut. From this subtracted library, 2984 clones were collected and screened. Of these clones, 637 clones are candidate differentially expressed genes within the PTM midgut. Sixty-nine cDNA clones were randomly selected for DNA sequencing. Tweleve clones were selected for further analysis using RT-PCR and Northern blot techniques. Eleven of the clones resulted in positive results for midgut expression. Five clones, showing homology with insect immune peptides, were used in the challenge experiment which revealed that these cDNAs are constitutively expressed in the midgut, as well as being up-regulated due to bacterial or viral challenge.

DEDICATION

To my wife Eman, thank you for your patience, support and everything you have done to help me to finish my dissertation.

To my parents, may Allah reward both of you for all you gave to me.

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CHAPTER I
GENERAL INTRODUCTION

1.1. General introduction

Potato is the fourth most widely produced food crop worldwide. Developing countries grow approximately 30% of the worldwide production of potato crop (Raman, 1994). In developing countries, potato production is inhibited by numerous insect and mite species. The potato tuber moth (PTM), *Phthorimaea operculella*, Zeller (Lepidoptera: Gelechiidae) is widely distributed and causes serious damage to potato tubers during storage, as well as to plants in the field. The larval stage mine the foliage and also infest the tubers. The number of PTM generations per year ranges from 2-12 depending on the environmental conditions. PTM is the most important pest on potato in the Middle East and in North and Central Africa. Despite the use of insecticides, potato damage may reach over 80% in some countries (Essmate et al. 1988).

The current control strategy in most areas is to protect the potato crop by applying insecticides in the field and during storage. Due to the development of resistance in PTM populations, high doses of insecticides are used. The number of insecticide applications ranges from 12-20 in the field during the growing season and from 3-4 applications during storage. In an attempt to reduce dependence on chemical insecticides, different control strategies have been successfully used by potato producers.

The first line of defense in the control of insect pests is often host plant resistance. Several wild species of potatoes with high glandular trichomes and low concentrations of glycoalkaloides were used in breeding experiments to develop insect-resistant cultivars (Raman, 1988a). In field experiments, glandular trichome clones showed a high level of resistance to potato pests, including PTM.

The biological control agents, *Bacillus thuringiensis* (Bt) and granulosis virus (GV), have also been used in different regions of the world to control PTM. Due to the success

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achieved with Bt, a variety of endotoxin genes are currently being used to genetically transform potato to develop PTM-resistant lines (Mohammed et al. 2000). Some of these potato lines have shown a high level of resistance to PTM during experimental field trials.

In addition, the PTM sex pheromone has been used as a tool for monitoring PTM populations under field and storage conditions. Pheromone traps are used to indicate the time at which insecticide applications are required, according to PTM population densities. In turn, the reduction of insecticide usage helps to protect the natural enemies of PTM in the field (Raman, 1988b).

Most of the research previously performed on PTM includes; evaluation of the efficacy of insecticides, population dynamics of the insect in the field, development of new insect resistant potato lines, investigation of the potential of Bt and GV as biopesticides and recently, the efficacy of the sterile insect technique as a tool to control PTM. Only a limited number of studies have concentrated on aspects of PTM biology, to gain more understanding of the insect and to develop a new control strategy or to prevent, or at least delay, the development of resistance in this insect.

1.2. Germline transformation of insects

Germline transformation technology for insects was developed more than 20 years ago. Following the success of the first germline transformation experiment in *Drosophila melanogaster* (Rubin and Spradling, 1982), several attempts have been made to apply the same system in different insect species. Many scientists around the world have used the germline transformation system in *Drosophila* for gene cloning and manipulation. The *P*-element transposon has been used to integrate transgenes into the *Drosophila* genome, and has been modified for transposon tagging, enhancer trapping and targeted transposition (see review Lukacsovich and Yamamoto, 2001)

In *Drosphila*, the *P*-element has been used as a mutagenic agent to transposon-tag genes, thus simplifying their cloning (Sentry and Kaiser, 1992). The transposable

element is randomly inserted into the insect genome, occasionally inserting into a gene, thus causing its inactivation or mutation. Due to this mutation, the phenotypic alteration would refer to its corresponding transposon tag, thus providing a method for identification of the function of that gene(s) and subsequent cloning. In enhancer trapping studies (Bellen et al. 1989), transposable elements are used to identify and analyze DNA elements that are involved in the regulation of gene expression. The identified control region can be cloned, fused with a reporter gene and introduced back into the insect genome by genetic transformation. Such studies result in an understanding of the regulation of gene expression under intrinsic and extrinsic conditions. Germline transformation has also been used in *Drosophila* for gene targeting, mediated by homologous recombination. Gene targeting is used for the specific modification of any genomic region that has been previously cloned. It facilitates the manipulation of an insect genome by the precise introduction of transgenes into targeted chromosomal sites. It has the potential to modify the target gene either by correction of a defect, or inactivation through disruption of the coding or regulatory sequences.

Unfortunately, the highly successful *P*-transposable element mediated transformation system does not function beyond *D. melanogaster* and its close relatives, and therefore cannot be used to develop stable germline transformation systems for non-drosophilid insects (Handler et al. 1993). This led to a search for other members of the class II family of transposable elements which transpose by a DNA only "cut and paste" mechanism (Finnegan, 1995). Four transposable elements, representing four different transposon families, have now been used to genetically transform non-drosophilid insects; *Minos* from *Drosophila hydei* (Franz and Savakis, 1991), *Hermes* from *Musca domestica* (Warren et al., 1994), *Mos*1 from *Drosophila mauritiana* (Medhora et al. 1991) and *piggyBac* from *Trichoplosia ni* (Fraser et al., 1995). With the application of these different elements, several successful, stable transformation systems for non-drosophilid insects have been developed. *Minos* has been used to transform the dipterans *Ceratits capitata* (Loukeris et al. 1995a), *D. melanogaster* (Loukeris et al. 1995b) and *Anopheles stephensi* (Catteruccia et al. 2000). It has also been shown to have

transpositional activity in *Anopheles gambiae* cell lines. *Hermes* has been used to generate stable transgenic lines for six insect species; *D. melanogaster* (Pinkerton et al. 2000), *Ceratitis capitata* (Michel et al. 2001), *Stomoxys calcitrans* (O'Brochta et al. 2000), *Tribolium castaneum* (Berghammer et al., 1999), *Aedes* a*egypti* (Jasinskiene et al. 1998) and *Culex quinquefasciatus* (Allen et al. 2001). The *Mos1* element has been to transform *D. melanogaster* (Lidholm et al. 1993) and *Ae. aegypti* (Coates et al. 1998). Finally, *piggyBac* has been used to genetically transform a large group of insects from different orders and it is expected that the number of these insects will be increased with more testing of the transpositional ability of this element (See review Handler, 2002).

The *piggyBac* element was isolated from a *Trichoplosia ni* cell line. Due to its insertion into the viral genome, it caused a plaque morphology mutation of *Galleria melonella* nucleopolyhedrosis virus that was being passage through the cell line (Fraser et al. 1983; Cary et al. 1989). *PiggyBac* is 2.5 kb in size and has 13 bp inverted terminal repeats (ITRs) that delimit the ends of the element. It contains a 2.1kb open reading frame that encodes a single open reading frame for the transposon protein. The excision of *piggyBac* from its donor sequence is absolutely precise and leaves no finger prints at the donor site (Fraser et al. 1996). Elick et al. (1997) examined the effect of the distance between the 5' and 3' ITRs on *piggBac* excision. They found that this distance is not an important factor used for excision and therefore, this feature could potentially be an advantage, allowing the insertion of large genes into the *piggyBac* transposon. However, in *Drosophila*, the transposition efficiency of *piggyBac* is influenced by the size of the internal DNA (Handler and Harrell, 1999). Increasing the vector size by one third in some experiments, served to decrease the *piggyBac* transformation frequency by 66%. Further analysis to define the limits for vector size and requisite *piggyBac* sequences is necessary for mediating efficient transposition of modified elements.

PiggyBac mediates germ-line transformation in *C. capitata* (Handler et al. 1998). The transformation frequency according to the fertility rate is 5% (number of G_0 producing transgenic G_1 individuals/number of fertile G_0 adults). All sublines contained at least one integration with several sublines containing additional integrations. Members

of the *piggyBac* family have been discovered in *T. ni* and outside the Lepidoptera. Sequence analysis of repetitive *B. dorsalis* genomic elements amplified using conserved *piggyBac* primers indicated the presence of highly conserved *piggyBac* elements (Handler and McCombs, 2000). This discovery is unusual since the *piggyBac* elements in both species are nearly identical, whereas the insect species are distantly related. The *piggyBac* transposon mediates germ-line transformation in *Bactrocera dorsalis,* despite the existence of closely related elements in its genome. However, the potential for autoregulation and cross-mobilization effects *piggyBac* mobility remains to be determined. One of the benefits of germline transformation is the potential for the development of genetic sexing technologies which would assist development of the sterile insect technique for insect control. Heinrich et al. (2002) transformed the Australian sheep blowfly, *Lucilia cuprina*, with a *piggyBac* vector and *EGFP* marker gene driven by the *D. melanogaster hsp70* promoter. Two more dipteran flies were transformed with the lepidopteran derived *piggyBac* transposon; the Caribbean fruit fly, *Anastrepha suspense*, (Handler and Harrell, 2001) and the house fly, *M. domestica*, (Hediger et al. 2001).

PiggyBac has also been successfully used to transform different mosquito species. The yellow fever mosquito *Ae. aegypti* was transformed with the *piggyBa*c transposable element vector containing the regulatory region of the *vitellogenin* (*Vg*) gene as a promoter and the antibacterial peptide, *Defensin A* (*DefA*), as a transgene (Kokoza et al., 2001), along with the *EGFP* marker gene regulated by the artificial, eye-specific 3xP3 promoter (Berghammer et al. 1999). Lobo et al. (2002) transformed the same species using a *cinnabar* marked *piggyBac* element. The phenomenon of multiple transposition events following an initial integration in the germline was observed in *Ae. aegypti*. Similar phenomena were also observed with the *piggyBac* transformation of *C. capitata* (Handler et al. 1998), *D. melanogaster* (Handler & Harrell, 1999) and *B. mori* (Tamura et al. 2000). The interpretations of such phenomenon is that remobilization of the transgene may occur due to replicative movement or a cut and paste mechanism followed by double stranded gap repair. The germline transformation of *An. gambiae*

and *An*. *albimanus* was achieved by using a *piggyBac* transposable element marked with the *EGFP* marker gene (Grossman et al. 2001; Perera et al. 2002).

Despite the *piggyBac* transposon being originally derived from lepidopteran insects, the *piggyBac* vector has been deployed to transform only two lepidopteran species. Transgenic lines of pink bollworm, *Pectinophora gossypiella*, were obtained using the *piggyBac* vector and *EGFP* marker gene driven by *BmA3* promoter (Thibault et al. 1999). The *piggyBac*-mediated germline transformation of the silkworm, *B*. *mori*, was first achieved by Tamura et al. (2000) using the *EGFP* marker gene under the regulation of the native *B*. *mori actin3* promoter (*BmA3*).

PiggyBac has proven mobility and utility for germline transformation experiments for a wide range of insect species. In addition to Dipteran and Lepidopteran species, *piggyBac* has also been successfully used to transform two insect species belonging to the Coleoptera and Hymenoptera. A remarkable transformation frequency of 60% was obtained using *piggyBac* in the red flour beetle, *Tribolium castaneum*, using *EGFP* under the regulation of the eye-specific universal promoter (3xP3), compared with a transformation rate of only 1% for the *Hermes* element (Berghammer et al. 1999). However, recent transformations experiment resulted in transformation frequencies of 4% and 1.5% in *T*. *castaneum* using a dual-marker *piggyBac* vector (Lorenzen et al., 2002). The dual-marker system was based on the *T*. *castaneum* eye-color gene *vermilion* (*Tcv*) and *EGFP*. Germline transformation was achieved at a rate of 5% in the hymenopteran *Athalia rosae*, using a *piggyBac* flanking two GFP-coding sequences (Sumitani et al. 2003). One of the *EGFP* marker gene was under the regulation of the *hsp70* promoter and the second *EGFP* marker gene was under the regulation of the *B*. *mori actin3* gene promoter.

While genetic transformation experiments have been successfully applied to a wide range of insect species, the technique still has a number of problems to be resolved to improve the efficiency of the systems. The transformation frequency, the number of G1 transgenic individuals/number of G_0 adult, is still low for most species. For example, the transformation frequency for the pink boll worm is 3.5% (Peloquin et al. 2000), 1-2% for

L. *cuprina*, and 5-10% of *Ae*. *aegypti* (Kokoza et al. 2001). The number of embryos that survive microinjection is generally low. The survival rate during transformation experiments for *Ae*. *aegypti* is 10-25%, however, in *D*. *melanogaster* the rate is routinely 50% (Atkinson et al. 2001). The transgenes integrated into the insect genomes can be affected by the surrounding sequences. For example, the *white* eye color marker gene suffers from genomic positional effect variegation, modifying its expression. Similar effects have been observed in *C*. *capitata* (Handler et al. 1998) and *A*. *aegypti* (Coates et al. 1998; Jasinskiene et al. 1998). The *EGFP* marker system appears to be less affected by positional effect than eye color marker gene. However, other transgenes of interest could suffer the same problem after integration into the insect genome. Depending on the site of insertion either near enhancer or suppressor elements, transgene expression could be either highly up or down regulated due to the presence of these control elements.

In addition to basic research applications such as the identification of novel genes and regulatory sequences, germline transformation has the potential to be used for field research applications. The sterile insect technique (SIT) is a species specific and non-polluting form of insect pest control. SIT relies on the release of large numbers of sterile-induced radiated males. The released males mate with wild females, reducing the insect pest population in the field. Mass rearing produces both sexes and the females are removed prior to release. Marking the released males with a genetic marker would facilitate the discrimination of released flies from nontransgenic, wild flies, (Robinson and Franz, 2000). Thomas et al. (2000) presented two methods that could be used to induce conditional lethality of the released males and tallow inheritance into the wild population, termed “release of insects carrying a dominant lethal’ (RIDL). Development of a refractory mosquito strain that prevents pathogen development or propagation in a vector has been a focus of mosquito research using germline transformation (see review James, 2000).

Recently, the application of germline transformation in lepidopteran species was performed in *B*. *mori*. The nuclear receptor Ftz-F1, which plays a role in the ecdysteroid

signaling pathway, was expressed in the silkworm (Uhlířová et al. 2002). The cDNA encoding BmFtz-F1 under the regulation of a *hsp70* promoter fused with the 3xP3-EGFP marker, flanked with the *piggyBac* vector was employed for the germline transformation. Using RT-PCR analysis, the transgene mRNA was detected after heat induction at 42°C for different time period over 6 hours. Further, the recombinant human type III procollagen was expressed in the cocoons of transgenic *B. mori* (Tomita et al. 2003).

1.3. Insect midgut

The lepidopteran midgut is a rich region for the identification of molecular targets that are involved in food processing. The insect midgut cells are actively involved in the production and secretion of digestive enzymes and in the absorption of digested food. The midgut is composed of a single layer of epithelial cells which rest on a continuous basement membrane. The midgut epithelium is composed of different cell types according to their functions; the predominant columnar epithelial cells are those responsible for food digestion and absorption (Billingsely and Lehane, 1996), goblet cells (Anderson and Harvey 1966), regenerative cells (Cioffi, 1979) and endocrine cells (Endo and Nishiitsutsuji-Uwo, 1982).

Three different processes provide targets for potential insect control strategies in the lepidopteran midgut; digestion, absorption and protection. Digestion includes the production and secretion of digestive enzymes. Large numbers of digestive enzymes have been identified and the molecular characterization of these enzymes is underway. The regulation of enzyme production and secretion, at the molecular level at least, is not fully understood. The absorption of digested food, water regulation and ion movement has gained a great deal of attention and there are many molecules that play an important role in these functions yet to be identified. The midgut of insects often contain a non-cellular semipermeable tube, the peritrophic matrix (PM), which separates the contents of the gut lumen from epithelial cells. Two types of PM have been defined in insect species based on their site of synthesis (Peters, 1992). Type I PMs are synthesized by the

midgut cells as a continuous tube, this being typically utilized in lepidopteran species. Type II PMs are synthesized from a specific organ, the cardia, located in the anterior portion of the midgut, this being typically utilized in dipteran insects. The PM is composed of peritrophine proteins (three types have been identified according to their solubility following treatment by solublizing agents), chitin and proteoglycans (for review see Tellam et al. 1999). The protection of the midgut cells from direct contact with food particles and microorganisms as well as recycling digestive enzymes, is the main function of the peritrophic matrix (Terra, 2001). Many of the components forming the matrix are suitable targets for insect control (Wang and Granados, 2001).

The digestive enzymes are hydrolases and are simply classified into peptidases, glycosidases, lipases, phospholipases and esterases. Peptidases are enzymes acting on peptide bonds and include exo- and endopeptidases. Serine, cysteine, aspartic and metallo proteinases are examples of endoproteinases and they are related by the active site amino acid (aa), involvement of the carboxyl residue, or the requirment of a metal ion for catalysis, respectively. Exopeptidases include enzymes that hydrolyse single aa's from the N-terminus (aminopeptidases) or from the C-terminus (carboxypeptidases). Serine proteinases have been the subject of more extensive research than the other types of digestive enzymes.

The serine proteinases include trypsin and chymotrypsin, with characteristic serine and histidine residues at the active site. Trypsin cleaves the peptide chain on the carboxyl side of basic L-amino acids such as arginine or lysine. Chymotrypsin is active on the carboxyl termini of hydrophobic amino acids as tyrosine, tryptophan, leucine or phenylalanine (Terra et al. 1996). Both of these enzymes have been isolated from many insect species, however, few of these have been identified and characterized in detail.

Lepidopteran larvae are continuously feeding insects. Thus, the constitutive secretion of digestive enzymes in the lepidopteran midgut is more likely than a tightly regulated secretion process. However, the expression and regulation of digestive enzymes in several herbivorous insect pests are influenced by diet containing Bt endotoxins, or plant proteinase inhibitors (Giri et al. 1998). Therefore, regulation of digestive enzyme

secretion is possible, especially in the case of insects that can develop resistance to either a proteinase inhibitor, or to midgut-related toxins. It is unclear whether trypsin or chymotrypsin, or both, play the predominant role in the lepidopteran response to the presence of dietary proteinase inhibitors (Leighton and Broadway, 2001a).

Carboxypeptidases hydrolyse single aa's from the C-terminus and are further divided into three classes; serine-, cysteine- and metallocarboxypeptidases, according to their catalytic mechanism (Terra et al. 1996).

Identification of serine proteinase genes from lepidopteran insects is currently a high priority in the midgut research field. However, the role played by these enzymes in the development of resistance is still unclear. Several trypsin and chymotrypsin-like proteins have been cloned and sequenced from different lepidopteran species; *C. fumiferana* (Wang et al., 1995); *Plodia interpunctella* (Zhu et al. 1997; 2000), *Scirpophaga incertulas* and *S. armigera* (Leighton et al. 2000); *A. ipslon* (Mazumdar et al., 2001); *G. mellonella* (Li et al. 2002); and *M. sexta* (Peterson et al. 1994). Aminopeptidase is an exopeptidase bound to the membrane of the midgut epithelial cells. The aminopeptidase of *S. furgiperda*, is bound to the microvillar membrane by a glycosyl phosphoatidylinositol (GPI) anchor (Jordão et al. 1999). Several aminopeptidases (Knight and Dean, 1995; Yaoi et al., 1999; Burton et al. 1999) as well as cadherin-like molecules (Jenkins et al., 2001) have been identified as *Bacillus thuringiensis* Cry toxin receptor candidates. Nakanishi et al. (2002) reported that 18 cDNA isoforms of aminopeptidase are registered in the genbank database from eight lepidopteran species; *B. mori*, *H. virescens*, *P. xylostella*, *H. punctigera*, *M. sexta*, *Limantria dispar*, *P. interpunctella* and *Epiphyas postvittana*.

Insects usually regulate ion concentration in the haemolymph using a Na^+/K^+-ATPase to drive solute and fluid absorption. However, the diet of herbivorous insects is characterized by low Na^+ and high K^+ precluding the usage of a sodium pump. The midguts of lepidopteran larvae lack a Na^+/K^+ ATPase, all solute fluxes being energized by what was initially thought to be a potassium pump (Harvey et al. 1981). It was discovered that the net pumping of K^+ was instead caused by the vacuolar type proton

ATPase (V-ATPase), first described in *M. sexta* (Wieczorek et al. 1992). The V-ATPase occurs in the apical membrane of the goblet cells. The voltage drived from pumping H^+ energizes secondary transport in the midgut epithelium. The net active transport of K^+ is the outcome of H^+-V-ATPase and K^+/H^+ antiport. The transapical voltage in the epithelial (produced by the H^+-V-ATPase) is more than 240 mV (Dow and Peacock, 1989) and drives the antiport exchange of $2H^+$ with 1 K^+. The secretion of K^+ leads to high alkaline of more than 11 in the lepidopteran midgut. The resulting potassium motive force is used to drive K^+ transport coupled with the uptake of nutrients such as amino acids (Wieczorek et al. 1999).

Application of germline transformation would have a significant impact on PTM midgut research. Putative midgut-specific promoters could be tested by expressing a marker gene under the regulation of the promoter in question. The expression pattern of the marker gene in the midgut compared to the whole body will reveal or confirm the tissue specificity of the promoter. Gene(s) that play a role in food processing in the midgut could be specifically knocked out in transgenic individuals and the regulation of such systems could be modified using inducible promoters.

1.4. Aim of the work

Both transposon tagging and enhancer trapping technologies in insects allow the efficient identification and isolation of genes and genetic systems involved in development, behavior and reproduction (Handler 2000). The analysis of such genes and their use in transgenic strains for laboratory experiments will provide new targets for insect control. Application of such techniques in PTM will result in a more complete understanding of the biological process at a molecular level and the identification of functional genes and DNA control regions.

There are two major goals of this research; 1) To establish a germline transformation system for PTM and 2) to isolate and characterize genes expressed in the PTM midgut. This research will advance our understanding of PTM biology, enabling a search for key

genes that are critically required for important biological processes. Investigation of gene products that are involved in a variety of biological processes will be of great value for the identification of novel targets to develop new control strategies.

To achieve germline transformation for PTM, three different components should be evaluated prior to initiating the transformation experiment; a) promoter, b) marker gene and c) transposable elements. Promoter activity is determined by expressing a reporter gene under the regulation of the desired promoter. Promoters that have been used in germline transformation in other insect species are likely to demonstrate transcriptional activity within PTM embryos. A luciferase reporter gene is used for the promoter assay. The choice of marker gene depends on the availability of that gene and the ease of determination of its activity. An eye color marker gene is not currently available for PTM and there are no recipient mutant strains. The enhanced green fluorescent protein (*EGFP*) has been widely used in insect species as transformation marker gene and it can be determined by transient expression analysis. Interplasmid transposition assays can be used to demonstrate the mobility of transposable elements within PTM embryos. The assays are based on the mobility of the element between plasmids within PTM embryos and hence transposition frequencies are determined. The transformation experiments are performed based on the results of the previous assays. Plasmids carrying the transposable element and marker gene under the regulation of specific promoters, are injected into PTM embryos. The surviving embryos are allowed to reach adult hood and are cross-mated with wildtype individuals. The progeny are screened for transgenic individuals according to the marker gene expression.

A midgut-specific subtracted library will be constructed using the Suppression Subtraction Hybridization (SSH) technology (Diatechnko et al. 1996). The carcass (whole body without the midgut) cDNAs are subtracted from the midgut cDNA population according to the SSH procedure. The SSH-PCR products are cloned into a appropriate plasmid vector and clones are identified using midgut-specific probes. Candidate cDNA clones are confirmed to be midgut-specific by molecular analysis, including RT-PCR and Northern blot analysis.

CHAPTER II
PROMOTER AND MARKER GENE ACTIVIY WITHIN POTATO TUBER MOTH EMBRYOS

2.1. Introduction

A complete germline transformation system for an insect species, based on a binary transposable element system, requires the accurate and efficient expression of the transposase protein, transformation marker genes and the transgene of interest. The transposase protein catalyzes the integration of the corresponding donor transposable element into the genome of the insect embryos (G_0 generation). The transformation marker gene provides a mechanism to identify the transgenic individuals in the G1 progeny. The transgene of interest is the target gene sequence to be incorporated into the insect genome, the expression of which is designed to produce a new strain with a desired phenotype(s). The transposase and transformation marker genes both need to be expressed at suitable levels to lead to successful transformation events. Promoters that provide the appropriate level of expression for each insect species, play a crucial role in insect transformation.

The knowledge gained from *Drosophila* genetic research has provided a number of promoters and regulatory sequences which have been used in both drosophilid and non-drosophilid transformation experiments. The *Drosophila* heat shock promoters have been utilized in germline transformation experiments for a number of different insect species. The *D. melanogaster hsp70* promoter has been used to control the expression of transposases and/or marker genes in successful transformation experiments in the following species (for review see Ashburner 1989), *Bactrocera dorsalis* (Handler and McCombs 2000), *Anastrepha suspensa* (Handler and Harrell 2001), *Anopheles gambiae* (Grossman et al. 2001), *Lucilia cuprina* (Heinrich et al. 2002) and *Aedes aegypti* (Lobo et al. 2002).

The *hsp82* promoter regions from four *Drosophila* species contain conserved DNA

sequences that are likely to be involved in the response to the heat shock. In contrast to the *hsp70* promoter, the regulatory regions of the *hsp82* promoter are also responsible for a high level of constitutive expression in *D. melanogaster*. In transient expression experiments, the heat shock promoter from the *D. pseudoobscura hsp*82 gene has been used to express the chloramphenicol acetyltransferase (CAT) gene from *E. coli* in the *Lucilia cuprina* embryos (Coates et al. 1996). The *D. melanogaster* polyubiqutin (pUB) promoter has been used to express *EGFP* linked in-frame with the SV40 nuclear localizing sequences (nls) in *D. melanogaster* (Handler and Harrell 1999), and *L. cuprina* (Heinrich et al. 2002). The *D. melanogaster* pUB promoter with nls was used to regulate two marker genes, *EGFP* and *white*$^{+}$, in *A. suspensa* (Handler and Harrell 2001). The baculovirus *ie1* promoter was used in transposition assays in cells and embryos, and germline transformation in the malaria vector *A. gambiae* (Grossman et al. 2000; Grossman et al. 2001). A universal artificial eye-specific promoter, 3xP3, drives the expression of *EGFP* in the eye of widely divergened insect species including the fruit fly, *D. melanogaster* (Berghammer et al. 1999; Horn et al. 2002), the flour beetle, *Tribolium castaneum* (Berghammer et al. 1999), *B. mori* (Thomas et al. 2002; Uhlířová et al. 2002), *Ae. aegypti* (Kokoza et al. 2001).

A critical component of a transformation system is the ability to select or identify transgenic from non-transgenic organisms such that pure transgenic lines can be established. Genes that confer antibiotic resistance have previously been used as selectable transformation markers. The bacterial neomycin phosphotransferase gene (*neo*) confers resistance to the aminoglycoside antibiotic, Geneticin (also known as G418 and neomycin) and was used in *Drosophila* and mosquito transformation experiments (Steller and Pirrotta, 1985; Miller et al. 1987). Genes involved in the production of eye color have also been used as phenotypic transformation marker genes. The *D. melanogaster white* (*w*) and *cinnabar* (*cn*) genes have been used to rescue the eye color in a mutant strain of *Ceratitis capitata* (Loukeris et al. 1995a; Handler et al. 1998; Michel et al. 2001) and *Ae. aegypti* (Coates et al. 1998; Jaseniskiene et al. 1998), respectively. A dual-marker gene system based on the *T. castaneum* eye-color gene

vermilion (*Tcv*) and *EGFP* was used to transform *T. castaneum* (Lorenzen et al. 2002). A gene encoding tryptophan oxygenase in *An. gambiae* rescued the eye color in the *vermilion* mutant of *D. melanogaster* (Besansky et al. 1997). A single copy of the gene produces eye coloration that is sufficient for identifying the transgenic individuals. This phenotypic marker gene system is easy to detect and heterozygous individuals can be distinguished from homozygous individuals (Sarkar and Collins, 2000). Lack of suitable recipient strains, positional effects and the genetic load on mutant strains limit the usage of eye color marker systems in some insect species.

The green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria* (Parsher et al. 1992) has been used for transformation experiments in a wide variety of animal and plants species. The GFP is autonomous and requires no host factors. The enhanced variant, EGFP, of the wildtype GFP was developed to eliminate problems associated with its expression, insolubility and excitation within the UV-spectrum. EGFP-based marker systems have been successfully used in almost all insect species in which successful germline transformation has been achieved (see table 1 in Horn et al. 2002). The successful history of EGFP-based constructs within insect species, belonging to three different orders, as a genetic marker (see review Horn et al. 2002) and its widespread activity among animal and plant kingdoms (see Chalfie and Kain, 1998) highylights the need to investigate EGFP expression in the PTM embryos.

This chapter focuses on the determination of the transcriptional activity of different promoters as well as the fluorescence activity of *EGFP* in PTM embryos.

2.2. Materials and methods

2.2.1. Plasmid construction

The transcriptional activities of promoters were determined using a luciferase assay from Promega®. Two plasmids are used for this assay; the control plasmid expressing *Renilla* (*Renilla reniformis*) luciferase under the regulation of a SV40 promoter and an

experimental plasmid expressing firefly (*Photinus pyralis*) luciferase controlled by the test promoter. Once the *Renilla* luciferase expression was found to be at a very low level within PTM tissues, the SV40 promoter was replaced by the *hsp82* promoter to produce a detectable level of *Renilla* luciferase.

2.2.1.1. Control plasmid for luciferase assay

The control plasmid was designed to express the *Renilla* luciferase under the regulation of the *hsp*82 promoter. The pBChsp82 plasmid was constructed by inserting the 1021bp *Hinc*II/*Kpn*I fragment from pKhsp82 (Coates et al. 1996) into the *Hinc*II/*Kpn*I sites of pBCKS+ (Stratagene, La Jolla, CA, USA). The SV40 promoter/enhancer fragment was removed from pRL-SV40 (Promega, Madison, WI, USA) by *Kpn*I and *Pst*I digestion, with the vector backbone being gel purified using the Gene Clean Spin Kit (Qbiogene, Montreal, Canada) according to the manufactures' instructions. The *Pst*I/*Hinc*II fragment containing the *hsp82* promoter was excised from pBChsp82 ligated with the linearized pRL-SV40 vector forming the control plasmid pRL-hsp82.

2.2.1.2. Experimental plasmids for the luciferase assay

Five different experimental plasmids were constructed to drive the expression of firefly luciferase under the regulation of the actin5C, hsp70, hsp82, IE1, or polyubiquitin promoters.

i. The heat shock promoter *hsp70* (150 bp) was cut from pHSHH1.9 (O'Brochta et al. 1996) using *Xho*I/*Not*I restriction enzymes then ligated into the corresponding sites of pBCKS+ forming pBC-hsp70. The DNA fragment containing the heat shock promoter *hsp70* was released from pBChsp70 using *Kpn*I/*HindI*II restriction enzymes then ligated into pGL2basic digested with the same enzymes (Promega), upstream of the firefly luciferase gene. Two micrograms of pBC-hsp70H were digested by 5units of *Kpn*I,

overnight at 37°C. The enzyme was heat inactivated at 85°C for 30 minutes. The digested plasmid was phenol : chloroform and chloroform extracted and re-precipitated by 1/10 volume of Na acetate and twice volume of 100% ethanol and spun for 10 min at maximum speed in a Microfuge 18 centrifuge (Beckman Coulter, Fullerton, CA, USA). The pellet was washed by 70% ethanol and dried in a speedvac concentrator model A160 (Savant, Holbrook, NY, USA) for 7 min at room temperature. The digested plasmid was resuspended in deionized double distilled H_2O and subjected to the second restriction enzyme digestion reaction. The *KpnI*-digested pBC-hsp70H was cut by 5units of *HindIII* for 4 hours at 37°C. The enzyme was heat inactivated and DNA fragments were precipitated as described above. The DNA fragments were resuspended in dd H_2O. The pGL2basic plasmid was equally treated as previously described for the pBC-hsp70H.

The *Kpn*I/*Hind*III-digested pBC-hsp70H was subjected to electrophoresis on a 0.7% agarose gel in 1X TBE buffer (89mM Tris-HCl, 89mM boric acid and 2mM EDTA) using a 100bp DNA marker (Promega). The expected band at 150bp molecular weight representing the *hsp70* promoter was excised from the gel and the DNA fragment was eluted using the Gene Clean Spin Kit (Qbiogene). The eluted fragment was ligated to the linearized pGL2 basic vector in a 3:1 Molar ratio using 9 units of the Promega T_4DNA ligase overnight at 16°C. The ligated plasmid DNA was introduced into electrocompetent *E. coli* cells, DH10B strain (Invitrogen, Carlsbad, CA, USA), using the Electro Cell Manipulator 600 (BTX, San Diego, CA, USA) set at 2.25 kv, 186 Ω and 25μFD. The cells were inoculated into 1ml SOC media (Sambrook *et al*. 1989) with shaking at 37°C for 1 hour and the cells were briefly collected and plated onto ampicillin selective 2YT media (100μg/ml) overnight at 37°C. A number of randomly selected colonies were inoculated into 1.5 ml of 2YT broth (16mg/ml pancreatic digest of casein, 10mg/ml yeast extract, 5mg/ml NaCl) with shaking at 37°C overnight. Plasmid DNA was prepared using Wizard MiniPreps (Promega) according to the manufactures' instructions. The plasmid construct was identified on the basis of the expected restriction enzyme digestion pattern using 1unit of *Pst*I enzyme.

ii. The *Sac*I/*Sal*I hsp82 fragment from pKhsp82 was ligated upstream of the firefly luciferase coding region in to a *Sac*I/*Xho*I pGL2basic vector. The pGL2-hsp82 plasmid was constructed essentially following the same procedures used to construct pGL2-hsp70.

iii. pGL2-IE1 was constructed to express the firefly luciferase protein under the regulation of the *ie1* promoter flanked with the *hr5* enhancer element. Both pIE1-3 (Novagen, Madison, WI, USA) and pBCKS+ were digested with *Bam*HI and *Bgl*II. The DNA fragment representing the *hr5-ie1* regulatory sequences was ligated into the linearized pBCKS+, forming pBC-IE1. To construct pGL2-IE1, the *hr5-ie1* fragment was released from pBC-IE1 by *Kpn*I and *Bgl*II digestion and ligated into the corresponding sites of pGl2-basic. The cloning procedure essentially followed the same conditions utilized for pGL2-hsp70.

iv. A 2.7-kb *Hind*III-*Sal*I fragment from pGL2-Basic, containing the firefly luciferase and the polyA signal, was inserted into the corresponding sites of pBCKS+ creating pBCLuc. A 2.7-Kb *Sma*I-*Sal*I fragment from pBCLuc was inserted into the *Sma*I-*Sal*I sites of pSLfa1180fa (Horn and Wimmer, 2000) forming pSLLuc. The *D. melanogaster actin5C* promoter was excised from pHermesA5CEGFP (Pinkerton et al. 2000) using *Pst*I and *Bam*HI digestion. The DNA fragment was then cloned into the corresponding sites of pSLLuc creating pSLAct5Cluc.

v. pSLPUbLUc was designed to express the firefly luciferase under the regulation of the *D. melanogaster polyubiqutin* (*pUB*) promoter. A 2kb fragment containing the *pUB* promoter was digested from pB[pUB-nls-EGFP] (Handler 2001) using *Kpn*I and *Bam*HI. This fragment was cloned into the same sites of pBCKS+ to make pBCPUb. The *Kpn*I/*Bam*HI fragment from pBCPUb was then cloned into the same two sites of pSLLuc to creat pSLpUbLuc.

2.2.2. Plasmid purification

All plasmids used for insect injections were purified using double CsCl separation gradients. The bacterial cells were grown overnight in 150 ml 2YT media with the appropriate antibiotic. The cells were collected by centrifugation at 6000 rpm in a high speed centrifuge model J2-21, using a JA-14 rotor (Beckman Coulter) for 5 min at 4°C. The following steps were performed on ice. The bacterial pellet was resuspended and washed in 30 ml TE buffer (10mM TrisHCl pH8.0 and 1mM EDTA) followed by repeated centrifugation. The bacterial pellet was re-suspended in 3.6ml sucrose buffer (25% sucrose and 50mM TrisHCl pH8.0) and 0.1mg/ml of lysozyme was added. The lysate was incubated on ice for 5 min. Six hundred micro-liters of 0.25M EDTA (pH 8.0) was added, followed by a 5 min incubation on ice. Nucleic acids were librated from the lysate by adding 5.6ml of detergent mix (10% tritonX-100, 62.5mM EDTA pH 8.0, 50mM TrisHCl) and incubating on ice for one hour. The cells debris was precipitated by adding 1ml of TE buffer and centrifugation for 40 min at 15,000 rpm. The nucleic acids were passed through two rounds of CsCl gradients. The first step of the gradient purification was performed by adding 8.5ml of nucleic acid supernatant to 8.1g of CsCl and 880µl of 10mg/ml ethidium bromide. The gradient separation was performed in an ultracentrifuge model optima L-70 with NVT 65-2 rotor (Beckman) over night at 45,000 rpm at 4°C. The plasmid DNA band was extracted from the tube using a 3 cm^3 syringe. The collected band volume was added to 5.1g of CsCl, 5ml TE buffer and 225µl ethidium bromide and subjected to the second round of purification. The ethidium bromide was extracted from the purified plasmid DNA using 5 washes in an equal volume of NaCl saturated isopropanol. The plasmid DNA was precipitated by adding 2 volumes of TE buffer, 1/10 volume of Na acetate and 2 volumes of 100% ethanol. After a 30 min incubation at –20°C, the plasmid DNA was precipitated by centrifugation at 10,000 rpm for 15 min. The plasmid DNA was washed with 70% ethanol and air-dried. The plasmid DNA was re-suspended in 400µl TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA).

2.2.3. Insect rearing

The rearing conditions were performed essentially as described by Mohammed et al. (2000). PTM larvae were reared to washed and dried tubers. The cleaned tubers and cardboard stacks, providing places for pupation, were added to plastic trays and the filter papers carrying eggs were added over the tubers. The cardboard stacks filled with pupae were collected and placed in glass jars supplied with a 50% honey solution. The jars were closed with a piece of mesh cloth and filter paper was provided over the top to serve as an oviposition site for the emerged adults. The eggs-containing filter papers were changed everyday and the jars were supplied with new filter papers. All insect stages were reared in constant conditions at 26 ± 2°C with 60-70% humidity in an incubator model 1-36VL (Precival Scientific, Inc. Boone, IA, USA) with light-dark periods of 16-8 hours.

2.2.4. Injections

Micro-injection was performed essentially as described by Morris (1997). For egg collection, the overnight filters were removed with new filter papers added for fresh ovipositions. After one hour, the filters were collected with the newly laid eggs. The eggs were picked up from the filter papers using wet hair-brushes. The eggs were lined on double-sided sticky tape placed on a coverslip. The eggs were submerged in Halocarbon oil series 700 (Halocarbon Products Corporation, Nj, USA). All injected plasmids were suspended in injection buffer (0.1mM NaPO4 pH6.8, 5mM KCl). Injected embryos were incubated at 26 ± 2°C in an oxygen-saturated chamber for at least 16 hours prior to any further manipulation.

Glass needles were pulled from 10 cm borosilicate glass capillaries, 1B100F-4, (World Precision Instruments, Inc., Sarasota, FL, USA). The pulling was performed using a Flaming/Brown Micropipette Puller model p-98 (Sutter instrument Co., Novato, CA, USA) under the following conditions; Heat 419, pull 10, velocity 15, time 80sec.

The needles were bevelled using a micro-pipette Beveler model BV-10 (Sutter instrument Co.,).

2.2.5. Luciferase Assay

The transcriptional activities of the promoters were determined using the Dual-Luciferase® Reporter Assay System (Promega). In this assay, two plasmids were co-injected into PTM preblastoderm embryos. The first plasmid is used as a control and expresses *Renilla* luciferase under the regulation of the *hsp82* promoter. The second is the experimental plasmid encoding firefly luciferase controlled by *actin5C*, *hsp70*, *hsp82*, *hr5-ie1* or *pUB*. Both control and experimental plasmids were co-injected at 0.5µg/µl each. Three replicates with 60 embryos each were performed for each promoter. All injected embryos were allowed to develop for at least 16 hours at 26 ±2°C in an oxygen-saturated chamber. For each heat shock promoter, *hsp70* and *hsp82*, an additional 3 replicates were heat shocked at 37°C for one hour, followed by another hour at 26 ±2°C, prior to embryo collection. The assay was performed as the manufactures' procedures. Sixty embryos were collected in a microfuge tube, snap frozen in liquid N_2 and homogenized in 100µl 1X Passive Lysis Buffer (PLB). The homogeniser was washed with an additional 100µl of 1X PLB. The homogenate was frozen in liquid N_2 and then placed on ice. Twenty microliters from the homogenate were introduced into an assay vial. The vial was inserted into a TD-20/20 Luminometer Ver. 2020-1E 1098 (Turner Designs, Sunnyvale, CA, USA) that automatically added the appropriate reagents and recorded the corresponding readings. A 100 µl of Luciferase Assay Reagent II (LARII) was injected into the homogenate and the firefly luciferase activity was recorded. 100µl Stop & Glo®Reagent was injected and the *Renilla* luciferase activity was measured. The ratio of firefly to *Renilla* luciferases expression was also calculated by the luminometer.

2.2.6. Characterization of *EGFP* as a marker gene within the PTM soma

A hundred PTM preblastoderm embryos were injected with 1µg/µl of pB[pUB-nls-EGFP] (Handler 2001) which contains the enhanced green fluorescence protein, *EGFP*, gene under the regulation of the *Drosophila* polyubiquitin promoter. The injected embryos were allowed to develop for 24 hours at 26 ± 2°C in an oxygen saturated chamber before visual examination using a UV microscope model M2Bio, Steinsvil (Carlzisse, Inc., Thornwood, NY, USA) and GFP-525/470 Filter.

3. Results

2.3.1. Promoter activity

All promoter-reporter plasmids were constructed using *Drosophila*-derived promoters, except the *ie1* promoter from the baculovirus. The promoter-reporter plasmids used the firefly *luc* gene as a reporter. The control plasmid was expressing the *Renilla* luciferase, under the control of the SV40 promoter, to provide baseline to standardize for injection volumes, embryo survival and plasmid recovery. However, the expression of *Renilla luc* regulated by the SV40 promoter demonstrated very low activity within PTM embryos (Table 2.1). The reading of the Renilla *luc* activity ranged from 3.99-5.53. The expression of Renilla *luc* gene within PTM embryos was enhanced by replacing the SV40 promoter with the hsp82 promoter, resulting in a higher level of expression as shown in Table 2.2. The number of injected embryos used for the Table 2.1 experiment was 110, whereas the Table 2.2 experiments used 60.

In Table 2.2, the results of the activity of the five promoters (*actin5C*, the heat shock promoters *hsp*70 and *hsp*82 with and without one hour heat shock at 37°C, *polyubiquitin* and *hr5-ie1*) are presented. The *hsp70* promoter had the lowest activity whereas the *hr5-ie1* enhancer-promoter had the highest. The *hsp70* promoter induced by a one hour heat shock results in a similar ratio (firfly/*Renilla luc* ratio) as the *actin5C* promoter, 0.0896 ±

0.06 and 0.0863 ± 0.03, respectively. The *hsp82* promoter resulted in a ratio of 1.631 ± 0.34, that slightly increased to 2.16 ± 0.36 following heat induction. The activity of the *pUB* promoter within PTM embryos is higher than the *actin5C* and heat shock promoters as presented in Table 2.2. The *pUB* promoter resulted in a ratio of 6.803 ± 1.3. The pGl2-IE1 construct showed the highest expression level of the firefly luciferase, which was beyond the detection of the luminometer sensitivity. Therefore, the volume of homogenate assayed from embryos injected with pRL-hsp82/pGl2-IE1, was reduced by 50%. Even with this volume reduction, the ratio for the *hr5-ie1* enhancer-promoter was 227.6 ± 58.2. The ratio average of all promoters is represented in Figure 2.1.

2.3.2. Marker gene expression

PTM embryos were microinjected with pB[pUB-nls-EGFP] and allowed to develop for 24 hours prior to detection. The *EGFP* expression is detected using a UV microscope, within the PTM embryos (Fig. 2.1 B). Out of 100 injected embryos, transient expression of *EGFP* was detected with variable fluorescence intensity in 57 embryos. The plasmid expression is seen as glowing green spots within the embryo. The *EGFP* expression shows different patterns in the 57 embryos. However, some of the dead embryos show a high auto-fluorescence background as indicated by the arrow in figure 2.2. B. Non-injected embryos, shown under UV light in Figure 2.2.A, are opaque and lack fluorescence.

Table 2.1: Firefly *luc*/*Renilla luc* ratio; *Renilla luc* expressed by SV40 promoter.

Plasmids	Firefly reading	Renilla reading	Firefly/Renilla *luc* Ratio
pGL2-hsp70 / pRL-SV40	246.3	5.535	44.51
pGL2-hsp70 / pRL-SV40	541.0	5.423	99.75
pGL2-hsp82 / pRL-SV40	568.0	3.990	142.4
pGL2-hsp82 / pRL-SV40	741.5	4.161	178.2

Table 2.2: Promoters activities within PTM embryos.

Plamsids	Firefly reading (test)	Renilla reading (control)	Firefly/Renilla *luc* Ratio
	6.139	73.62	0.083
pSLAct5Cluc /pRL-hsp82	4.331	80.09	0.054
	16.89	138.6	0.122
Average			0.086
	0.696	15.06	0.046
pGL2-hsp70/pRL-hsp82	2.116	44.70	0.047
	1.725	26.39	0.065
Average			0.052
	2.096	15.19	0.138
pGL2-hsp70/RL-hsp82	0.763	44.21	0.017
(with heat shock)	1.620	14.27	0.114
Average			0.089
	8.102	4.194	1.932
pGL2-hsp82/pRL-hsp82	12.82	10.19	1.258
	29.79	17.49	1.703
Average			1.631
	15.97	9.031	1.768
pGL2-hsp82/pRL-hsp82	20.59	9.266	2.222
(with heat shock)	35.76	14.35	2.492
Average			2.16
	2262	7.726	292.7
pGL2-IE1/pRL-hsp82 *	1627	9.012	180.5
	1167	5.565	209.6
Average			227.6
	264.5	47.29	5.594
pSLPUbLUc /pRL-hsp82	555.0	83.82	6.622
	968.3	118.2	8.194
Average			6.80

* Only 10µl of embryos/ homogenate were used for this promoter assay

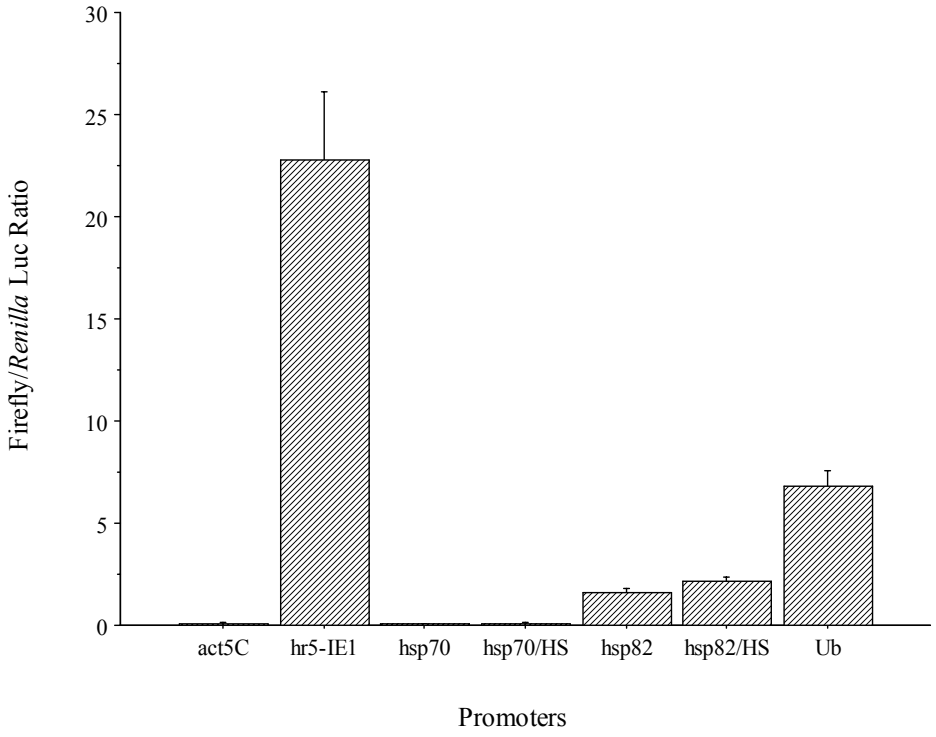
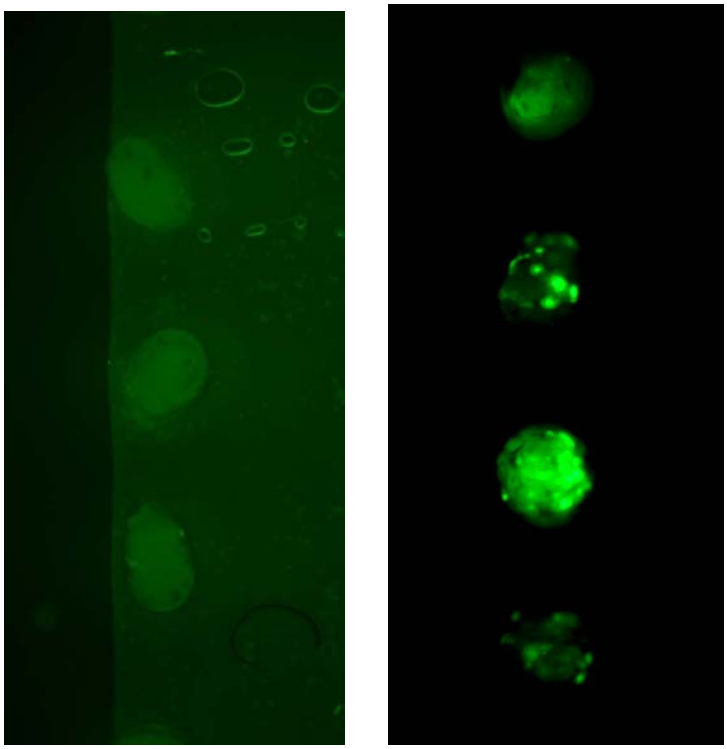


Fig.2.1: Comparison of the transcriptional activity of the exogenous promoters. Promoters (x axis) activities were determined according to the firefly/*Renilla luc* ratio (y axis) within PTM embryos. The firefly/*Renilla luc* ratio for the *hr5-ie1* enhancer-promoter represents 1/10 of the actual results to fit the scale.



A **B**

Fig. 2.2: Transient expression of *EGFP* within PTM embryos. The PTM preblastoderm embryos were injected with the pB[pUB-nls-EGFP] plasmid. The injected embryos were allowed to develop for 24 hours at 26^{o}C before visual examination using a UV microscope. A) Non-injected embryos and B) Injected embryos, both are shown under UV illumination.

2.4. Discussion

In the luciferase assay, the use of the *hr5-ie1* enhancer-promoter resulted in the highest level of firefly *luc* expression compared to the other tested promoters. The *hsp82* promoter replaced the SV40 promoter in the plasmid control to express the *Renilla* luciferase. The ratio is close to 1 when both firefly and *Renilla* luciferases are expressed by the same promoter, *hsp82*, and is slightly increased by heat induction. In a CAT assay, the *hsp*82 promoter elicits a higher level of expression of the cat gene in *L. cuprina* embryos compared with *hsp*70, and *actin5C* promoters (Coates et al. 1996). The heat shock has slightly increased the transcriptional activity of both *hsp*82 and *hsp*70 promoters within *P. operculella* embryos. The non-significant effect of heat shock induction on both *hsp*82 and *hsp*70 has previously been observed in CAT assays for both *D. melanogaster* and *L. cuprina*. Atkinson & O'Brochta (1992) suggested that a 'stress response' due to embryo manipulation during microinjection, may stimulate a stress-induction of the heat shock promoters. In addition, a high level of constitutive expression of the *hsp*82 promoter was observed in *D. melanogaster*. The 5' regulatory region of the *hsp*82 gene includes an overlapping array of heat shock consensus sequences (Bienz & Pelham 1987; Xiao & Lis 1989) which are responsible for the high level of constitutive expression in *Drosophila*.

The *D. melanogaster polyubiquitin* promoter resulted in a higher expression level of firefly *luciferase* within PTM embryos compared to the other *Drosophila* promoters. In *L. cuprina* embryos, the *polyubiquitin* promoter has driven transient expression of *EGFP* in 40% of injected embryos whereas neither *hr5-ie1* nor *actin5C* promoters showed transcriptional activity of EGFP (Heinrich et al. 2002). The transformed caribbean fruit fly, *A. suspense*, has been marked with polyubiquitin-regulated *EGFP* (Handler and Harrell 2001). In this study, the transient expression of *EGFP* regulated by *polyubiquitin* promoter has been detected within 57% of the injected *P. operculella* embryos. These results reveal that the *polyubiquitin* promoter from *Drosophila* has a wide activity range across insect species, in dipteran as well as in lepidopteran insects. However, it is likely that the promoter activity varies from one species to another. The *hr5-ie1* regulatorty

sequences have been used to drive the expression of *EGFP* as a marker gene for germline transformation of *A. gambiae* (Grossman et al. 2000). The high activity of the *hr5-ie1* ehancer-promoter within PTM embryos could be due to several factors. All promoters, excluding *hr5-ie1*, are originally cloned from drosophilid insects, which are distantly related to lepidopteran. In addition, the drosophilid promoters in this assay lack enhancer sequences. The *hr5* enhancer sequence has been shown to increase the transcriptional activity of *ie1* promoter. Both the *hr5* enhancer element and the *ie1* promoter were cloned from the *Autographa californica* nuclear polyhedrosis virus (AcNPV). AcNPV is infectious to lepidopteran insects and utilizes the host genetic machinery to replicate its genomic DNA. Upon cell infection, the immediate early genes are transcribed by host factors, as demonstrated by transient expression assays (Guarino and Summers 1986; 1987). The immediate early protein (IE1) can function as a transcriptional activator of early promoters (Guarino and Summers 1986; Nissen and Friesen 1989), allowing the viral replication cycle to continue. Eight homologous regions (*hrs*) are dispersed throughout the AcNPV genome. The *hr* elements function as origins of replication for the viral genome (Pearson et al. 1992) as well as enhancer elements for the early genes (Rodems and Friesen 1993). The 484bp *hr5* enhancer consists of six 26-to 30-bp imperfect palindromes. The smallest functional unit of *hr5* is the central 28bp repeat and is capable of orientation and positional-independent transcription activation (Rodems and Friesen 1995). The IE1 protein has demonstrated binding activity with the *hr5* sequence (Guarino and Dong, 1991). Further investigation indicated that the C-terminus of the IE1 protein is required for DNA binding activity and the N-terminus is involved in transcriptional activity (Kovacs et al. 1992).

The *polyubiquitin*-regulated EGFP demonstrates fluorescence activity within potato tuber moth embryos. The transient expression was not even throughout the embryos as shown in Fig. 2.2. That could be due to embryo death during injection, or plasmid breakdown within the embryo that leads to weak or no fluorescence. Once the fluorescent protein is expressed in the host tissue, it requires no host factors for its maturation following a certain period of time (Horn et al. 2002). Moreover, a phenotypic

genetic marker based on rescue of eye color in a mutant strain was not used for silkworm or pink bollworm transformation, currently the only transformed lepidopteran insects. Eye color genes have limitation for use as genetic markers. Available genes that complement the null mutation of the eye color should be workable in the desired species with an expression level suitable for visual screening. Furthermore, the mutant stain should be available with a fitness level that aids the continuation of the experiment generation after generation. In the case of releasing transgenic individuals, the fitness of the mutant strain should meet the requirements of competition with the wild type insects. Both of these components are not available for the potato tuber moth. There is a lack of both the recipient mutant stain and cloned gene(s) in the PTM, as in many other insect species. This favors deploying the EGFP-based marker system for germline transformation experiments. In addition, EGFP-fluorescene could be detected during the early stages of the insect life cycle, including developed embryos and neonate larvae, thereby eliminating the labor cost of rearing G1 individuals to screen them at adulthood as in the case of eye color markers.

The conclusion from these results is that both the *hr5-ie1* enhancer-promoter cassette, along with the *EGFP* marker gene present promising tools to be used for the transformation experiment of the PTM.

CHAPTER III
INTERPLASMID TRANSPOSITION ASSAY

3.1. Introduction

Germline transformation is tedious work and requires great effort. To adequately prepare to produce gemline transformants of PTM, it is necessary to perform an assay to demonstrate the mobility of the transposable element in the embryonic soma prior to initiating a transformation experiment. Four transposable elements (*Hermes*, *Mos*1, *Minos* and *piggyBac*) have been used to transform a number of insect species. Two mobility assays; excision and interplasmid transposition have previously been used to assess transposable element activity (Rio et al. 1986; O'Brochta and Handler, 1988; O'Brochta et al. 1994).

An excision assay measures the ability of a transposable element to excise from a donor plasmid within the host. These assays are based on the detection of either a gain or loss of a genetic marker. In *Drosophila*, an excision assay was utilized to examine the mobility properties of the *P* element (Rio et al. 1986). These assays are typically based on the injection of two plasmids into the insect embryos; a helper and indicator plasmid. In *Drosophila* experiments, the helper plasmid expresses the transposase and the indicator plasmid is designed to contain the *P* element within the peptide-coding region of the *LacZ* gene. Excision of the element restores the *LacZ* open reading frame and hence leads to the expression of the gene product which is easily detected The excision properties of *Hermes*, *mariner* and *piggyBac* elements has been measured in a variety of insect species (Atkinson et al. 1993; Coates et al. 1995; 1997; 1998; Jasinskiene et al. 1998; Thibault et al. 1999).

Interplasmid transposition assays are based on the movement of a transposable element from a donor plasmid into a target plasmid, within the insect embryos in the presence of a helper plasmid. This is essentially a routine assay to prior performing transformation experiment for a new insect species. The target plasmid usually has a

selectable marker gene and is incapable of replicating within *E. coli* cells, whereas the donor plasmid contains a second selectable marker gene in addition to a ColE 1 origin of replication delimited within the left and right arms of the transposable element. The helper plasmid provides a source of the transposase. A transposition event into the target yields a plasmid that is capable of replicating within *E. coli* cells and that can be selected on two antibiotics. The plasmid is further analyzed by restriction enzyme digestion and DNA sequencing of the insertion sites. This type of assay has been utilized for several elements. Atkinson and O'Brochta (2000) used transposition assays to show that *Hermes* is mobile in at least 12 insect species. The *Mos*1 *mariner* element has demonstrated mobility through transposition assays in *L. cuprina* and *Ae. aegypti* (Coates et al. 1995; 1997; 1998). The mobility of *piggyBac* was measured in *T. ni*, (Lobo et al. 1999); *Ae. Albopictus, Ae. triseriatus* (Lobo et al. 2001); *An. gambiae* (Grossman et al. 2000) and *P. gossypiella* (Thibault et al. 1999).

Lu et al. (1997) demonstrated the transcriptional coactivation of BmIE1 and *hr3* on the *actin* promoter. Transfection of Bm5 cells with pBmA.cat shows lower CAT activity than those cells transfected with the same plasmid combined with the *hr3*-based construct. Quantitative analysis revealed enhancement by two orders of magnitude in the *hr3*-transactivated experiment relative to the control level of expression. The IE1 protein has demonstrated binding activity with the *hr5* sequence (Guarino and Dong, 1991). Further investigation indicated that the C-terminus of the IE1 protein is required for DNA binding activity and that the N-terminus is involved in transcriptional activition (Kovacs et al. 1992). The helper plasmid used for the transposition assays for PTM produces transposase under the regulation of the *hr5-ie1* enhancer-promoter regulatory sequences. Incorporation of a plasmid expressing the IE1 protein may act as a "transactivator" to increase the transcriptional activity of the *hr5-ie1* enhancer-promoter casette. Increased transcriptional activity of the *hr5-ie1* enhancer-promoter will lead to higher expression of the transposase, which may increase the frequency of transposition events.

The existence of endogenous transposable elements in the host genome may cause

cross mobility, hence destabilizing elements being used in transformation experiments either during or following integration. Screening the host genome for the presence of homolgous transposable elements prior to initiating transformation experiments is highly recommended.

In this chapter, an interplasmid transposition assay is used to determine the mobility frequency of three transposable elements within the potato tuber moth embryos. The PTM genome was also screened for the presence of *Mos*1 and *piggyBac* related elements.

3.2. Materials and methods

3.2.1. Plasmid construction

Three helper plasmids were designed to drive the expression of the transposase coding regions of the *Hermes*, *Mos1*, and *piggyBac* transposable elements under the regulation of the *hr5-ie1* enhancer-promoter sequences (Chapter II section 2.2.1.2.iii). The three open reading frames (ORFs) were amplified using specific primers and then cloned into the pGEMT vector (Progema). From the pGEMT plasmids the ORFs were then cloned into pIE1-3 in front of the *hr5-ie1* enhancer-promoter. The primers were designed to have restriction enzyme recognition sites on their ends to facilitate directional cloning. *Mos1* and *piggyBac*-specific primers have *Sac*II and *Bam*HI restriction sequences on the 5' and 3' ends, respectively. *Hermes*-specific primers have *Sac*II and *Not*I restriction sequences on the 5' and 3' ends, respectively. The primer sequences are as follow;

i. *Hermes*-specific primers:

5'HERMSII: 5'-CCGCGGAGTAGAGATTAGATGCAGAAAATGG-3'

3'HERMNI: 5'-GCGGCCGCTCCAGTCCAAAATTTATTTA-3'

ii. *Mos1*-specific primers:

5'MOSSII: 5'-CCGCGGTCTAGTGCAGTCAACATGT-3'

3'MOSBHI: 5'-GGATCCATTTATTCAAAGTATTTGCC-3'

iii. *PiggyBac*-specific primers:

5'PIGSII: 5'-CCGCGGATAAAATGGGTAGTTCTTTAGACGA-3'

3'PIGBHI: 5'-GGATCCTAATTAGCTTAACTTATACA-3'

Four units of Vent® polymerase (Biolabs, USA) were used to amplify the ORFs of *Hermes*, *Mos*1 and *piggyBac* from pBChsp70H, pKhsp82MOS (Coates *et al*., 1995) and phspBac (Handler and Harrell, 1999), respectively. The amplification conditions were as follows; preheating period for 5 min at 94°C, followed by two cycling conditions. The first type of cycles was 94°C for 15 sec, 40°C for 15 sec and 72°C for 2 min repeated 5 times. The second type of cycle 15 repeats of 94°C for 15 sec, 45°C for 15 sec and 72°C for 2 min followed by 5 min at 72°C and the reaction ended at 4°C. Because of the T/A cloning strategy of the pGEMT vector and Vent polymerase yielding blunt ended products, the PCR products were A-tailed by the addition of 2mM dATP and 0.25 units of Promega *Taq* ploymerase for 30 min at 72°C. The PCR fragment was cloned into the pGEMT vector by a ligation reaction utilizing 5 units of T_4DNA ligase, overnight at 16°C. One microliter of the ligation reaction was used for electroporation into DH10B electrocompetent cells. The cells were placed in 1 ml SOC media (Sambrook *et al*., 1989) with shaking at 37°C for 1 hour and the cells were briefly collected and plated onto ampicillin selective 2YT media (100µg/ml) supplied with Xgal/IPTG (40µg/ml and 20µg/ml) overnight at 37°C. The white colonies were screened by PCR as follows. The white colonies were transferred, by a single touch using a toothpick, into a 48-well PCR master plate (Multiplate™ MJ Research, Inc., Waltham, MA, USA) The PCR screening was performed using T_7 and SP_6 primers that are specific to the T_7 and SP_6 promoters on the vector sequence (Promega). The DNA inserts were amplified by 0.25 unit of Promega *Taq* polymerase under the following conditions; 25 cycles of 94°C for 15 sec, 50°C for 15 sec and 72°C for 2 min, followed by 5 min at 72°C and the reaction ended at 4°C using a Peltier Thermal Cycler model PTC-200 (M J research, INC. Watertown, MA, USA).

The DNA fragment, expressing *Hermes* transposase, was cut from pGEMT by the

*Sac*II-*Not*I restriction enzymes using standard digestion reaction conditions. The restricted DNA was electrophoresed on a 0.7% low melting agarose gel. The expected size DNA band was excised and purified from the agarose gel. The extracted DNA band was cloned into the multiple cloning site of pIE1-3 that was previously cut with the same restriction enzymes. The pIE1-HerORF plasmid construction was confirmed by restriction analysis, using the *Hind*III restriction enzyme.

Unlike the *Hermes* ORF cloning strategy, both the *Mos1* and *piggyBac* ORFs cloning procedures had an intermediate cloning step. The *Mos1* ORF was cut from pGEMT by *Sac*II-*Bam*HI digestion. The DNA band was gel purified and then ligated into pBCKS+ that had been linearized with the same enzymes. The *piggyBac* ORF was cut by *Kpn*I-*Sac*I restriction enzymes. The DNA band was gel purified and then ligated into pBCKS+ that had been linearized with the same enzymes. The pBCMosORF and pBCPigORF were used to clone *Mos*1 and *piggyBac* ORFs, respectively into pIE1-3. The ORFs were released using *Sac*II-*Bam*HI digestion and the DNA fragments were purified from the agarose gel, then cloned into pIE1-3 that had been previously cut with the same enzymes. The pIE1-MosORF and pIE1PigORF plasmids were confirmed by restriction analysis using *Sac*I-*Nar*I and *Sac*I-*Aat*II, respectively.

3.2.2. Plasmid purification

All injected plasmids were purified as described in chapter II, section 2.2.2. The pGDV1 plasmid is carried within a *Bacillus subtillus* strain which is grown under different conditions to *E. coli* cells. The bacterial cells were grown overnight at 37°C with shaking in 250ml of terrific media (12mg/ml pancreatic digest Casein, 24mg/ml Yeast extract, 9.4mg/ml Dipotasium phosphate, 2.2mg/ml Monophosphate and 4ml Glycerol). The bacterial pellet was washed in 50ml TE buffer. After precipitation, the pellet was re-suspended in 6ml of sucrose buffer, 1ml of 10mg/ml lysozyme was added, followed by 2ml of 0.25M EDTA and finally 9ml of detergent mix. The plasmid DNA was purified through two CsCl gradients and ethanol precipitated as described in chapter

II section 2.2.2.

3.2.3. Insect rearing and injections

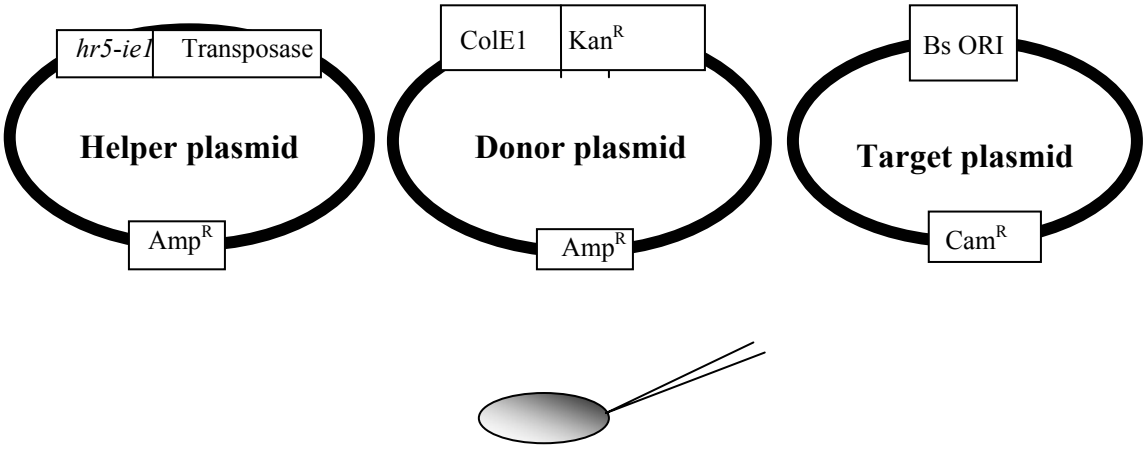
Rearing and injection conditions were as described in chapter II, sections 2.2.3 and 2.2.4.

3.2.4. Plasmid DNA rescue from injected embryos

For each plasmid rescue, 60 embryos, representing one replicate of each experiment, were homogenized in 50µl of lysis buffer (0.6% SDS, 0.01M EDTA; Hirt 1967) with debris on the homogeniser washed into the tube with an additional 50µl of buffer. The homogenate was incubated at 65°C for 30 min, after which 14µl of 8M potassium acetate was added. The homogenate was mixed gently and incubated on ice for 30 min. The cell debris was collected by maximal centrifugation for 10 minutes. The supernatant was transferred into a new Eppendorf tube, the supernatant volume recorded and the nucleic acids were precipitated by the addition of two volumes of 100% ethanol and 2 min incubation at room temperature, followed by maximal centrifugation for 10 min at room temperature. The pellet was washed with 70% Ethanol, briefly dried and resuspended in 5µl deionized ddH_2O. The plasmid DNA was introduced into either DH10B or XL1 blue (Stratagene) electrocompetent cells. The cells were inoculated into 1ml of SOC media and allowed to grow in a shaking incubator for 1 hour at 37°C. To record the number of donor plasmids recovered from the embryos during injection, 5µl, representing 0.5% of the total transformed cells, were plated onto ampicillin (100µg/ml) selective 2YT media. To recover the transposed plasmids, the remainder of the transformed cells were gently spun down and plated onto 2YT media containing 25µg/ml of chloramphenicol and 10µg/ml of kanamycin (Cam/Kan). To avoid plasmid contamination among independent experiments, aerosol-resistant pipette tips were used at all times.

3.2.5. Interplasmid transposition assay

The mobility of transposable elements (*Hermes*, *Mos*1, and *piggyBac*) was assessed within the PTM soma. For each transposable element assay, three plasmids were microinjected into the PTM preblastoderm. The transposition assay (Coates et al. 1997) is outlined in Figure 3.1. The total concentration of microinjected plasmid DNA was 2µg/µl; 1µg/µl of target plasmid and 0.5µg/µl each of donor and helper plasmids. Both helper and donor plasmids are ampicillin resistant. The helper plasmid expresses the transposase that catalyzes transposition of the corresponding inverted terminal repeats from the donor plasmid, into target sites on pGDV1 plasmid. The pGDV1 target plasmid, is chloramphenicol resistant and possess a *Bacillus subtilus* origin of replication and is thus incapable of replication within *E. coli* cells. Integration of inverted terminal repeats including the ColE1 origin of replication and kanamycin resistant gene into pGDV1 results in the ability of the pGDV1 transposition product to replicate within *E. coli* and grow on both 10µg/ml of chloramphenicol and 25µg/ml kanamycin containing media. The helper plasmid was designed to express the transposase under the regulation of the *ie1* promoter, flanked by the *hr5* enhancer element. Each replicate represented either different injection experiments or independent embryo collections. *Hermes* and *Mos*1 experiments were replicated three times and *piggyBac* five times. For each replicate, 60 embryos were injected. To ensure replication quality, the embryos were either collected from different adult groups or on different days. The injected embryos were allowed to develop at 26 ±2ºC in an oxygen-saturated chamber for 24 hours. The oil was removed from the slides and the embryos were collected using a brush. Each replicate was collected in a sigle sterile 1.5 ml Eppendorf tube and the plasmids were rescued from the embryos and transformed into competent cells as described in the previous section.



Injection of the three plasmids into preblastoderm embryos

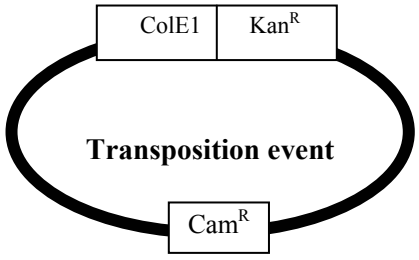


Fig. 3.1: Interplasmid transposition assay. Three plasmids are injected into the insect preblastoderm embryos; helper, donor and target plasmids. The helper, ampicillin resistant, expresses the transposase enzyme which cuts the transposable element from the donor plasmid and inserts it into the target plasmid. The donor plasmid, also ampcillin resistant, carries a kanamycin resistance gene and the *E. coli* ORI limited by the transposable element ITRs. The target plasmid, chloramphenicol resistant, carries a *B. subtilus* ORI. The three plasmids are recovered from the embryos after 24 hours, transformed into E. coli electropcompetant cells. 0.5 % of the cells are selected on Amp selective media to determine the number of helper and donor plasmids injected into the embryos. The remainder of the cells are selected on Cam/Kan selective media to determine the number of target plasmids that gained Kan^R and ColE1ORI followed by molecular analysis to determine the number of transposition events.

The bacterial colonies that grow on either Amp or Cam/Kan plates were counted. The colonies that confer resistance to Cam/Kan were reselected as two exact plate copies on Amp and Cam/Kan. The colonies that grow on both plates were excluded from the experiment as these likely represent the product of recombination rather than transposition. Only those did not grow on ampicillin were chosen for further molecular analysis.

In an independent experiment, we determined the transposition activity of the *piggyBac* element in the presence of a "transactivator". We repeated the interplasmid transposition assay for *piggyBac* in the presence of a fourth plasmid, pIE1/153, (Lu *et al*. 1997) to increase the expression level of *piggyBac* transposase. Five different ratios of the helper : transactivator plasmids were used (1, 0.1, 0.02, 0.01 and 0.005).

3.2.6. Molecular analysis of the plasmids recovered from the transposition assay

The plasmid DNA, from Cam/Kan only resistant bacterial colonies, was prepared using Wizard MiniPreps (Promega) according to the manufactures' instructions. The plasmid DNA was analyzed using restriction enzyme digestion and DNA sequencing.

3.2.6.1. Restriction enzyme digestion analysis

The pGDV1 plasmid has a unique restriction site for the *Bam*HI enzyme at position 2001. Also, the donor cassette of both transposable elements, *Mos*1 and *piggyBac*, has a unique site for *BamHI*. The plasmid DNA was digested using 0.5 units of *Bam*HI for at least two hours at 37°C. The digested plasmid DNAs were then subjected to electrophoresis on a 0.7% agarose gel in 1X TBE buffer against a 1kb DNA marker (Promega). The pGDV1 plasmid is 2.5kb in size and the *piggyBac* donor cassette, including the Kanamycin resistant gene, ColE1 origin of replication and *LacZ* gene is 5.5kb. The combined molecular weight of any restriction fragments of a transposed element, is expected to be 8.0kb (2.557kb + 5.5kb). Restricted DNA samples that

produced different molecular weights than expected, were excluded. The *Mos*1 donor cassette is 4.2kb, so the expected restricted bands for pGDV1 transposed by *Mos*1 should to be 6.7kb (2.557kb + 4.2kb).

3.2.6.2. DNA sequencing

Candidate clones were selected for further analysis by DNA sequencing, using specific primers. The primers were designed to the left and right arm sequences of the *piggyBac* donor and were oriented toward the inverted terminal repeats, thus allowing the DNA sequence at the junction point of the element and the pGDV1 target plasmid to be determined. Only those transposition products from the assay with the transactivator plasmid at a 1:0.02 ratio (Helper : Transactivator) had their DNA sequence determined in both directions. For other assays, experimental clones were grouped according to their restriction enzyme patterns and one or more representative(s) of each group were confirmed by DNA sequencing using the right arm-specific primer (RTR) only. The outward facing primers were designed according to Thibault *et al*. (1999) as follows.

RTR: 5'-ACCTCGATATACAGACCG-3'

LTR: 5'-CGGATTCGCGCTATTTAG-3'

The plasmid DNA was sequenced using either manual-based sequencing using Sequenase Version 2.0 DNA Sequencing kit (USB, Cleveland, OH, USA) or automated-based sequencing using the ABI Prism BigDye™ Terminator Cycle sequencing ready reaction kit (Applied Biosystem, Foster city, CA).

The automated DNA sequencing was performed as follow. Reactions were performed in a total volume of 5ul containing 2ul of BigDyemix, 400ng of DNA template and 1pmole of primer. All reactions were carried out in a Peltier Thermal Cycler model PTC-200. Amplification began with 95°C for 4 min, followed by 35 cycles of 95°C for 15 sec; 50°C for 15 sec; and 60°C for 4 min. Reactions were resolved by an Applied BioSystems automated DNA sequencer at the Gene Technology Lab, Texas A&M University.

3.2.6.3 DNA sequence analysis

The insertion point within the target plasmid was determined by matching the DNA sequence results against the pGDV1 sequence using the Vector NTI Suite (InforMax, North Bethesda, MD, USA).

3.2.7. Screening the PTM genome for transposable element sequences

3.2.7.1. PTM genomic DNA isolation

PTM genomic DNA was prepared according to Ausubel et al. (1992). The fourth larval instars of PTM were collected from the tuber. Approximately 300 mg of larvae was snap frozen in liquid Nitrogen and ground to a fine powder with a mortar and pestle. The ground tissues were transferred into 15 ml Falcon tubes, and 3.6 ml of digestion buffer (100mM NaCl, 10mM Tris-Cl pH 8.0, 25mM EDTA pH 8.0, 0.5% SDS and 0.1μg/ml of freshly prepared proteinase K) was added. The cellular homogenate was incubated overnight at 65ºC. The genomic DNA was extracted using an organic buffer (phenol/chloroform). An equal volume of the phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added to the lysate to extract the DNA from the cell debris and cellular proteins. To separate the two phases, the mixture was spun for 10 min at 3000 rpm. The aqueous phase was transferred into a new fresh tube, then one volume of chloroform/isoamyl alcohol (24 : 1) was added to the aqueous phase to remove traces of phenol from the DNA solution. Again the solution was spun for 10 min at 3000 rpm to separate the two phases, the aqueous phase containing the DNA was transferred into a new fresh tube. Finally, the DNA was precipitated by the addition of 0.5 volume of 7.5M ammonium acetate and two volumes of 100% ethanol followed by centrifugation for 10 min at 14000 rpm. The DNA was washed with 70% ethanol to remove traces of salt from the DNA pellet. The DNA was air dried and then re-suspended in 500 μl of TE buffer (10mM Tris-Cl pH 8.0 and 1mM EDTA).

3.2.7.2. Degenerate PCR

The PTM genomic DNA was screened for *piggyBac* and *mariner*-related elements using degenerate primers. The *mariner* degenerate primers were designed according to Robertson (1993) as follows;

MAR124F: 5'-TGGGTNCCNCAYGARYT-3'

MAR276R: 5'-GGNGCNARRTCNGGNSWRTA-3'

The *piggyBac* degenerate primers were designed according to the conserved region of the *piggyBac* open reading frames that originated from *T ni* and *B*. *dorsalis*, as follows.

PIG155degF: 5'-GTNAARTGGACNAAYGCNGA-3'

PIG563degR: 5'-GGRCARTANGTRCARTANGT-3'

The degenerate PCR was performed on genomic DNA from the PTM along with genomic DNA from the honey bee, *Apis mellifera*, and *T. ni*, which were used as positive controls for *mariner* and *piggyBac* elements, respectively. The amplification conditions were conducted as described in Robertson (1993). In a 25µl total reaction volume, 0.25 units of Promega *Taq* polymerase, 1x *Taq* buffer, 2.5mM $MgCl_2$, 200µM dNTPs and 20 pmoles of each primer were mixed with 20-100 ng genomic DNA. The reaction started with an initial denaturation step of 95°C for 2min followed by 40 cycles of 1 min denaturation at 95°C, 15 sec at 50°C and 1 min extension at 72°C. The reaction was electrophoresed on a 1% agarose gel, stained with Ethidum Bromide (Eth Br) and visualized with ultraviolet light.

The PCR product produced with the *mariner* degenerate primers was gel purified and cloned into the TOPO T/A cloning vector (Invitrogen). Using the M13 universal primers, the PCR product was sequenced as described above. DNA sequence fragments were compared to the Gene Bank databases using BLAST-X and tBLAST-X algorithms (Altschul et al. 1997).

3.3. Results

3.3.1. Transposition assay

The choice of a transposable element for use in a transformation experiment depends in part upon the transpositional activity of each element within the PTM embryonic germline. In an attempt to estimate relative activities, transposition events that occur between plasmids in the soma were recorded for the *Hermes*, *Mos*1 and *piggyBac* elements. The total number of colonies recovered on Cam/Kan plates from the three experiments for *Mos*1 and five experiments of *piggyBac* was 13 and 31, respectively, (Table 3.1). In contrast, no colonies were recovered from the *Hermes* experiments. However, further analysis using *Bam*HI restriction enzyme digests (Fig. 3.2) revealed that no transposition events were recovered following the recovery of 724,700 donor plasmids from the *Mos*1 experiments (Table 3.2). The only transposable element for which transposition products were recovered was the *piggyBac* elemet; 20 of 31 plasmids from five experiments showed the expected *Bam*HI restriction enzyme pattern, with a total size of 8.0kb. Further DNA analysis confirmed the transposition of *piggyBac* into the target plasmid pGDV1. DNA sequencing of the insertion sites using *piggyBac* specific primers revealed the duplication of a TTAA insertion site, the hallmark of *piggyBac* transposition, for all 20 transposition productds. The total transposition frequency for three experiments, is calculated by dividing the total number of transposition events, by the total number of recovered donor plasmids.

Table 3.1: Interplasmid transposition assay results. Total number of helper and donor plasmids are estimated from the number of colonies recovered on Amp.

Hermes		*Mos*1		*piggybac*	
Helper and Donor Plasmids	Target colonies on Cam/Kan	Helper and Donor plasmids	Target colonies on Cam/Kan	Helper and Donor plasmids	Target colonies on Cam/Kan
79,200	0	230,400	1	524,200	1
8,400	0	331,800	8	74,200	2
120,800	0	887,200	4	272,800	1
				27,200	24
				59,200	3
Totals: 208,400	0	1,449,400	13	957,600	31

Table 3.2: Number of transposition events of the *Mos1* and *piggyBac* elements following *Bam*HI restriction enzyme analysis

*Mos*1		*piggyBac*		
No. of Donor plasmids	Total number of transposition events	No. of Donor plasmids	Total number of transposition events	Transposition frequency (total) (Event/Donor plasmids)
115,200	0	262,100	0	
165,900	0	37,100	0	
443,600	0	136,400	0	$4.2X10^{-5}$
		13,600	19	
		29,600	1	

3.3.2. Transposition assay in the presence of a transactivator plasmid

The transposition assay was also performed in the presence of a fourth plasmid, pIE1/153, that expresses the IE1 protein. The IE1 protein, as mentioned in the previous chapter, binds to the *hr5* enhancer element. The IE1/*hr5* complex elevates the transcriptional activity of the associated promoter. The helper plasmid is designed to express the *piggyBac* transposase under the regulation of the *hr5-ie1* enhancer-promoter and is thus expected to be up-regulated in the presence of the IE1 protein and the level of transposase expression should increase. The pIE1/153 plasmid was mixed with the other three plasmids (helper, donor and target) and used in the transposition assay. The optimal amount of the pIE1/153 plasmid to be included in the assay was unknown. Five ratios of helper (pIE1-PIGORF) : transactivator (pIE1/153) were used and all demonstrated a higher transposition frequency (Table3.3) than when the *piggyBac* helper plasmid was used in isolation in the previous experiment. The highest transpositional activity was achieved using a 1:0.01 ratio (transposition frequency of 4.3 $X10^{-4}$) followed by the 1:0.02 ratio. These two ratios (1:0.01 and 1:0.02) of pIE1/153 plasmid included in the assay enhanced the transpositional activity by 10 and 9 times, respectively, compared to the results of helper plasmid alone.

Sequencing of the insertion sites confirmed that transposition of the *piggyBac* element had occurred. The duplication of the target site, TTAA, a hallmark of *piggyBac* transposition, was present in the vast majority of the recovered products. Figure 3.3 presents the sequence data recovered from the 1:0.02 ratio of helper : transactivator experiments and shows the duplication of TTAA and the insert site positions on pGDV1. The insert sites mapped on pGDV1 are represented in figure 3.4.

Table 3.3: Number of transposition events of the *piggyBac* element in the presence of transactivator plasmid.

Helper (pIE1-pigORF) : Transactivator (pIE1/153) ratio	Exp. No.	No. of Donor plasmid s	Total number of transposition events	Average transposition Frequency
1:1	1	306,700	1	$7.1X10^{-5}$
	2	125,500	26	
	3	203,200	18	
1:0.1	1	113,500	0	$1.8X10^{-4}$
	2	37,600	17	
	3	92,700	27	
1:0.02	1	47,400	18	$3.8X10^{-4}$
	2	5,200	31	
	3	106,100	11	
1:0.01	1	101,000	32	$4.3X10^{-4}$
	2	20,200	11	
	3	16,700	17	
1:0.005	1	60,000	6	$9.6X10^{-5}$
	2	186,000	5	
	3	171,500	29	

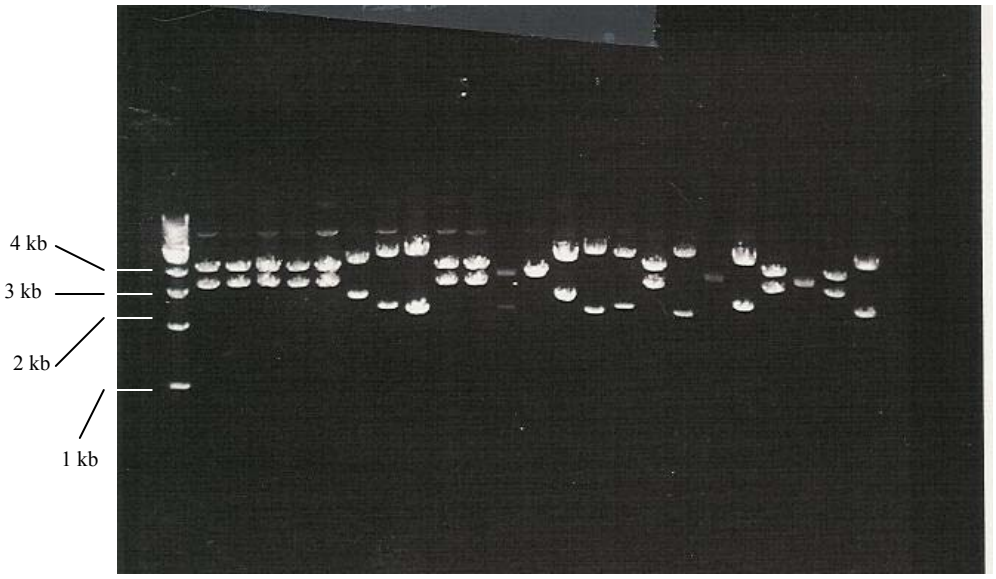


Fig 3.2. Restriction enzyme analysis of the candidate transposition products from *piggyBac* experiments using *Bam*HI.

```
 85  gagcgattccTTAA   5   TTAAacgaaattga
 101 gaaattgagaTTAA   3   TTAAggagtcgatt
245   tcaaccagaaTTA   1   TTAAttctggttgag
 363 caaacaaagtTTAA   2   TTAAagctaaagca
453  cgtgttgaatTTAA   1   TTAAtccaaataaac
 491 aatgctttggTTAA   2   TTAAaacaaaatat
 603 gataaagcagTTAA   1   TTAAgaaaactatt
 945 aagcaattgaTTAA   3   TTAAagaaatatct
 968 tccaattgatTTAA   3   TTAAcggaattaat
977  attaacggaaTTAA   2   TTAAtgaaatcgact
 992 gaaatcgactTTAA   4   TTAAaagagaatga
2447 atacaaatgcTTAA  2   TTAAataaaaaaag
2500 tagtgttataTTAA  1   TTAAtaacaaaata
```

Fig. 3.3: Insertion point sequencing of the transposition plasmids using *piggyBac*-specific primers.The numbers on the left side of the sequences represent the positions on the target plasmid in which the *piggyBac* element was inserted. The number in between sequences represent how many times the same site was hit during the three experiments. The TTAA sequence is the insertion site and they are duplicated in each case. The small letters are the target plasmid sequence.

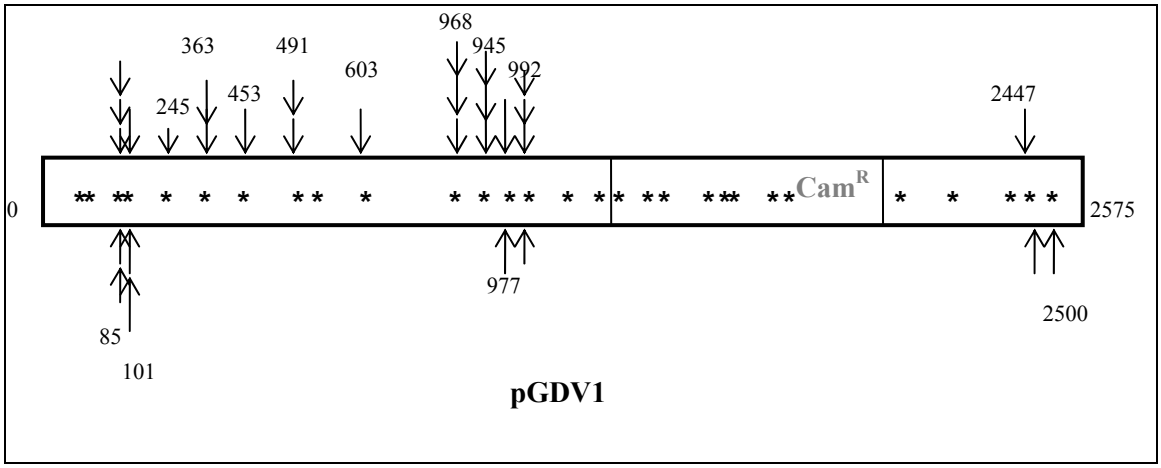


Fig. 3.4: pGDV1 map. The plasmid is represented by a bar, the black stars represent the potential TTAA target sites for *piggyBac* insertion, the numbers are the target site positions on the plasmid, the arrows represent the number of insertions into the same site by *piggyBac*. The arrows above the bar indicate that the *piggyBac* insertion is in a 5'-3' orientation with respect to the Cam resistance gene. The arrows below the bar indicate that the *piggyBac* insertion is in the opposite orientation with respect of the Cam resistance gene.

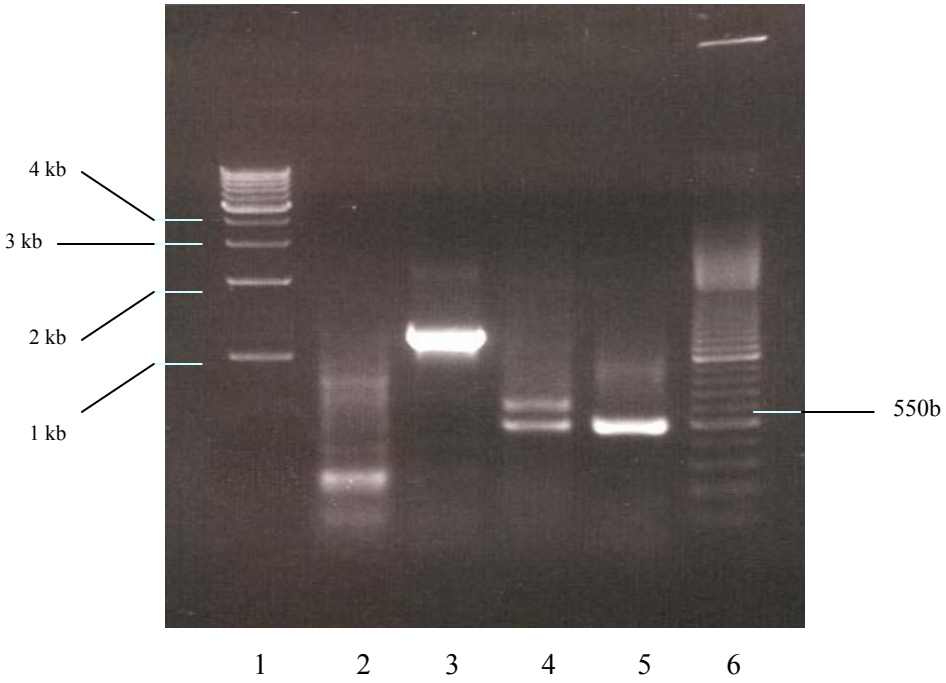


Fig 3.5: Degenerate PCR screening of the PTM genomic DNA for *mariner* and *piggyBac* elements. Lane1: 1kb DNA marker, lanes 2 & 3: PTM and *T. ni* genomic DNA using *piggyBac* primers, lanes 4&5: PTM and honeybee genomic DNA using *mariner* primers, and lane 6: 100bp DNA marker.

GGTGCCAGNATCGGGNAGTATATGGTGGGTGAGGCAGGATAGACACATTTTTTCTACTAAAAAAAT
CAAGTGTTCTGGCGGACGTGTGCGGCGAGGCATTGTCATGGTGCCAAAGAAGGTGCCGGATTCCTG
ACTTCGGACGCTTCTCACACCAGGATGATAATACTGCTGGGGCACAAACTGATACATACCACTCCG
AATTAACTGTTTTTTGATCCTCTAGCACCACAGTAGCGATATGACCGGTCTTACAAAAAAACGAGG
CAACCATTTGTTTCTGCGTGCTGCGCGTTCGACGAAACTTTGTTGGTGTTGGTTCATCATCGAAGC
ACCATACGTCGATTGCCGTTTTGTTTCCGGGTCGTAGTTATACAGCCATGTTTCGTCACCGGTGAG
TATATCGTATACGTTTTTTTTCTCGCCCCCGTTGAATTTTTCAATCATAAATCGGCACCAATCTAC
CCGGCGGGCTTTTTGTTCATCGGTCAGTTCGTGGGGCACCCA

Fig 3.6: DNA sequence of the PCR product from PTM genomic DNA using *mariner* degenerate primers.

3.3.3. Genome screening for transposable elements

Degenerate primers were designed for the conserved regions of the *mariner* open reading frame (Robertson 1993) and *piggyBac* (this study). The PCR is expected to yield 0.5kb and 1.224kb products for *mariner* and *piggyBac* related elements, respectively. The honeybee and *T. ni* genomic DNAs were used as positive controls for *mariner* and *piggyBac* PCR reactions, respectively. Using the PTM genomic DNA as a template yielded 2 PCR products with the *mariner* degenerate primers, of 0.5 and 0.6Kb, compared to a single product of 0.5kb for the positive control (Fig.3.5). Using the *piggyBac* degenerate primers resulted in no products with the PTM genomic DNA as a template. The positive control template, *T. ni* genomic DNA, yielded a PCR product at 1.2Kb as expected.

The PCR product generated using the *mariner* primers was cloned into TOPO T/A cloning vector and the DNA sequence determined. The DNA sequence (Fig. 3.6) matched with the *Epinotia vertumnana* clone 19.1 *mariner* transposase gene (Robertson and MacLeod, 1993) with a BLASTn prediction value of 8e-41 (Acc. # U04458).

3.4. Discussion

The mobility of transposable elements within the insect soma is typically determined by two assays, excision and interplasmid transposition assays. The excision assay demonstrates the ability of the transposable element to excise from a donor plasmid. The transposition assay determines the ability of a transposable element to transpose into a recipient plasmid. Using these assays, the mobility of transposable elements within certain insect species can be determined. We performed transposition assays to compare the mobility of three transposable elements belonging to three different families; *Hermes* from *M. domestica*, *Mos*1 from *D. mauritiana*, and *piggyBac* from *T. ni*, belonging to the *hat*, *mariner* and TTAA-specific families, respectively. The transposition assays for *Hermes* and *Mos*1 did not result in the recovery of any transposition events (Table 3.1 and 3.2). However, further analysis is required to confirm that these two transposons

lack mobility within the embryonic soma of PTM. Only two out of five experiments for *piggyBac*, resulted in the recovery of transposition products (Table 3.2). The transposition assay is highly variable among different experiments within the same species and the results differ according to several parameters including the injection conditions and egg batches. In the present study, we used twice the concentration of injected DNA compared to which is typically used by other researchers in different species. Transposition assays have been used demonstrate the mobility of *Hermes* in several different insect species, including the lepidopteran *Helicoverpa armigera* (Pinkerton et al. 1996). Similar successful transposition assays were performed for the *mariner* element in non-drosophilid insects (Coates et al. 1997).

Using a degenerate PCR assay, Robertson (1993) was able to detect *mariner* elements in 10 insect species related to six orders. It is believed that the overwhelming abundance of *mariner* among different insect orders is due to horizontal transfer events (Hartl, 2001). Our data show the presence of a *mariner*-like element in the PTM genome. Therefore, the use of the *Mos1* element in PTM germline transformation experiment may be unwise.

The *piggyBac* element has variable mobility rates in different insect species. Lobo et al. (1999) compared the mobility of *piggyBac* in embryos from different insect families using a transposition assay. They found that the rate of transposition in dipteran species is higher then occurs in *T. ni*, which harbors the *piggyBac* transposon. In *Drosophila*, use of the hsp70 helper results in 3-4 times more efficient transposition than the constitutive promoter *α 1-tublin*. In other dipteran species, *piggyBac* is capable of precise transposition in *Ae. albopictus, Ae. triseriatus* (Lobo et al. 2001) and *An. gambiae* (Grossman et al. 2000). Transposition assays in the lepidopteran, *P. gossypiella*, also demonstrated precise transposition of the *piggyBac* element (Thibault et al. 1999). In the present study, *piggyBac* mobility was determined to occur with a frequency of 0.0042%, comparable with that in *P. gossypiella* (0.006%). The significance of this result is that *piggyBac*, rather than *Hermes* or *Mos*1, represents the best choice for germline transformation experiments in the PTM.

We were able to increase the transpositional frequency of the *piggyBac* element in the embryonic soma of the PTM, by deploying a “transactivator” plasmid in the assay. The *ie1* product is a "multifunctional protein" that stimulates the activity of genes associated with *hr* enhancers and transregulates the expression of transfected genes (Rodems and Friesen 1995).

The experiment used a wide range of ratios of helper : transactivator plasmids, to determine the optimal concentration of transactivator to be included to achieve maximal transposition. Ratios ranging from 1:1 to 1:0.005 of helper : transactivator were used, surprisingly, the middle ranges resulted in the best results. The highest ratio (1:1) yielded the lowest transposition events result among experiments using transactivator. This result is more likely due to the reduction of the amount of the helper used in the experiment. Since the total concentration of plasmid DNA injected into embryos is always the same (2µg/µl) across all experiments, the transactivator was added at the expense of the helper plasmid only. Therefore, the amount of the helper plasmid used in this treatment was half that used in the original (helper only) experiment.. However, the transposition frequency is 1.7 times higher than the experiment which lacks the transactivator. Ten times more transposition efficiency was achieved using a 1: 0.01 ratio of helper : tansactivator.

A strong promoter with germline specific activity would help achieve a higher rate of transformation. A combination of a strong germline-specific promoter with transactivator may increase the frequency of integration of transposable elements into insect genome and hence the number of transformants. However, there is likely to be an optimal level of transposase within the injected embryos to result in an ideal activity of the transposable element. More experiments are required to determine the optimal combinations of promoter, transactivator, DNA concentrations and the resultant expression levels for each species. Mechanisms of *mariner* regulation have been studied by introducing *Mos*1 elements into *D. melanogaster*. It was suggested that *mariner* behavior within host species is cotrolled by a variety of parameters such as overproduction inhibition, missense mutation effects, competitive inhibition, dominant

negative complementation and transposase titration (Hartl et al. 1997a &b). The 1:0.1 ratio assay yielded 4.3 times the number of transpositions as the helper only experiments but 2.4 times lower than the 1:0.01 experiment. The amount of injected helper may not play a major role in this instance however, the reduction in the transpositional activity may due to due an overproduction inhibition effect. Further investigations of the regulation of *piggyBac* activity and a more complete understanding of its behavior within the host will likely improve the utility of this element as a transformation tool. Preliminary examination of the PTM genome for the presence of *piggyBac* or related elements has not revealed any such elements in the insect genome. In addition to degenerate PCR experiments, negative results were also obtained using low stringency hybridization (data not shown) of the *T. ni piggyBac*-based probe sequence with PTM genomic DNA. These experiments suggest the lack of a close relative of the *T. ni-piggyBac* element within the PTM genome. Furthermore, even the existence of related *piggyBac* elements in some insect species has not prevented successful germline transformation. Handler and McCombs (2000) discovered closely related *piggyBac* elements in the Oriental fruit fly genome and were still able to transform the fly with the *piggyBac* transposon, stable transgenic lines being maintained for 15 generations.

Of the twenty-nine potential target sites (TTAA) for *piggyBac* insertion on the pGDV1 target plasmid, eight of these are in the chloramphenicol resistant gene and thus transposition events into these 8 sites will not be recovered, due to selection on Cam/Kan. The *piggyBa*c element reveals an insertion preference for some sites. The high number of transposition insertions revealed from these experiments (a total of 269 insertions) will allow for further investigation of *piggyBac* insertion preference, based on the primary sequence and/or DNA conformation. The DNA sequence analysis of putative transposition products revealed the duplication of TTAA insertion sites for those clones sequenced in both directions and the TTAA site for those sequenced in only one direction. Only the 0.2 ratio experiment was subject for DNA sequencing in both directions. Of the 21 available TTAA sites on the pGDV1 target plasmid, *piggyBac* integration was recovered from 13 sites. Thibault et al. (1999) defined the role of

insertion multiplicity a “Multiple *piggyBac* insertions at a site are considered unique if they were recovered from separate pools of injected animals or from separate electroporation experiments”. Therefore, the maximum multiple insertion in the present experiment, three replicates, would be six (three for each orientation with respect of Cam resistance gene) regardless how many times the site was “hit” by the *piggyBac* element in each replicate. The multiple insertions were recorded as follows; 5 in one site (85), 4 in one site (992), 3 in three sites (101, 945 and 968), 2 in four sites (363, 491, 977, and 2447) and 1 in four sites (245, 453, 603, and 2500). In *P*. *gossypiella*, *piggyBac* uses nine sites for insertion (Thibaul et al. 1999). *PiggyBac* transposition in the PTM and pink bollworm experiments, combined together, occurred in only 15 sites. This suggests that the configuration of the plasmid DNA may facilitate the accessibility of *piggyBac* transposase to these sites. Previous studies have shown that the *piggyBac* insertion preference does not extend beyond TTAA site sequence (Elick et al. 1996). Out of 60 insertions, only one has a single insertion of the right arm into position 85 on the pGDV1 and the left arm is still linked with the donor sequence. This may be due to imprecise excision of the left arm following synapse formation. Similar phenomena have been demonstrated previously for *piggyBac* in other insect species. In *An*. *gambiae* cells and embryos, 44% of the transpositions recovered resulted in partial deletion of the target sequence (Grossman et al. 2000). It was proposed that this was due to either multiple insertions of the transposon into the target plasmid, followed by recombination between inserted elements, or perhaps an overabundance of the transposase that resulted in nicking and multiple excisions.

Using an interplasmid transposition assay, we have shown that the *piggyBac* element is the element of choice for transformation experiments, rather than *Hermes* or *Mos1*. The transpositional activity was shown to be highly increased due to activation by the IE1 transactivator protein. Thus, the transformation experiment may be supplemented with the pIE1/153 plasmid expressing IE1, the transactivator protein, to increase the transformation frequency within the PTM embryos.

CHAPTER IV
GENETIC TRANSFORMATION EXPERIMENT

4.1. Introduction

The use of transposable elements to integrate foreign DNA into insect genomes has been successfully performed in a wide range of insect species, including disease vectors, agricultural pests and beneficial insects. With the discovery of transposable elements in addition to the *P* element, the number of non-drosophilid insects that have been successfully transformed has been increased. The *piggyBac* element has been successfully used to transform several insect species, belonging to three insect orders (Lepidoptera, Diptera and Coleoptera). *PiggyBac* was isolated from a *Trichoplosia ni* cell line during a baculoviral infection of the cells. It was observed that *piggyBac* causes a phenotypic plaque mutation upon insertion into the viral genome. *PiggyBac* is a class II transposable element, these elements inserting themselves into host genomes through a DNA-only intermediate. *PiggyBac* is 2476 bp in length, with 13 bp perfect inverted terminal repeats and encodes a single open reading frame. The hallmark of a *piggyBac* target site is the tetranucleotide, TTAA, that is duplicated upon insertion. Precise integration into the host genome has been demonstrated in almost all insect species that have been transformed with the *piggyBac* transposon; *Ae*. *aegypti* (Kokoza et al. 2001; Lobo et al. 2002), *An*. *albimanus* (Perera et al. 2002), *An*. *gambiae* (Grossman et al. 2001), *An*. *stephensi* (Catteruccia et al. 2000; Nolan et al. 2002), *C*. *capitata* (Handler et al. 1998), *D*. *melanogaster* (Handler and Harrell 1999; Li et al. 2001), *B*. *dorsalis* (Handler and McCombs, 2000), *M*. *domestica* (Hediger et al. 2001), *Anastrepha suspense* (Handler and Harrell, 2001), *L*. *cuprina* (Heinrich et al. 2002), *B*. *mori* (Tamura et al. 2000; Uhlířová et al. 2002; Thomas et al. 2002), *P*. *gossypiella* (Peloquin et al. 2000), and *T*. *castaneum* (Berghammer et al. 1999; Lorenzen et al. 2002). Handler (2002) summarized the germline-transformation of insect species using the *piggyBac* transposon along with the marker genes used and the regulatory promoters. Mobility of

the *piggyBac* element has been demonstrated in embryos of the PTM using the transposition assays described in the previous chapter. Use of the transactivator plasmid in coinjection with the IE1-pigORF helper plasmid increased the transposition frequency. *PiggyBac* constructs were used in attempts to transform the germline of the PTM. The data from the experimental trials to transform this insect pest are presented in this chapter.

4.2. Materials and methods

4.2.1. Plasmids

The helper plasmid used for all transformation experiments was pIE1-pigORF (as described in chapter III). Two constructs were used as donor plasmids in different experiments; pK[BIGα] (Grossman et al. 2000) was used for experiments I through V, and pB[pUB-nls-EGFP] was used for experiments VI and VII. The pIE1/153 transactivator plasmid was used for experiments II, IV, V and VII.

4.2.2. Plasmid purification

All injected plasmids were purified as described in chapter II, section 2.2.2.

4.2.3. Insect rearing

The rearing conditions were the same as described in chapter II, section 2.2.3.

4.2.4. Genetic transformation experiments

Seven transformation experiments were performed during this study. Experiments I & III were performed under the same promoter conditions (helper and donor plasmids

only). Preblastoderm embryos were injected with pIE1-pigORF and pK[BIGα] at a concentration of 0.5μg/μl for each plasmid. For experiments II & IV, the preblastoderm embryos were also injected with a 1:0.02 ratio of helper : transactivator plasmids. The concentrations of the injected plasmids were 0.5, 0.49 and 0.0098μg/μl for pIE1-pigORF, pK[BIGα] and pIE1/153, respectively. For experiment V, the preblastoderm embryos were injected with concentrations of 0.5, 0.46 and 0.046μg/μl for pK[BIGα], pIE1-pigORF, and pIE1/153, respectively, (1:0.1 ratio of transactivator : helper). The preblastoderm embryos for experiments VI &VII were injected with a total DNA concentration of 2μg/μl and with a different donor plasmid, pB[pUB-nls-EGFP]. For experiment VI, the helper pIE1-pigORF and the donor pB[pUB-nls-EGFP] were injected at 1μg/μl each. For experiment VII, a 1:0.02 ratio of the helper : transactivator plasmids was used. The final concentrations used were 1, 0.99 and 0.0198μg/μl for pB[pUB-nls-EGFP], pIE1-pigORF, and pIE1/153, respectively. The plasmids and their concentrations for the transformation experiments are summarized in Table 4.1.

Table 4.1: Summary of the plasmids used in the transformation experiments.

Experiment No.	Donor plasmid	Helper plasmid (pIE1-pigORF)	Transactivator plasmid (pIE1/153)
I	pK[BIGα] 0.5μg/μl	0.5μg/μl	----
II	pK[BIGα] 0.5μg/μl	0.49μg/μl	0.0098μg/μl
III	pK[BIGα] 0.5μg/μl	0.5μg/μl	----
IV	pK[BIGα] 0.5μg/μl	0.49μg/μl	0.0098μg/μl
V	pK[BIGα] 0.5μg/μl	0.46μg/μl	0.046μg/μl
VI	pB[pUB-nls-EGFP] 1μg/μl	1μg/μl	----
VII	pB[pUB-nls-EGFP] 1μg/μl	0.99μg/μl	0.0198μg/μl

The injection conditions were the same as for Chapter II, section 2.2.4, with the exception that the embryos were injected without the prior addition of halocarbon oil. The injected embryos were kept in sealed Petri-dishes, provided with a small piece of wet paper towel as a source of humidity and were allowed to hatch in the Petri-dishes. The hatched larvae were picked up using a wet camel hair brush and transferred onto sliced tubers. One hundred neonate larvae were reared on one sliced tuber, kept individually in a single rearing cup. Individual pupae were collected from the tubers and kept in singular vials. Adults were sexed as they emerged. G_0 adult males were mated individually with two virgin wild-type females to form individual families. For experiment VI only, the families were formed from one male and three virgin females. G_0 adult females were mated in groups of 5, with 3 virgin wild-type males to form pools. All families and pools were reared in plastic containers supplied with 50% honey solution and covered with a small piece of mesh cloth and a 4.5-cm circular filter paper to provide an oviposition site. The filters carrying eggs were collected every two days and kept individually in 5-cm Petri-dishes. Eggs were counted and hatched G_1 larvae were screened for EGFP fluorescence using a UV Stereo-microscope. The non-hatched eggs were counted to estimate the number of screened larvae. The screening was performed on neonate larvae, except for experiment VI for which pupae were screened. For experiment VI, the filters carrying G_1 eggs were added to sliced tubers. Each family and each pool was added to a single sliced tuber. The pupae were collected, counted for each family and pool, and screened for EGFP expression.

4.3. Results

The genetic transformation experiment proceeded with initial trials to achieve optimal survival of neonate larvae that were able to feed and develop throughout the complete insect life cycle.

4.3.1. Pre-experimental trials to achieve optimal larval survival following embryo injection

Seven experiments were performed during this study to develop a germline transformation system for PTM. Before conducting these experiments, modifications of the injection conditions were trialed to attain a high proportion of larvae that hatch from the injected embryos. A total of 2955 PTM embryos were injected that either failed to hatch, or died within the oil. Prior to injection, the embryos are covered with oil to balance needle penetration forces and embryo internal pressure, to minimizing the extrusion of yolk through the injection site. Halocarbon oil series 700 is used for mosquito embryo injections. However, for other insect species, the halocarbon oil is toxic to the injected embryos as reported for the honeybee, *Apis melifera*, (Charles et al. 1988) for which paraffin oil can be safely used. For *Anopheles gambiae*, embryo microinjection survival was improved using an aqueous solution of 25 mM NaCl to cover the embryos during injection (Grossman et al. 2001). Initially, PTM preblastoderm injections were performed within the first four hours of development, under the halocarbon oil series 700, with the embryos being allowed to develop in a oxygen-saturated chamber. Non-fully developed embryos were observed inside the egg shells that failed to hatch and the few hatched larvae were unable to survive. To reduce the viscosity of the oil, a 1: 1 ratio of halocarbon oil series 70: 700 was used to cover the PTM embryos during injection. This mixture failed to improve survival, the few-hatched larvae being hindered by oil droplets attached to the larvae cuticle. To minimize oil contact with neonate larvae, the slides carrying the injected embryos were kept in

vertical positions to remove of as much of the oil as possible. However, high frequencies of mortality due to failed hatching were still observed. The results from experiments in which the PTM embryos were injected indicate that the main factor inhibiting larval survival is the oil itself.

To avoid the usage of oil, the PTM embryos were injected under air with no covering solution. Injection under air forced some modifications to the rearing conditions for the embryos. The oxygen-saturated chamber was not used. To enable rearing in 100% RH, the embryos were kept in Petri-dishes along with wet pieces of paper towel, sealed with Parafilm for the first 48 hours to avoid any desiccation of the eggs. At the third day post-injection, the embryos were moved to the normal 60-70% RH rearing conditions, thus reducing fungal growth within the Petri-dishes.

4.3.2. Genetic transformation experiments

Following the results of the luciferase and interplasmid transposition experiments, the helper plasmid expressing the *piggyBac* transposase under the regulation of the *ie1* promoter and *hr5* enhancer element was used for all transformation experiments. Table 4.2 summarizes the results of the seven experiments and table 4.3 represents the total number of individuals used for this study.

4.3.2.1. Experiments I and III

Experiments I & III were performed with the same plasmid combination; the helper plasmid, pIE1-pigORF, along with the donor plasmid, pK[BIGα], at a concentration of 0.5μg/μl each were coinjected into PTM preblastoderm embryos. A total of 1700 embryos were injected for experiment I. Of these, 326 larvae hatched (19.1%) and 198 of these survived to adulthood (92.1%). Due to a low number of available wild type females, only 90 families were produced from the 118 G0 males, these being outcrossed individually with either two or one wild-type female. The 80 G_0 females were mated in

16 pools, each including 5 G0 females outcrossed to 3 wildtype males. Egg clusters were collected, counted and kept in small Petri-dishes separately for each family and pool. The adults of all families and pools laid a combiend 26,243 eggs, of which 84.9% hatched, representing the highest percentage for all experiments. A total of 22,302 G1 larvae were screened; 12,641 from injected male outcrosses and 9,661 from injected female outcrosses. None of the screened larvae showed any EGFP expression.

The number of injected embryos for experiment III was 2,028, of which 13.1% hatched. One hundred and four adults emerged from this experiment (84.5%). All injected males were outcrossed with either 2 or 3 wild-type females forming 57 families. Injected females were outcrossed in pools of 5 with 3 wild-type males forming 10 pools, except for pool number 10 which had 2 females. Of 9,701 laid eggs, 7,070 neonate larvae hatched and were screened for EGFP expression. EGFP expression was not detected in any larvae.

4.3.2.2. Experiments II and IV

For these experiments, a third plasmid, p153, was coinjected along with the helper plasmid pIE-pigORF and the donor plasmid pK[BIGα] into the preblasotderm embryos. The p153 plasmid provides the transactivator protein and was used with the helper plasmid at a ratio of 1:0.02. The total number of injected embryos was 2,120 and 2,046 for experiments II and IV, respectively. The hatching rate for both experiments was similar, 10.1% for experiment II and 11.9% for experiment IV. Although the number of hatched larvae in experiment IV was slightly higher than that of experiment II, experiment II had 15 more emerged adults than experiment IV. A total number of 76 families and 59 pools (pool number 59 had only 4 females) were formed for experiment II, resulting in a total of 17,891 eggs. The hatched larvae in experiment II were not counted but all neonate larvae were screened under the UV microscope for EGFP expression. The number of screened larvae for line II in Table 4.1 is estimated based on the average of the hatching percentage of the other six lines. Visual examination under

the UV microscope showed no EGFP expression in any of the individuals. The number of families and pools for experiment IV was 46 and 11, respectively. Pool number 11 contained only 2 injected females outcrossed with 3 wildtype males. Due to a transient lack of wildtype adults, some additional injected adults were sibling mated in equal numbers, forming 3 family/pool (F/P) groups. Each F/P contained 10 adults, except for F/P3 which had 2 females and 1 male. The numbers of eggs produced in experiment IV adults was the lowest among the seven experiments. From 6,613 eggs, 3,741 (56.5%) hatched and were screened as neonate larvae for EGFP expression. No EGFP expression was observed in the screened larvae.

4.3.2.3. Experiment V

The PTM embryos in experiment V were injected under essentially the same conditions as for experiments II and IV, except for a different transactivator/helper plasmid ratio was used, 1:0.1, in this instance. A total DNA concentration of 1.06µg/µl was injected. Of 2,029 microinjected embryos, 413 larvae hatched (20.3%)and 270 of them reached adulthood (87.6%), representing the highest number of larvae and adults for these experiments. 121 families and 25 pools were established, in addition to 3 F/P groups. F/P3 was formed with 2 females and 2 males. An unexpected low number of eggs was laid (21,170). Of these, 15,169 larvae hatched (71.6%). None of these larvae showed any EGFP expression.

4.3.2.4. Experiments VI & VII

The *D. melanogaster polyubiquitin* promoter expressing the *EGFP* gene in the donor plasmid, pB[pUB-nls-EGFP], was used for experiments VI & VII. The total DNA plasmid concentration injected into the embryos was 2µg/µl. In experiment VI, 1µg/µl each of donor and the helper plasmids was injected. The transctivator plasmid p153 was coinjected for experiment VII at a transactivator : helper plasmid ratio of 1:0.02. A total of 1,570 and 1,660 embryos were injected for experiments VI and VII, respectively.

Table 4.2: Summary of the transformation experiments.

	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V	Experiment VI	Experiment VII
Injected embryos (G0)	1700	2120	2028	2046	2029	1570	1660
Hatched larvae	326 (19.1%)	214 (10.1%)	267 (13.1%)	245 (11.9%)	413 (20.3%)	255 (16.2%)	375 (22.5%)
Pupae	215	150	123	131	308	102	228
G0 adults (males/females)	198 (60.7%) (118 /80)	135 (63.1%) (76/59)	104 (38.9%) (57/47)	121 (49.4%) (57/64)	270 (65.3%) (133/137)	86 (38.2%) (46/40)	185 (49.3%) (90/95)
Families/pools	90/16	76/12	57/10	46/11 (+3 F/P)	121/25 (+3 F/P)	46/8	90/19
G1 eggs	26,243	17,891	9,701	6,613	21,170	18,438	27,151
Screened individuals	22,302 (84.9%)	13,025*	7,070 (72.8%)	3,741 (56.5%)	15,169 (71.6%)	10,585 (pupae)	21,415 (78.8%)
EGFP expressing individuals	0	0	0	0	0	0	0

***** The screened larvae were not counted during the experiment and the number represented here is the average of the screened individuals for the other six experiments**.**

Table 4.3: Number of PTM individuals used throughout the transformation experiments.

	Experiments I thru VII
Injected embryos	13,153
Hatched larvae	2,095
G0 adults	1,099
Families/pools	526/101
G1 eggs	127,207
Screened individuals	93,307
Transgenics	0

225 larvae hatched (16.2%) in experiment VI and 86 (38.2%) of these survived to adultood. The injected males were used to form 46 families and the injected females were used to form 8 pools. 18,438 G_1 larvae were reared on sliced potato tubers until pupation. Of these, 10,585 pupae were collected and screened under the UV microscope for EGFP expression.

Experiment VII represents the highest hatching percentage among the experiments, 22.5%, and 185 emerged as adults (49.3%). The injected males were backcrossed with non-injected females to form 90 families. The injected females were collected in 19 pools and mated with non-injected males. The total number of G_1 eggs was 27,151, with 78.8% hatching. The total number of G_1 neonate larvae was 21,415, and showed no expression of EGFP.

4.4. Discussion

A total of seven transformation experiments were performed on the PTM, resulting in no transformants. Prior to these experiments, there were a number of trials to achieve a high proportion of injected individuals that hatched and developed into adulthood. The halocarbon oil which is typically used during embryo injection to neutralize the pressures of injection vs. internal egg fluid, has a negative effect on the hatching rate of PTM embryos and on the survival of neonate larvae. Injections of potato tuber moth embryos under air resulted in hatching percentages ranging from 10.1 to 22.5. The rate following injection is always variable across transformation experiments, even for the same species, due to a variety of parameters during injections. In lepidopterans for example, injected *B. mori* embryos hatched at rate of 65.6 and 32.5 % in 2 experiments (Tamura et al. 2000) and those of *P. gossypiella* hatched at rates of 36.5 and 14.9 % in 2 experiments (Peloquin et al. 2000). For *B. mori*, the injection hole is sealed with a small drop of cyanocrylate glue. The *P. operculella* embryos were not treated after injection with any sealing materials. Usage of cyanocrylate to seal the puncture hole following microinjection may enhance embryo survival and in turn, increase the number of hatched

larvae. Across the seven experiments, 1,099 injected embryos reached adulthood. These adults were backcrossed with wild-type individuals, except for a few cases in which wild-type adults were not available. In some cases, as in experiments IV & III, the fertility of the adults was extremely low. However, we cannot assume that the low fertility was due to the injections since fecundity is variable among the wild-type moths. The number of individuals screened for EGFP expression for some experiments is relatively low and insufficient in others. In these instances, it is difficult to expect the recovery of transgenic individuals, especially if the transformation frequency for this genome is relatively low.

PiggyBac mobility within the embryonic soma of *P. operculella* genome is relatively low as shown by the transposition assay results in the previous chapter. Furthermore, the positive results in this assay are not necessarily indicative of germline activity. Two lepidopteran species have been successfully transformed using the *piggyBac* vector. The stable germline transformation of the pink bollworm, *P. gossypiella*, (Peloquin et al. 2000) and the silkworm, *B. mori*, (Tamurai et al. 2000; Thomas et al. 2002) were achieved with the *piggyBac* transposon carrying EGFP. The unsuccessful results of these transformation experiments described here do not necessarily indicate that *piggyBac* is unable to transform the PTM.

Generally, there is a lack of basic knowledge about the biology of the PTM. Therefore, any conclusion about the current transformation failure in PTM would be based on assumptions from previous results in other species. Atkinson et al. (2001) proposed different factors that inhibit insect transformation, including the physical handling during microinjection, the volume of the injected DNA, interruption of the internal cellular organization, and species-specific factors that might inhibit the genomic transposition of the transposable element. Peloquin et al. (1997) used an electromechanical actutor to deliver DNA at high velocities into *P. gossypiella* embryos through a micropipet. Moreover, they injected the embryos using a computer-controlled system that minimized the handling of the embryos before and after injection. The use of this advanced microinjection system resulted in a five-fold increase in embryo survival (Peloquin et al. 1997). In this instance, the handling of the PTM embryos for

microinjection is relatively primitive compared with the previous example. The highest hatching percentage of PTM was 22%, compared to 36.5% in the pink bollworm. Moreover, only 86 fertile adults emerged in the pink bollworm transformation experiment and resulted in 3 transgenic animals, compared to 185 adults for PTM in experiment VII. However, the number of fertile adults in the transformation experiments was not calculated. The total concentration of injected DNA used for microinjection in different insect species ranges from 0.5 to 1µg/µl (Tamura et al. 2000; Lorenzen et al. 2002; Thomas et al. 2002; Uhlířová et al. 2002). Two different DNA concentrations were used in these transformation experiments, 1 and 2µg/µl. These were chosen according to those used in other germline transformation experiments and to mimic that used in the transposition assay. These results suggest that neither the handling of the embryos nor the DNA concentration is the reason behind the unsuccessful transformation of the PTM.

An internal activity factor could be the reason for the failure of genomic transposition in the PTM. Position-effect variegation has been detected in *Ae. aegypti* (Coates et al. 1998) and *D. melanogaster* (Henikoff, 1996). This phenomenon affects the expression of the transformation marker within transformants, producing variable expression levels among transformed individuals. The *EGFP* gene is less susceptible to position effect variegation than the eye color marker (Higgs and Lewis, 2000). Lack of an element(s) closely related to *piggyBac* in the PTM genome (previous chapter) reduces the possibility of destabilization of *piggyBac* integrations by an endogenous transposable element. The *piggyBac* transposon is automonous and is thought to require no host specific factors (Atkinson and James, 2002).

However, host factors may affect the ability of *piggyBac* to integrate into the PTM genome. These possibilities require further experiments to determine their existence and mechanisms of action. The injections were performed in 4-hour embryos. In the embryonic development of the corn earworm, *Heliothis zea*, the cleavage nuclei enter the periplasm froming the blastema between 4.5-5 hours following egg laying (Presser and Rutschk, 1957). The time at which the blastema is formed in PTM is unknown. Injection within the first 2 hours may increase the likelihood of *piggyBac* integration into PTM

genome. In addition, alternative parameters could be tried in the future for transformation experiments such as different DNA concentrations, transposons, promoters and marker genes.

Another possibility is that *hr5-ie1* enhancer promoter regulatory sequence is inactive germline cells. The *hr5-ie1* enhancer promoter has shown the hieghest transcriptional activity compared to other tested promoters (Chapter II) and also shows transcriptional activity during interplasmid transposition assay by expressing the transposase enzyme (ChapterIII). However, these experiments demonstrate the transcriptional activity of the promoter within the somatic cells rather than gernline cells. On the other hand, transformation experiments mainly depend upon the transcriptional activity of the regulatory sequences within the germline cells.

Germline transformation is a relatively new research field in the entomological sciences and only a limited number of species have been successfully transformed, only two of which are lepidopteran. More efforts and trials to genetically transform PTM are required, using different parameters to enhance the survival rates of injected embryos and using different components to increase the mobility of the transposable element within PTM.

CHAPTER V
ISOLATION OF DIFFERENTIALLY EXPRESSED cDNAs FROM THE POTATO TUBER MOTH MIDGUT

5.1. Introduction

Many of the most common and destructive agricultural pests belong to the family Lepidoptera. The larval stages chew plant tissues using their mandibles and pass them into the foregut and then into the midgut. The midgut cells are responsible for the production and continuous secretion of digestive enzymes into the gut lumen for digestion purposes. The larval midgut is characterized by a high K ion concentration and is highly alkaline. The midgut provides a rich environment for the identification of molecular targets involved in each of these processes; enzyme production and secretion, absorption and ion movement, that could be used to design new control strategies.

The processes of cellular growth, differentiation, tissue specificity and organogenesis in higher eukaryotes are controlled by differential gene expression. Identification of genes exclusively expressed in specific organs and/or tissues, or those that play a role in determining environmental responses, would allow a greater understanding of the biochemical and physiological bases of these processes at a molecular level. Identification and cloning of such differentially expressed genes has been a long term research focus. A variety of methods have been used as molecular approaches to identify and clone cDNAs from differentially expressed genes, including differential display (DD), representational difference analysis (RDA), and microarray. Differential display uses a combination of a limited number of short arbitrary primers with anchored oligo-dT primers to amplify most of the mRNA molecules. This method resulted in a significant number of false positives which makes it difficult to identify rare transcripts (Stein et al. 1997). Representational difference analysis of cDNA is a PCR-based subtractive hybridization techniques allow the selective amplification and cloning of transcripts that differ in abundance between two populations (Lisitsyn et al. 1993). This method requires

multiple subtraction rounds and fails to equalize the number of rare and highly abundant transcripts. Microarray techniques are powerful for identifying differentially regulated genes on a large scale, yet rare transcripts may not be detected (Dessens et al. 2000). The cDNA subtraction method was improved by Diatchenko et al. (1996). They deployed "suppression PCR" along with subtractive cDNA hybridization to selectively amplify target cDNAs and simultaneously suppress the non-target cDNAs, a process they termed Suppression Subtractive Hybridization (SSH). When Diatechnko et al. (1996) evaluated the subtraction efficiency of the SSH method, the target viral DNA fragments were enriched by 100-, 1000, and 5000-fold compared to samples containing 0.1%, 0.01% and 0.001% of viral DNA, respectively. Analysis of the expressed sequence tags (ESTs) from subtracted cDNA libraries of the hindgut and Malpighian tubules (HMT) of *Ctenocephalides felis* revealed significant enrichment of cDNAs encoding proteins involved with inorganic ion transport, organic ion transport, and vesicle-associated proteins, in the subtracted library compared with the unsubtracted library (Gaines et al., 2002). Kung et al. (1998) compared gene expression in estrogen receptor (ER)-positive and ER-negative breast cancer cell lines by differential screening and SSH. Differential screening failed to identify new genes whereas the SSH procedure resulted in the identification of 123 sequences, including three known genes and one novel gene.

We performed SSH to compare PTM midgut and carcass gene expression, to identify differentially expressed genes that could be deployed in future control strategies.

5.2. Materials and methods

5.2.1. RNA extraction

The PTM midgut was dissected in dissection buffer, 300mM Mannitol, 5mM EGTA and 17mM Tris-HCl pH 7.5, (Escriche *et al*. 1994). The midgut as well as the rest of the body (carcass) was immediately immersed in liquid nitrogen and stored at -80°C. Total RNA was extracted from both midgut and carcass homogenates, using the Triazol® reagent (Invitrogen). PolyA$^+$ RNA was subsequently purified from the total RNA using either the Micro-FastTrack 2.0 mRNA isolation kit (Invitrogen), or the polyATact® mRNA Isolation System III (Promega), and eluted in 50µl of RNase-free water, then stored at -80°C for subsequent use. Purification of poly A$^+$ RNA from the total RNA was based on using an oligo(dT) primer to hybridize, at high efficiency in solution, to the 3′ poly(A)+ region present in most mature eukaryotic mRNAs. Both total RNA and polyA$^+$ RNA were extracted as described in the manufacture's instructions provided with each kit.

5.2.2. Suppression subtractive hybridization (SSH)

The carcass cDNAs were subtracted from the midgut cDNAs according to the user manual for the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA). The SSH method is summarized in figure 5.1 as provided by the manufacture's manual. Both tissues were used to synthesize the cDNA populations as follow. The first strand cDNA was synthesized from 2µg polyA$^+$ RNA, using 20units of AMV reverse transcriptase, 10µM oligo dT primer and reaction buffer as supplied by the SSH Kit. The second strand cDNA was synthesized using 24units of DNA polymerase I, the RNA template was removed by RNaseH (1 unit), and the gaps were joined by 4.8units of *E. coli* DNA ligase. The two cDNA populations were digested with the *Rsa*I enzyme, generating blunt ends. The midgut cDNA fragments (tester) were split into two equal portions, each was ligated

with a different cDNA adaptor, I or II, using 400units of T4DNA ligase. No adaptors were ligated to the carcass (driver) cDNAs.

The highly expressed cDNAs were normalized to the low expressed cDNAs, and differentially expressed transcripts were ‘identified’ by two rounds of hybridization. In the first round, both adaptor-ligated cDNA (tester) pools were heat denatured at 98°C for 1.5 min, as was the driver cDNA pool. An excess of driver cDNAs were added to both adaptor-ligated cDNA pools, the two reactions being allowed to anneal at 68°C for 8 hours in the presence of a hybridization buffer. In the second hybridization round, the two samples were mixed together and fresh denatured driver cDNAs were added and allowed to anneal at 68°C overnight. Addition of denatured driver cDNAs in the second round reduces the presence of non-differentially expressed sequences as suitable templates for downstream amplification. Different cDNA hybrid molecules exist following these two rounds of hybridization, including differentially expressed sequences that have the two adaptor sequences on alternative ends. The differentially expressed molecules are targeted for amplification by two rounds of PCR. The first round of PCR is started with a pre-incubation step at 75°C for 5 min to fill in the strands that are missing the complementary sequences of the adaptors. This extension reaction creates a binding site for PCR primers. The molecules that have two different adaptors are exponentially amplified and molecules with the same adaptor are suppressed due to the formation of hairpin structures. The second PCR round is used to reduce the background and to amplify the targeted molecules using nested primers (adaptor/primer sequences and the reaction conditions are described in the manual).

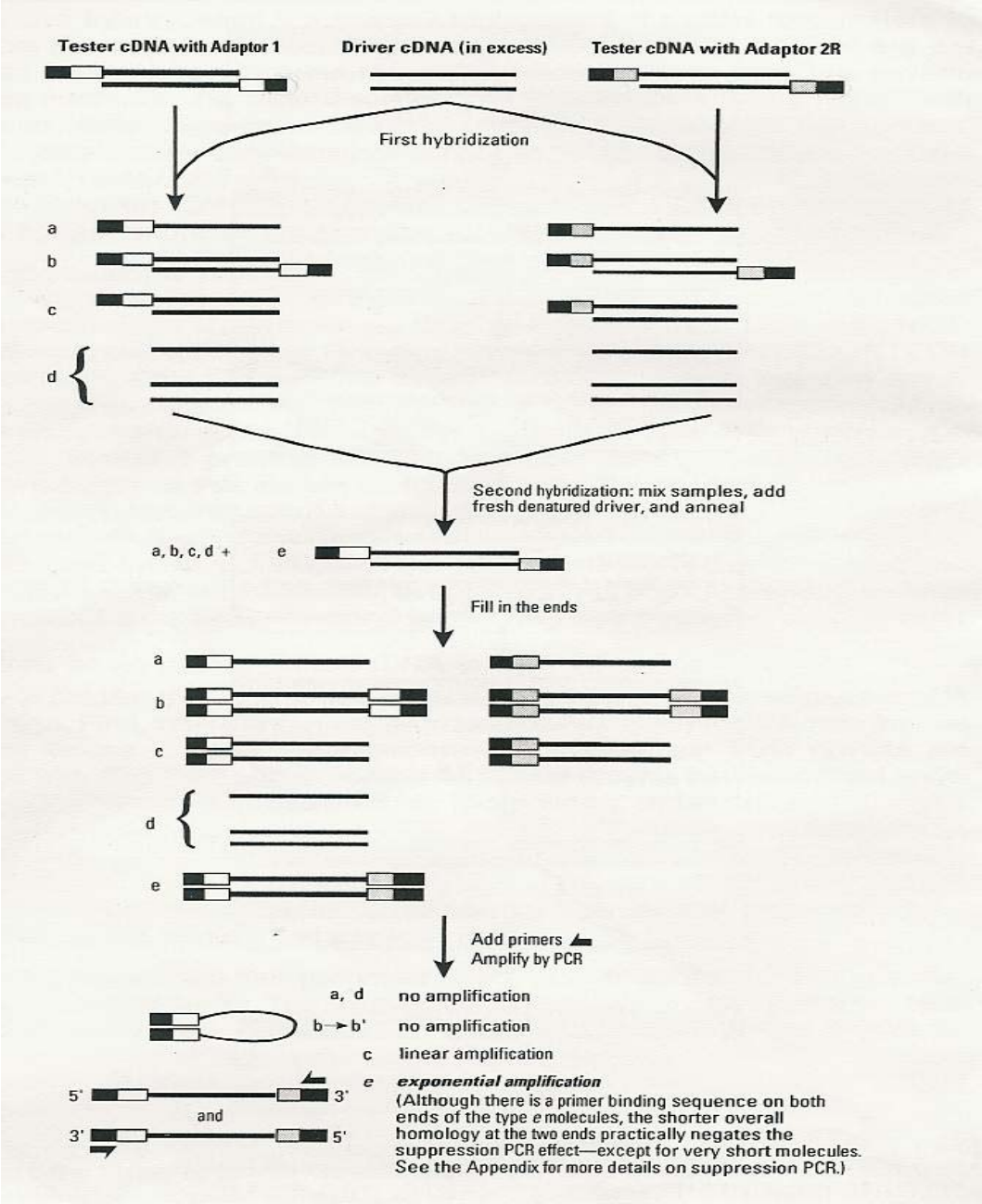


Fig 5.1: Scheme of the SSH method. Both tester and driver cDNAs are represented by solid lines. The two groups of tester cDNAs are ligated to different adaptors as represented by boxes on both ends of the cDNAs. The shaded boxes represent the outer parts of the adaptors. The first hybridization round is performed by adding heat denatured driver cDNAs to both denatured cDNA populations, annealling resulting in 4 types of cDNA molecules (a, b, c, and d). The second hybridization round is performed by mixing the two tester cDNA populations and additional heat denatured driver cDNA. This step results in a new type of cDNA molecule (e) which is a differentially expressed and normalized cDNA among the tester population. After filling in the ends of all molecule types, the first PCR reaction is used to amplify the differentially expressed cDNAs (molecule type e) and suppress the amplification of other products. The second PCR amplification using nested primer pairs is used to reduce the background and further enrich the differentially expressed cDNAs. (Source: Clontech PCR-Select cDNA Subtraction Kit User Manual).

5.2.3. Screening of the amplified molecules

The SSH-PCR products were cloning into the T/A cloning vector, pCR® 2.1-TOPO®, (Invitrogen). Ligation products were transferred into either DH5α-T1^R cells by chemical transformation, or into DH10B cells by electroporation. The cells were grown on LB-agar media under kanamycin selection and plated onto 222mm X222mm Q-trays followed by blue-white screening. The white colonies were picked and inoculated into 384-well plates using the Q-bot robotics system (Genetix, Genetix, New Milton, UK), according to the parameters described in the manual. The robot (fig. 5.2) inoculated 11 384-well plates, each slightly below full capacity. The cultures were allowed to grow overnight in these plates, each well containing 70µl of freezer media (90% of 2YT media, 8.5% of 5mg/ml Na citrate, 0.1mg/ml $MgSO_4$, 9mg/ml $(NH_4)_2SO_4$, 44ml Glycerol and, 1.5% of 1.8mg/ml KH_2PO_4 and 6.3mg/ml K2HPO_4) with 50µg/ml of kanamycin.

5.2.3.1 PCR screening

PCR screening was performed using the M13 forward and reverse primers that are specific to the vector sequence. The DNA inserts were amplified by 0.25units of Promega *Taq* polymerase using 25 cycles of 94°C for 15 sec, 50°C for 15 sec and 72°C for 2 min, followed by 5 min at 72°C and the reaction ended at 4°C.

Fig. 5.2: Q bot robotics system. The system is capable of picking up to 3500 colonies/hour and inoculates them into either 96 or 384 well plates. The system has a CCD camera through which it picks the colonies and also is capable of gridding the colonies from 96 or 384 well plates onto large (22.2 X 22.2 cm) nylon membrane sheets.

5.2.3.2. Colony hybridization

The colonies were transferred onto Nylon membranes using the Q-bot robotics system. Two plates were transferred onto each Nylon membrane, with 2 replicates for each colony on duplicate membranes. The eleventh plate (XI) was transferred onto duplicate membranes without a companion plate. The membranes were laid on kanamycin LB-agar media in Q-trays and the colonies were allowed to grow on the membranes overnight at 37°C. The membranes were processed in three solutions to extract the plasmid DNA from the bacterial cells as follows. The membrane was laid over 2 Whatman paper filters soaked in solution 1 (0.5M NaOH and 1.5M NaCl) for 4 min at room temperature. The membrane was then baked for 4 min at 85°C. The membrane was then laid over 2 Whatman paper filters soaked in solution 2 (1M Tris-HCl and 1.5M NaCl) for 4 min at room temp. Finally, the membrane was submerged in 30ml proteinase K solution 3 (50mM TrisHCl pH8.5, 50mM EDTA, 100mM NaCl, 1% N-Laurylsacrosine, 250μg/ml protease K) for 30min at 37°C. The extracted DNA was cross-linked to the membranes using a UV Stratalinker® 2400 (Stratagene). Duplicate membranes were screened with two probes, derived from the midgut and the carcass. The probes were prepared from ds cDNAs synthesized from the midgut and the carcass. The mRNA (500ηg) was used to prepare ds cDNA. The mRNA was mixed with 2pmoles of Oligo dT primers and heated to 70°C for 10min and immediately chilled on ice. The reaction was performed by adding 1X 1st strand buffer, 10mM DTT and 0.5mM dNTPs and incubated at 42°C for 2min and finally 20units of SuperScriptII (GibcoBRL) was added. The reaction was incubated at 42°C for 50 min before inactivation at 70°C for 15 min. The RNA template was removed from the 1st stand cDNA by 2units of *E. coli* RNaseH (Promega) for 20 min at 37°C. The second stand was synthesized by the action of 6units of DNA polymerase (Clontech) and 1.2units of DNA ligase in 1X buffer for 2hours at 16°C. Another 3units of DNA polymerase was added to the reaction, for 30min at the same temperature. The reaction was terminated by the addition of 4μl of 4x EDTA/Glycogen (Clontech). The ds cDNA was extracted with a 1 : 1 phenol : chloroform solution, followed by ethanol precipitation and re-suspension in ddH_2O before labeling. The probes were labeled by

incorporation of 50μCi of (α-^{32}P)-dATP (Aersham Pharmacia Biotech, Piscataway, NJ, USA) by random priming labeling using the Prime-a-gene® labeling system (Promega). The random priming was performed according to the manufacture's instructions. Each probe was hybridized to one copy of each duplicate membrane overnight at 65°C in 6x standard saline citrate (SSC), 0.5% SDS, 5x Denhardt's solution and 100μg/ml salmon sperm DNA. After hybridization, each membrane was sequentially washed for 2x 30 min periods in 2x SSC/0.5% SDS at room temperature, followed by 2x 30 min periods in 0.2x SSC/0.5% SDS at 65°C. The membranes were exposed to X-ray films (Kodak) for 24-48 hours and then developed using a Konica medical film processor, Model QX-130A plus (Konica corporation, Tokyo, Japan).

5.2.4. DNA Sequencing

Plasmid DNA clones had their DNA sequence determined in both directions using M13-forward and M13-reverse primers and the ABI Prism BigDye™ Terminator Cycle sequencing ready reaction kit. The sequencing gel was run and analysis performed by the Gene Technology Lab (GTL), Texas A&M University. DNA sequences were compared to the GenBank database using BLASTX-nr and BLASTN-est algorithms (National Center for Biotechnology Information) and BLASTN-3'UTR (Bioinformatics and Genomic Group-C.N.R) (Altschul et al. 1997).

5.2.5. Molecular analysis

Twelve SSH clones representing five gene groupings were subjected to northern blot and RT-PCR analysis to confirm midgut-specific expression. Clones representing digestive enzymes are II-M12, VII-A17, V-B17, and V-A15, showing similarity with trypsin, chymotrypsin, aminopeptidase and carboxypeptidase enzymes, respectively. The second group, containing immune-related sequences, were represented by IX-P2, X-M12, IX-K21, IX-C8 and VI-L3, which matched defensin, attacin, hyphancin, gloverin and lipase1 genes, respectively. The third group, midgut-related sequences, was represented

by VI-A2, a putative microvilli membrane protein. The fourth group, miscellaneous sequences, was represented by IX-A24 (Sensory appendage protein). The fifth group was represented by II-B23 (hypothetical protein).

5.2.5.1. Northern blot analysis

Total RNA was extracted from both midgut and carcass homogenates using the Triazol® reagent as described previously. Six to ten micrograms of total RNA from the carcass was run in adjacent lanes with a similar amount of midgut total RNA on 1% denaturing formaldehyde agarose gels. The gel was soaked in solution 1 (0.5M NaOH and 1.5M NaCl) shaking for 30 min at room temperature, followed by solution 2 (1M Tris-HCl and 1.5M NaCl) for 20 min at room temp. The RNA was transferred overnight onto a Nylon membrane using a pressure-Blotter (Stratagene) at room temperature. The RNA was cross-linked to the membranes using a UV crosslinker (Stratagene).

For each clone to be analyzed, the insert was removed from the vector by digestion with 0.5 units of *EcoR*I for 2 hrs at 37°C, electrophoresed on a 0.7% agarose gel and then gel extracted (as described in chapter 2 section 2.2.1.2). The DNA fragment representing each clone was randomly labeled and hybridized to the membrane as described in chapter V section 5.2.3.

5.2.5.2. RT-PCR analysis

First strand cDNA, prepared from mRNA from the midgut and carcass, was used as a template for RT-PCR analysis. The mRNA (500ng), from both tissues was mixed with 2pmoles of the Oligo dT primers and heated to 70°C for 10min and immediately chilled on ice. The reaction was performed by adding 1X 1st strand buffer, 10mM DTT and 0.5mM dNTP and warmed at 42C for 2min and finally 20units of SuperScript II (GibcoBRL) was added. The reaction was incubated at 42°C for 50 min before inactivation at 70°C for 15 min. The RNA template was removed from the 1st stand

cDNA by 2units of *E. coli* RNaseH (Promega) for 20 min at 37°C. The 1st strand cDNA was mixed with 0.25units Promega Taq polymerase in 1x reaction buffer, 0.5M dNTPs, 25mM MgCl2 and 25pmoles primers. The amplification conditions were 94°C for 2 min followed by 40 cycles of 94°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec followed by 4 min at 72°C. The RT-PCR amplification was performed for each clone using its corresponding primer set as shown in figure 5.3.

Ptmdef-IXP2 (5'); 5'-CCGTGGTGTCTTCTGCTCCA-3'
Ptmdef-IXP2 (3'); 5'-GCGCAGAACGTGTGGTTCCA-3'

Ptmatt-XM 12 (5'); 5'-ACTACTGTTGGTGCTAGTGTCAACG-3'
Ptmatt-XM 12 (3'); 5'-GTGCGGCGTTGTCATTCTAC-3'

Ptmglov-XIC 8 (5'); 5'-GAGTCTCCGTAGGGCCCCAAAA-3'
Ptmglov-XIC 8 (3'); 5'-ACTTTTTGGCAAGGGCGGCT-3'

Ptmhyf-IXK 21 (5'); 5'-TTGAGGCTTGTCCAACTACTTG-3'
Ptmhyf-IXK 21 (3'); 5'-CAGCCAATCCACGATGGAAC-3'

Ptmtryp-IIM 12 (5'); 5'-ACCGATTTCTGCAGGTGTTCAA-3'
Ptmtryp-IIM 12 (3'); 5'-CCCCAATTTAACAGGAACAACT-3'

Ptmchem-VIIIA 17 (5'); 5'-CGTGTCTGATCTGCGCGTCTGT-3'
Ptmchem-VIIIA 17 (3'); 5'-GCAAGTCGTTATGCATTCTGGC-3'

Fig. 5.3.: cDNA specific primers.

Ptmcarb-VA 15 (5’); 5'-ACAGAATCATACTTCCGTAACT-3’
Ptmcarb-VA 15 (3’); 5'-CTTTACCTTCACTCCTAATGGT-3’

Ptmamin-VB 17 (5’); 5'-AAGACCCCAGTTTTCCATAGCACC-3’
Ptmamin-VB 17 (3’); 5'-CCTGATCGCCATTCTTGTAGGA-3’

Ptmmirco-VIA 2 (5’): 5'-ACCAACTTCTCAGCATTCATAA-3’
Ptmmirco-VIA 2 (3’); 5'-GATGTCTTATCCAATTAATCCG-3’

Ptmlip-VIL 3 (5’); 5'-GAAGACCGTCGCGAAGAATGCC-3’
Ptmlip-VIL 3 (3’); 5'-CCGGTCAGAAGGGTTACTCCAA-3’

Ptmshc-IIB 23 (5’); 5'-GAATTCATCCTCGGCCAGTTCG-3’
Ptmshc-IIB 23 (3’); 5'-CACCATGAAAGCGTCAGGGAGC-3’

Ptmsens-IXA 24 (5’); 5'-GTCCTTGTGGCTGCTCG-3’
Ptmsens-IXA 24 (3’); 5'-GGCAAGACTCGTTTGAG-3’

Fig. 5.3. continued.

5.3. Results

5.3.1. RNA extraction from the larval midgut

The midguts of fourth larval instars were dissected and separated from the rest of the larval body (carcass). The malpigian tubules were detached from the midgut as much as possible to avoid contamination of the midgut RNA. The amount of total RNA from both the midgut and carcass was determined by spectrophotometrie analysis, calculating the optical density (O.D.) at 260 and 280nm, as well as by visualization on 1% denaturing formaldehyde agarose gels using ethedium bromide staining and UV transillumination to ensure that the quality and quantity of the extracted RNA from both tissues was suitable (Fig. 5.4). The amount of purified polyA$^+$ RNA, using either purification method, was approximately 1.25% of the total RNA input. Both total RNA and polyA$^+$ RNA were kept at –80°C and visualized on a denaturing agarose gel before further use to avoid using degraded RNA in downstream procedures.

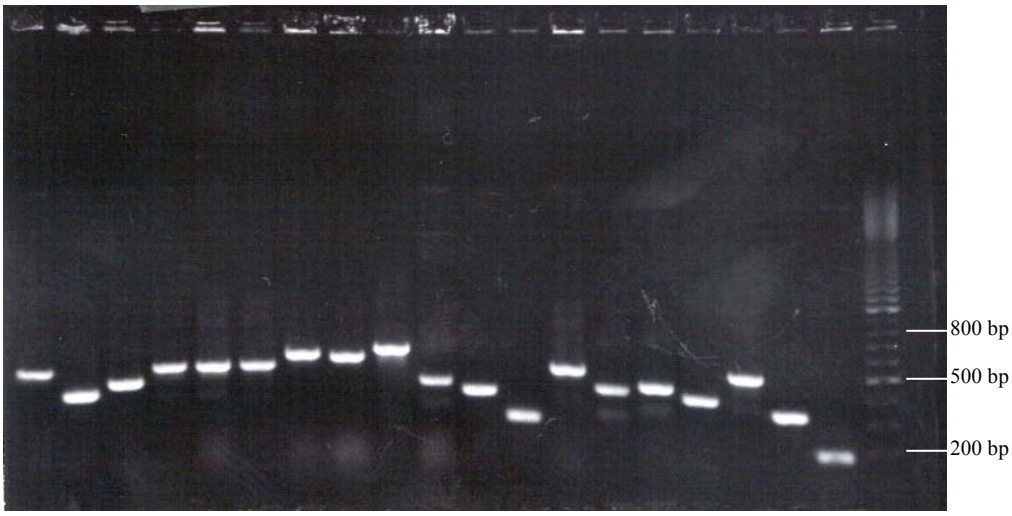
5.3.2. Suppression subtractive hybridization (SSH)

Two micrograms of polyA$^+$ RNA from both tissues was used for the SSH procedures. The SSH-PCR products ranged in size from 0.2 to 0.9kb and were cloned into the TOPO T/A cloning vector. The insert size was determined by PCR screening using vector-specific primers (M13). Inserts of 0.15 to 0.7kb are represented in figure 5.5.

Eleven 384-well plates were inoculated with white colonies from the Q-trays using the Q-bot robotics system. The plates were not inoculated to full capacity 2,984 clones being collected and screened in two replicates with midgut and carcass-derived probes. On the basis of differential hybridization signals, 637 clones are candidates for differentially expressed genes within the PTM midgut. These putative clones hybridize only with probes derived from midgut ds cDNAs and not with probes prepared from carcass ds cDNA (Fig 5.6).

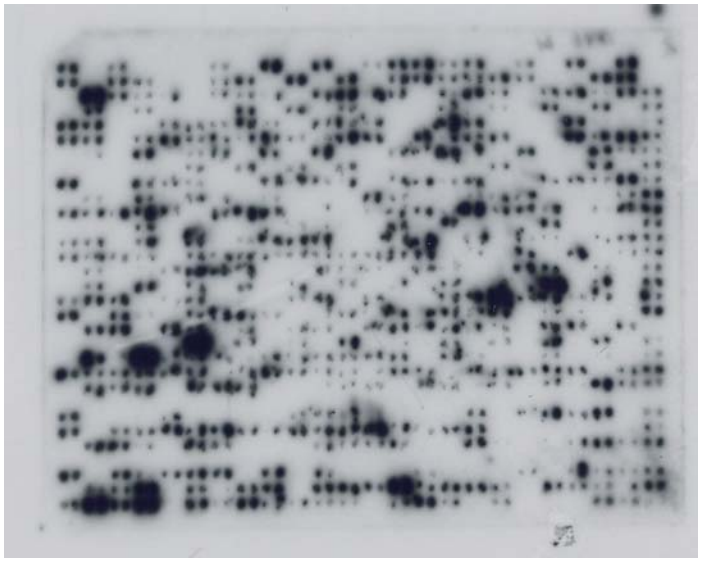
M C

Fig 5.4: Total RNA from the PTM midgut and carcass. The total RNA was electrophoresed on a 1% denaturing formaldehyde agarose gel. M: total RNA from PTM midgut; C: total RNA from PTM carcass

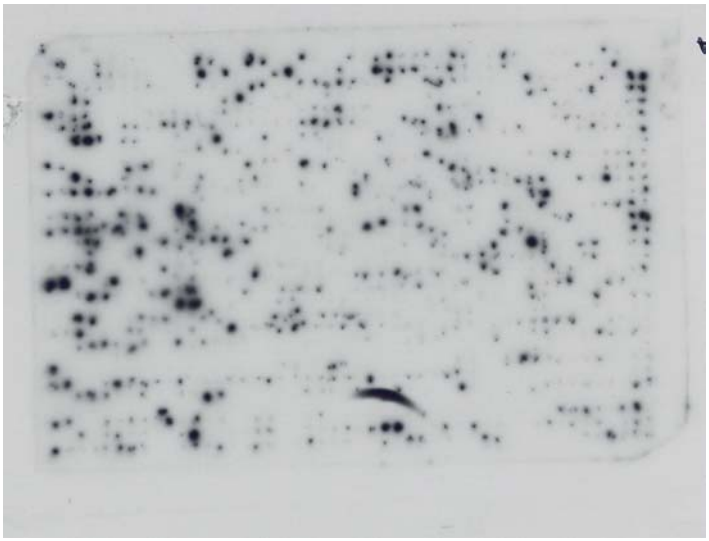


1 19 M

Fig 5.5: PCR screening of SSH cDNA inserts within the TOPO vector. The SSH-PCR products were cloned into the TOPO T/A cloning vector as described. The white colonies were screened by PCR using vector-specific primers (M13 forward and reverse). M is the 100bp DNA marker. Lanes 1 through 19 are the PCR amplification bands representing the insert sizes of the cDNA clones, ranging from 150bp to 700 bp.



A



B

Fig 5.6: Autoradiograph of colony hybridization from differential screening of SSH clones. The white colonies were picked up from 384-well plates and transferred onto Nylon membranes using the Q-bot robotics system. Two plates were transferred onto one Nylon membrane, with 2 replicates for each colony on each duplicate membrane. The colonies were allowed to grow overnight by laying the membranes on a kanamycin 2YT agar plate. The plasmid DNA was extracted from the colonies and fixed onto the membranes by a UV-crosslinker. Each of the two replicate membranes were allowed in hybridize to a specific radioactive probe under the same conditions. The membranes were exposed to x-ray film for 24-48 hours.

A) The probe used in the hybridization was prepared from the midgut cDNA.

B) The probe used in the hybridization was prepared from the carcass cDNA.

5.3.3. DNA sequencing

Sixty-nine clones (11%) were randomly selected for DNA sequencing. The sequence results were analyzed for homology to the GeneBank database, based on the conceptual translation of the partial nucleotide sequence to amino acid in the query. Clones that have no similarity within the database accessed by BLASTX-nr, were further matched against the BLASTN-est EST database. Sequences that appear to contain a polyA tail, were matched against the BLASTN-3'UTR database at C.N.R. The clones were grouped according to sequence data analysis (Table 5.1). The putative digestive enzymes are represented by 34.8% of the total number of sequenced clones. Molecules that are thought to play a role in insect immunity, unexpectedly, constitute a similar percentage to the digestive enzymes. Other molecules that may be involved in midgut activity, referred to as midgut-related sequences, are present at 4.3% and other miscellaneous sequences are present at 8.7 %. Sequences that have significant similarity with hypothetical proteins, 3' untranslated (3'UTR) regions, or non-significant similarity to database entries, represent 17.4% of the total sequenced clones.

Group I, putative digestive enzymes, comprises 24 cDNA clones as follow; 14, 4, 4 and 2 cDNA clones showing significant homology to trypsin or trypsin-like proteases, chymotrypsin, aminopeptidases and carboxypeptidases, respectively.

Group II, immune related peptides, contains 24 cDNA clones. The highest proportion of this group revealed homology with defensin (14 cDNA clones). Four cDNA clones showed homolgy with attacin, and one cDNA clone with each of hyphancin, gloverin, and lipase-1. Two cDNA clones were identified as matching bacterial-induced genes from *M. sexta*.

Three cDNA clones were referred to as group III, midgut-related sequences and have homology with allergin, microvilli, and vacuolar V-ATPase proteins.

Table 5.1: Database search results of SSH-cDNA clones from the PTM midgut.

Clone	Length (bp)	Gene product	Data base	Organism	e-value	Reference
Group 1:						
II-D12	632	Trypsin	BLASTX-nr	*C. fumiferana*	5e-11	Wang et al. (1995)
III-P18	683	Trypsin	BLASTX-nr	*C. fumiferana*	5e-33	Wang et al. (1995)
IV-B5	628	Trypsin	BLASTX-nr	*C. fumiferana*	9e-25	Wang et al. (1995)
VI-A16	431	Trypsin	BLASTX-nr	*C. fumiferana*	2e-41	Wang et al. (1995)
III-F21	402	Trypsin	BLASTX-nr	*C. fumiferana*	7e-27	Wang et al. (1995)
IX-F2	190	Trypsin	BLASTX-nr	*C. fumiferana*	2e-06	Wang et al. (1995)
V-P1	157	Trypsin-like protein	BLASTX-nr	*G. mellonella*	3e-07	Li et al. (2002)
III-C6	300	Trypsin-like protein	BLASTX-nr	*G. mellonella*	2e-17	Li et al. (2002)
II-D7	257	Trypsin, alkaline A	BLASTX-nr	*M. sexta*	4e-04	Peterson et al. (1994)
I-H11	642	Trypsin, alkaline B	BLASTX-nr	*M. sexta*	3e-13	Peterson et al. (1994)
II-M12	366	Trypsin-like protease	BLASTX-nr	*H. armigera*	3e-32	Bown et al. (1997)
V-P1	387	Trypsin-like protease	BLASTX-nr	*H. armigera*	4e-11	Bown et al. (1997)
V-B12	409	Trypsin precursor	BLASTX-nr	*L. oleracea*	1e-26	Unpublished
VII-E4	360	Trypsin	BLASTN-est	*M. digitata*	8e-99	Unpublished
II-C12	141	Chymotrypsinogen	BLASTX-nr	*A. ipslon*	7e-13	Leighton and B. (2001b)
VIII-A17	382	Chymotrypsinogen	BLASTX-nr	*A. ipslon*	6e-15	Leighton and B. (2001b)
VI-F9	386	Chymotrypsinogen	BLASTX-nr	*A. ipslon*	1e-19	Leighton and B. (2001b)
V-E6	248	Chymotrypsin-like	BLASTX-nr	*H. armigra*	3e-09	Bown et al. (1997)
III-M1	230	Aminopeptidase N3	BLASTX-nr	*B. mori*	3e-09	Unpublished
V-B17	531	Aminopeptidase	BLASTX-nr	*E. postvittana*	2e-37	Simpson et al. (2000)
V-O7	300	Amniopeptidase N	BLASTX-nr	*H. armigra*	1e-32	Unpublished
V-O3	425	Aminopeptidase N	BLASTX-nr	*P. xylostella*	2e-34	Chang et al. (1999)
V-A15	405	Carboxypeptidase A	BLASTX-nr	*H. armigra*	1e-24	Bown et al. (1997)
VII-K1	347	Carboxypeptidase A	BLASTX-nr	*D. heteroneura*	8e-09	Unpublished
Group 2:						
VI-L3	384	defensin	BLASTX-nr	*D. melanogaster*	7e-13	Unpublished
III-A7	382	defensin	BLASTX-nr	*D. melanogaster*	3e-13	Unpublished
I-I8	385	defensin	BLASTX-nr	*D. melanogaster*	4e-13	Unpublished
III-I7	386	defensin	BLASTX-nr	*D. melanogaster*	5e-13	Unpublished
V-A3	384	defensin	BLASTX-nr	*D. melanogaster*	3e-12	Unpublished
X-H5	386	defensin	BLASTX-nr	*D. melanogaster*	7e-13	Unpublished
IX-P2	384	defensin	BLASTX-nr	*D. melanogaster*	3e-13	Unpublished
II-O21	375	defensin	BLASTX-nr	*D. melanogaster*	2e-09	Unpublished
I-E12	394	defensin	BLASTX-nr	*D. melanogaster*	2e-13	Unpublished
V-E17	385	defensin	BLASTX-nr	*D. melanogaster*	9e-14	Unpublished
V-N11	383	defensin	BLASTX-nr	*D. melanogaster*	2e-13	Unpublished
IV-C8	380	defensin	BLASTX-nr	*D. melanogaster*	4e-13	Unpublished
III-B24	382	defensin	BLASTX-nr	*D. melanogaster*	5e-13	Unpublished
II-A22	221	defensin	BLASTN-est	*M. digitata*	2e-99	Unpublished
VI-H2	169	defensin	BLASTN-est	*M. digitata*	3e-58	Unpublished
III-D23	383	Attacin	BLASTX-nr	*S. cynthia ricini*	4e-16	Kishimoto et al. (2002)
VI-H24	332	Attacin	BLASTX-nr	*S. cynthia ricini*	5e-11	Kishimoto et al. (2002)
X-M12	269	Attacin	BLASTX-nr	*S. cynthia ricini*	8e-12	Kishimoto et al. (2002)
III-D17	388	Basic attacin	BLASTX-nr	*H. cecropia*	2e-16	Sun et al. (1991)
IX-K21	268	Hyphancin IIIG	BLASTX-nr	*H. cunea*	1e-10	Unpublished
XI-C8	352	Gloverin	BLASTX-nr	*H. cecropia*	2e-17	Axen, et al. (1997)
VI-L3	697	Lipase-1	BLASTX-nr	*B. mori*	9e-05	Unpublished
I-L22	532	Bacteria-induced gene	BLASTN-est	*M. sexta*	4e-47	Unpublished
IX-C4	108	Bacteria-induced gene	BLASTN-est	*M. sexta*	7e-98	Unpublished

Table 5.1 continued

Clone	Length (bp)	Gene product	Data base	Organism	e-value	Reference
Group 3:						
VI-A2	550	microvilli protein	BLASTX-nr	*A. aegypti*	3e-05	Unpublished
VI-B18	483	Major allergen	BLASTX-nr	*B. germanica*	7e-04	Pomes et al. (1998)
III-B7	580	Vacuolar ATPase	BLASTN-3'UTR	*M. sexta*	3e-25	Unpublished
Group 4:						
IX-A24	320	Sensory append. prot.	BLASTX-nr	*A. gambiae*	2e-18	Biessmann et al. (2002)
IV-C16	405	Put. transcript. factor	BLASTX-nr	*P. Americana*	6e-16	Marie et al. (2000)
X-B5	288	40S ribosomal protein	BLASTX-nr	*S. frugiperda*	1e-05	Unpublished
VIII-E5	425	Biphenyl dioxygenase	BLASTX-nr	*Rhodococcus sp.*	6e-08	Masai et al. (1995)
VII-D6	596	Lazarillo precursor	BLASTX-nr	*S. americana*	6e-04	Ganfornina et al. (1995)
VII-B22	326	Biliverdin bind-protein	BLASTX-nr	*S. cynthia ricini*	4e-08	Unpublished
Group 5:						
VI-C7	371	Gene product	BLASTX-nr	*A. gambiae*	3e-05	Unpublished
II-B23	213	Gene product	BLASTX-nr	*A. gambiae*	2e-10	Unpublished
II-F2	553	Gene product	BLASTX-nr	*D. melanogaster*	7e-27	Unpublished
Group 6:						
IIE24	309	Midgut cDNA library	BLASTN-est	*H. armigera*	9e-04	Unpublished
X-O4	278	Unknown polyA tail	BLASTN-est	*B. mori*	1e-70	Unpublished
IV-K22	459	3'UTR	All data bases			
V-D17	653	3'UTR	All data bases			
VI-E3	390	Unknown	All data bases			
II-N19	588	Unknown	All data bases			
VI-E17	384	Unknown	All data bases			
VI-N8	326	Unknown	All data bases			
I-D6	185	Unknown	All data bases			

SSH-cDNA clones were used as queries against the GenBank databases; BLASTX-nr, BLASTN-EST and 3'UTR.

Six cDNA clones that revealed homology to either housekeeping genes or semmingly non-related to the midgut were collected in group IV. Biliverdin-binding protein, lazarillo protein, biphenyl dioxygenase, sensory appendage protein, putative transcription factor and 40S ribosomal protein are represented by single cDNA clones.

Hypothetical proteins were represented by 3 cDNA clones in group V. Nine cDNA clones were gathered in group VI and were referred to as unknown sequences. Two of these clones matched, using the EST database, with clones from a *H. armigera* midgut

cDNA library and an unknown polyA tail transcript from *B. mori*. Seven cDNA clones, showed no homology with any sequences from the three databases, two of them having apparent polyA tail.

Some of the cDNA clones that matched with trypsin/trypsin-like proteases apparently represent different genes. These clones show differences in their nucleotide sequences and in turn, the deduced amino acid sequences. Alignment of the deduced amino acid sequences of these clones with the lepidopteran *Choristoneura fumiferana* trypsin (accession number: U12917) is shown in figure 5.7. The clones shown in the figure match to different parts of the *C. fumiferana* trypsin and overlap each other in some cases. The cDNA clones belong to the remainder of digestive enzyme group; chymotrypsin, aminopeptidase and carboxypeptidase, are shown in appendix B.

The number of cDNA clones that have homology with defensin was unexpected. However, while they include the termination codon and the polyadenlation signal (Fig. 5.8), they lack both the initiation codon and polyA tail. The 14 clones have high homology among them and presumably reveal the extent of polymorphism for this gene among the colony individuals. The average length of the defensin molecules is 383bp.

Clones X M12, III D23, VI H24 and III D17 have 100% similarity with each other, suggesting that they represent a single transcript. An assembly of the 4 clone sequences results in a sequence length of 433bp. The deduced amino acid sequence shows homology to the attacin peptide from the lepidopteran, *Samia cynthia ricini*, (accession number: AB059394) and reveal that these cDNA clones together represent the 3'half of the transcript (120aa), with the presence of a stop codon at position 361 of the nucleotide sequence (Fig. 5.9).

The deduced amino acid sequence of IX-K21 (268bp) and XI-C8 (352bp) clones revealed similarity with the lepidopteran antibacterial peptides, hyphancin and gloverin, respectively, in the BLASTX-nr database. Furthermore, the antiviral peptide, from *B. mori* (accession number: AB076385), showed homology with the IV-L3 cDNA clone. Two more cDNA clones related to the PTM immune system were identified from the EST database. Clones I-L22 and IX-C4 match with a bacterial-induced gene from *M.*

sexta. The nature of these genes, however, is identified by neither the genbank database nor publication entries.

5.3.4. Northern blot

Total RNA sample from both the midgut and carcass of the fourth larval instars were electrophoresed side-by-side (Fig. 5.10) transferred to membranes, and allowed to hybridize with radio-labeled probes prepared from the insert sequences (SSH product). Each probe represents one of the selected sequences. Northern blot analysis demonstrates that a majority of these cDNA clones detect a mRNA transcript from the PTM midgut, not from the carcass. Northern blot results are shown in figure 5.11. Of the 12 examined clones, 9 have shown strong hybridization signals only with the midgut total RNA. A strong signal with total RNA from both tissues, midgut and carcass, was obtained using the defensin cDNA clone. The hyphancin clone showed a stronger signal with midgut RNA than that of carcass. The cDNA encoding the putative sensory appendage protein resulted in a negative signal for both RNA samples. Clones of the trypsin, defensin, gloverin, lipase-1 and hypothetical protein have resulted in two band signals, revealing the probability of the existence of more than one gene responsible for each of these clones, or alternative transcript forms in the PTM.

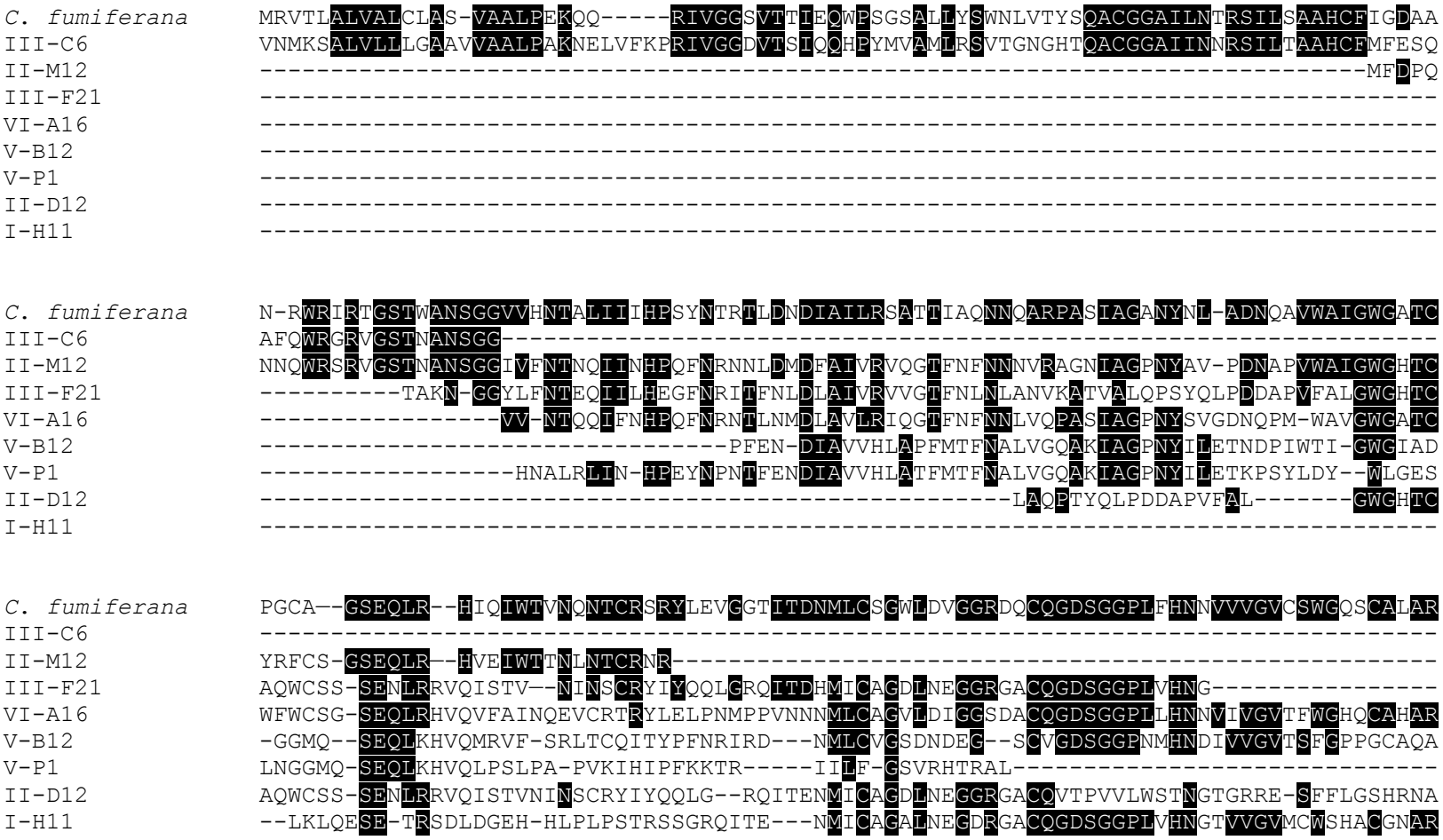


Fig. 5.7. Amino acid alignment of trypsin cDNA clones. Deduced amino acid of eight cDNA clones from PTM midgut with trypsin from *C. fumiferana* (Acc. # U12917). The shaded areas represent the identical amino acids between the clones and the trypsin.

```
C. fumiferana      YPG-VNARVSRFTAWIQANA-
III-C6             ---------------------
II-M12             ---------------------
III-F21            ---------------------
VI-A16             YPG-VNARVFIASNWIGSNV-
V-B12              GKPSVTTRVSSYVAWIEDNAF
V-P1               ---------------------
II-D12             GQPPRNTPWENKAASAIP---
I-H11              YPG-VSARIPAVSNWIAANT-
```

Fig. 5.7. continue: Amino acid alignment.

```
                 *                                                  *
CTGGCTATGGCTGCCGTGGTGTCTTCTGCTCCACCAAAATATGAAGAACCCATCGAAGTTCTCCCTTCTGAATACGAGCTTAGCGACGTAAATGTCGT
  L  A  M  A  A  V  V  S  S  A  P  P  K  Y  E  E  P  I  E  V  L  P  S  E  Y  E  L  S  D  V  N  V
   *                                                                 *                          *
AGAAGACCTCCAGAACTTTCTAGAAGAAGCTTTGACTGTGGAAGAAGCTGAGGCTCGTCAGGCTCGAGTGACATGCGACCTGCTGTCGGGTATCGGCTG
V  E  D  L  Q  N  F  L  E  E  A  L  T  V  E  E  A  E  A  R  Q  A  R  V  T  C  D  L  L  S  G  I  G
                    *                                                                          **
GAACCACACGTTCTGCGCCGCGCATTGCATCTTCAAGGGTTACAAGGGAGGTGCTTGCAACAGCAAGGGAGTTTGTGTGTGCCGCAGATAAGCTGGAGC
W  N  H  T  F  C  A  A  H  C  I  F  K  G  Y  K  G  G  A  C  N  S  K  G  V  C  V  C  R  R
  * *  * *     * *    **       *    **    *   *       *      *                 **
AGCTCTCGACTATATGTCTTTAGAAATAATGTTATTTTATTGTTAAGACTTTTGTATTGGGGAAAATGAAAATAAAGTGAGAATTTGT
```

Fig. 5.8. PTM defensin sequence and its deduced amino acid. The termination codon is shown in black box and the polyA signal is underlined.
*: indicates the amin o acids which show polymorphism among cDNA sequences.

```
CTGCAGCATATACCGAAGATGGGCAACCAAGCGTCAGCAGCAGCCGGCATCGGCGTGGGCAGCGAGACGCATCGGGTTGGCGTTGATGCCTTCGCGACGCGT
```

Fig. 5.9. Partial PTM attacin sequence and its deduced amino acid. The sequence is an assembly of four cDNA clones (X-M12, VI-H24, III-D23 and III-D17). The termination codon is shown in black box

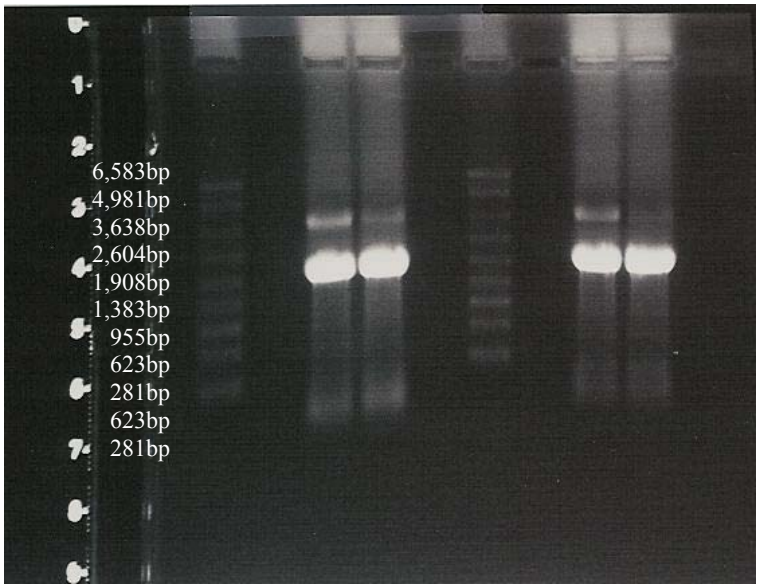


Fig. 5.10: Total RNA from midgut (M) and carcass (C). The total RNA was electerophoresed on a 1% denaturing formaldehyde agarose gel. RL is the RNA marker (281, 623, 955, 1,383, 1,908, 2,604, 3,638, 4,981 and 6,583 bases)

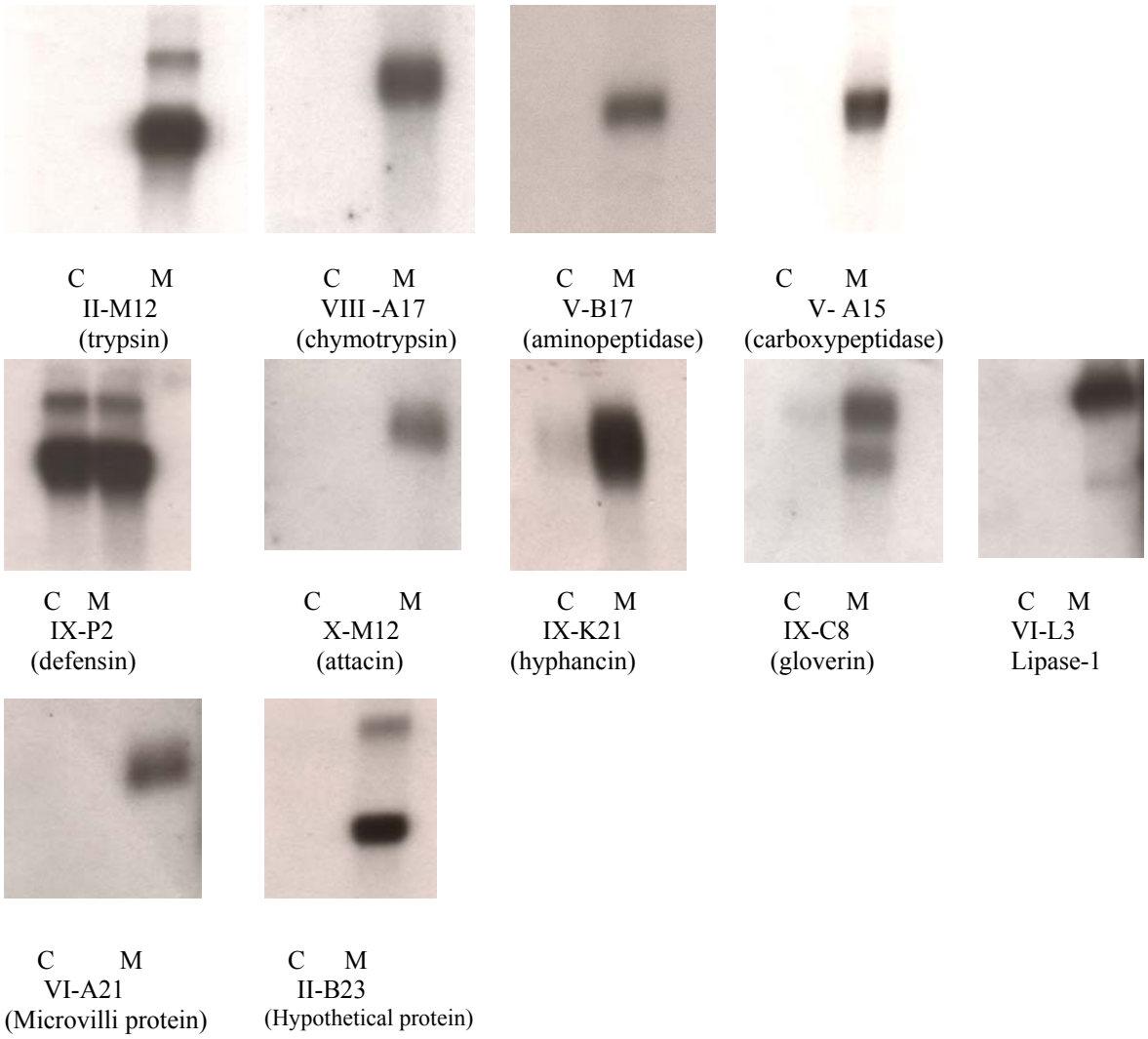
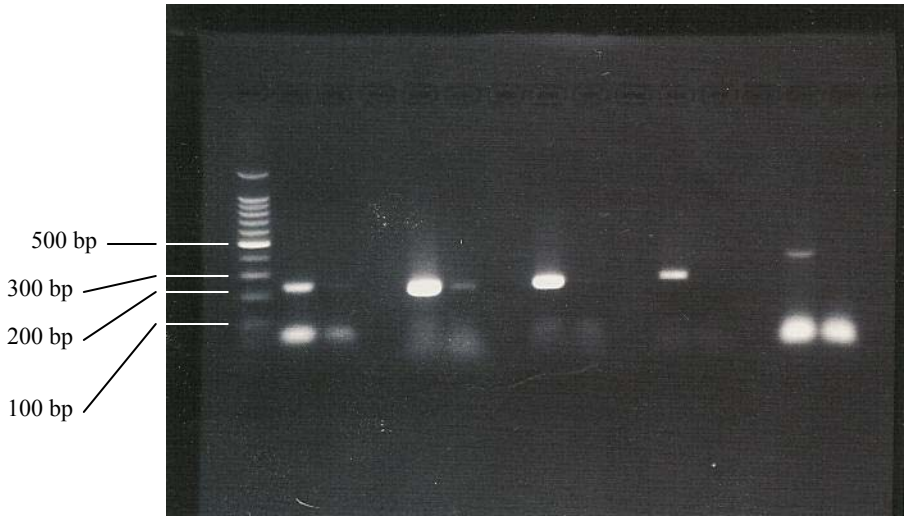
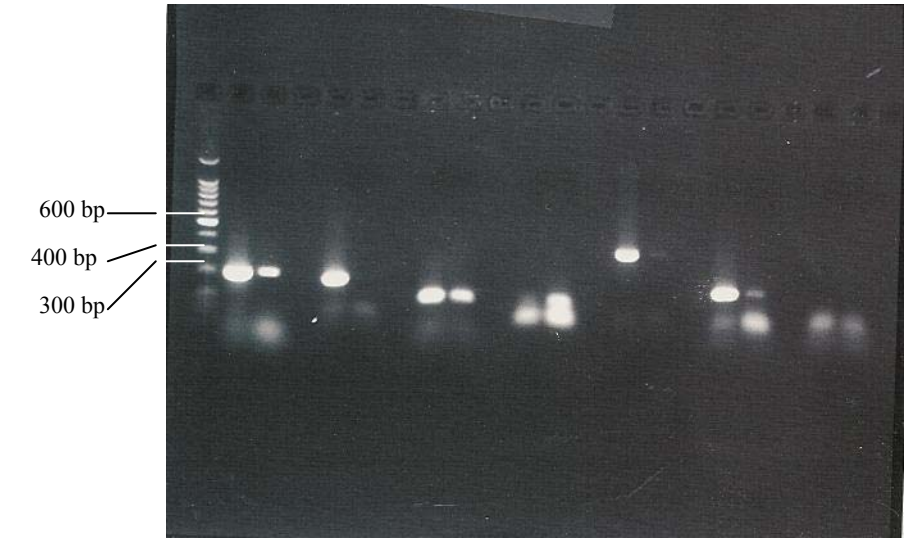


Fig 5.11: Northern blot hybridizations of SSH cDNA clones using midgut-derived probe vs carass-derived probe. Six to eight micrograms of PTM larval total RNA from both carcass (C) and midgut (M) were electrophoresed on a 1% formaldehyde agarose gel and then transferred onto nylon membranes. The hybridizations were performed with probes designed to the SSH-cDNA clones.



(A)



(B)

Fig 5.12: RT-PCR analysis of twelve SSH cDNA clones. The PCR was performed using 1st strand cDNA, prepared from mRNA from midgut and carcass, as template and sequence-specific primers for each clone. DL is the 100bp DNA marker. M is the 1st strand cDNA prepared from the midgut. C is the 1st cDNA prepared from the carcass. For each reaction, the sequence-specific primers are designated on the corresponding lanes. The expected PCR band sizes are as follows; trypsin (240bp), chymotrypsin (210bp), aminopeptidase (240bp), Carboxypeptidase (270bp), lipase-1 (440bp), defensin (200bp), attacin (180bp), hyphancin (110bp), gloverin (100bp), microvilli protein (350bp), hypothetical protein (160bp), and sensory appendage protein (170bp).

5.3.5. RT-PCR analysis

PCR analysis (Fig. 5.12) has shown essentially the same results as the Northern blot experiment further supporting that these cDNAs show increased expression in the larval midgut of the PTM. The sensory appendage protein clone (IX-A24, 170bp) was also negative in the Northern blot analysis. In addition to defensin (IX-P2, 200bp), clones of hyphancin (IX-K21, 110bp), chymotrypsin (VIII-A17, 210bp) and hypothetical protein (II-B23, 160bp), also show positive results with both templates. In contrast to the northern blot results, chymotrypsin and hypothetical protein also showed a PCR amplification product for RNA from the carcass. cDNA clones showing positive results only with midgut templates using RT-PCR are trypsin (II-M12, 240bp), aminopeptidase (V-B17, 240bp), carboxypeptidase (V-A15, 270bp), lipase-1 (VI-L3, 440pb), attacin (X-M12, 180bp), gloverin (XI-C8, 100bp) and microvillli protein (VI-A2, 350bp).

5.4. Discussion

The SSH technique was used to generate a PTM midgut-enriched cDNA library. SSH eliminates most of the housekeeping transcripts by subtracting the cDNA population of the control tissue from the target tissue. In addition, the low abundant transcripts in the target tissue are normalized with the high abundant transcripts, increasing the probability of their identification. From a total of 2,984 subtracted library clones, 637 clones are candidates for being differentially expressed genes within the PTM midgut. These putative clones hybridize only with probes derived from midgut ds cDNAs and not with probes prepared from carcass ds cDNA. The proportion of positive clones (21%) may indicate that the common transcripts between the driver and tester cDNA populations are not completely excluded during the two hybridization steps. Therefore, the SSH method may enrich for the differentially expressed sequences compared to the housekeeping genes, without eliminating the later completely from the library.

The positive clones were sorted into three groups according to their hybridization

signals; strong, moderate and weak signal groups. The weak signal group represents only 5% of the total screened clones and is most likely to comprise differentially expressed transcripts of low abundance. However, clones matching with trypsin genes in the GenBank database, also show different hybridization signal intensities. Stein et al. (1997) estimated the exposure time needed to detect high abundant transcripts as being 4-12 hours, whereas more than 4 days may be required as an exposure time for the detection of low abundant transcripts. In this study the exposure time ranged from 24- 48 hours, which may not be adequate to detect many of the low abundant transcripts present in the library.

Unsurprisingly, transcripts representing digestive enzymes constitute the highest portion of the randomly selected clones. Digestive enzymes genes are highly expressed in the insect midgut cells and their products are secreted into the lumen. The predominant protease enzyme, trypsin, is represented by redundant clones in the library, indicating that the normalization of the SSH procedure is not perfect. However, the deduced amino acid sequences of several trypsin clones have high similarity but are not identical, revealing that they likely belong to different genes. Other digestive enzymes such as chymotrypsin, carboxypeptidase and aminopeptidase are less represented in the subtracted library compared to trypsin. Trypsin and other serine proteinases such as chymotrypsin and elastases are hydrolytic endopeptidases that are used by lepidopteran insects for the digestion of plant tissues. Because of their importance in gut physiology, the genes encoding serine proteinases are currently being researched for their potential use in developing new control strategies. Serine proteinases from the midguts of several lepidopteran species have been isolated and characterized; *H. zea* and *A. ipsilon* (Leighton et al. 2001 a&b; Bown et al. 1997), *C. occidentalis* (Valaitis et al. 1999), *C. fumiferana* (Wang et al. 1995), *M. sexta* (Peterson et al. 1994), *G. mollnella* (Li et al. 2002), *P. interpunctella* (Zhu et al. 1997).

Northern blot analysis demonstrated that the cDNAs encoding these enzymes were exclusive expressed in the midgut. However, RT-PCR analysis demonstrated the expression of chymotrypsin in the carcass as well as the midgut. In similar results,

chymotrypsinogen-like RNA is also present at low levels in the non-midgut tissue of *P. interpunctella* (Zhu et al. 1997). Conversely, the trypsin-like gene is expressed in the midgut and is not detected in the non-digestive tissues of *C. fumiferana* (Wang et al. 1995). Moreover, Peterson et al. (1995) also investigated the expression pattern of trypsin and chymotrypsin in the *M. sexta* midgut. Chymotrypsin mRNA was expressed in the anterior and middle portions, whereas trypsin-like mRNA was expressed at higher levels in the middle and posterior portions of the midgut.

The presence of redundant clones related to defensin was unexpected. Moreover, other cDNA clones with similarity to additional antibacterial peptides such as attacin, hyphancin and gloverin were also present. Identification of antibacterial peptides has not been previously reported in naïve lepidopteran midguts. Kishimoto et al. (2002) isolated the antibacterial attacin from immunized larval hemolymph of the eri-silk worm, *Samia cynthis ricini*. The attacin transcript was not detected in the naïve fat body, whereas in the bacteria-injected larvae, it was strongly expressed in the fat body, hemocyte and Malpighian tubules, however little expression was detected in the midgut. Similarly, bacterial infected *T. ni* larvae have been shown to strongly expresse gloverin in the fat body and hemocytes, and not in the gut, whereas in the naïve larvae, gloverin expression was not detected in any tissue (Lundström et al. 2002). However, immune-related peptides have been reported in the gut of blood-sucking insects such as the mosquitoes; *An. gambiae* (Vizioli et al. 2001) and *Ae. aegypti* (Lowengerger et al. 1999), and the soft tick, *Ornithodoros moubata*, (Nakajima et al. 2001; Nakajima et al. 2002). Two cDNA clones matched with lepidopteran bacterial-induced genes in the EST database. These genes are not clearly identified or characterized. They may represent either a new category of immune peptides, or an existing type with low sequence similarity to known genes. RT-PCR analysis of cDNAs encoding the immune peptides found within the PTM midgut has shown differential expression levels among these clones. Defensin and hyphancin are expressed in both the midgut and carcass, whereas gloverin and attacin are only detected in the midgut.

The cDNA clones encoding proteins that play a putative role in midgut physiology

were grouped as midgut-related cDNAs. The nature of the putative microvillar protein is not clearly known, however it may be a membrane-bound protein involved in either digestion or absorption. The cDNA related to the plasma membrane V-ATPase was identified using the 3' UTR database. The vacuolar proton pumps, V-ATPases, are ubiquitous among eukaryotes (Dow 1999). In the midgut of lepidopteran larvae, the V-ATPase in the apical cell membranes of the goblet cells plays a role in amino acid absorption, by energizing the plasma membrane through pumping H^+ ions/proton into the goblet lumen. The cDNAs encoding the V-ATPase subunits of *M. sexta* were identified and characterized (Wieczorek et al. 1999; Merzendorfer et al. 2000). Allergens are soluble low molecular weight proteins, or glycoproteins, that cross-link with IgE antibodies after diffusion through mucosal surfaces. Many allergens are produced by cockroaches and cause asthma. Pomés et al. (1998) cloned three allergen cDNA clones from the hindgut of *Blattela germanica* and *Periplaneta* a*mericana* and speculate that these allergen molecules may have a digestive function.

The miscellaneous group includes cDNAs encoding proteins that may be involved in midgut physiology, other larval tissues, or they may be housekeeping genes. The SSH library does include a housekeeping gene encoding the 40S ribosomal protein. One of the cDNA clones matched the putative neural transcription factor from *P. americana* (Marie et al. 2000). This cDNA may encode a transcription factor, however there is no evidence that this factor is midgut specific. Lazarillo is a highly glycosylated small protein with internal disulfide bonds, linked to the extracellular surface of the plasma membrane by a GPI-tail and has significant similarity with members of the lipocalin family (Ganfornina et al. 1995). Lazarillo is expressed by subset of neurons in the nervous system of the grasshopper. However, Lipocalins are a very diverse family of lipid-binding proteins. These include fatty acid binding proteins located in the cytoplasm and nuclear receptors. It is believed that lipocalins have a variety of biological roles including lipid transport and metabolism, in addition to functions in signaling pathways. More studies are required for cDNAs encoding such proteins, to determine if they play a role in either lipid absorption or signal trafficking in the midgut. Biliverdin binding

proteins are one of the major classes of hemolymph proteins and are classified into three groups according to the molecular weight of their subunits. The cDNA encoding the putative biliverdin binding protein may thus be a false positive.

All identified cDNAs in the library have similarity with proteins originating from insects, except for one cDNA which has shown similarity with the biphenyl dioxygenase from the bacterium *Rhodococcus sp* strain RHA1, which is a gram-positive polychlorinated biphenyl degrader (Masai et al. 1995). The polychlorinated biphenyls are widely used in industrial materials and because of their chemical stability, cause environmental pollution. Bacterial genes encoding enzymes that degrade biphenyl and polychlorinated biphenyl, have been characterized for use in bioremediation (Abramowicz, 1990). Similar enzymes from different gram negative bacteria degrade DDT. For example, a non-specific monooxygenase hydroxylates DDT at the 2- and 3-positions (Masse et al. 1989). The biphenyl degrader from *Alcaligenes sp*. JB1 may degrade DDT by attacking its aromatic ring. Growth of *A*. *eutrophus* A5 on biphenyl induces the conversion of DDT into DDT-cis-2-,3-dihydrodiol by dioxygenase (Nadeau et al. 1998). The cDNA from the SSH PTM library showing similarity to this enzyme may have a role in pesticide resistance. Another possibility is that this may represent a transcript from a midgut-associated bacterial symbiotic. One of the surprising results is the similarity of a cDNA clone with the sensory appendage protein from *An*. *gambiae* (Biessmann et al. 2002). However, the expression profile of this protein could not be confirmed by either RT-PCR or Northern blot analysis. The results of further molecular analysis may indicate that this cDNA clone is a false positive.

Three cDNA clones matched hypothetical proteins belonging to two insect species, suggesting that these cDNAs encode for uncharacterized proteins. Furthermore, nine cDNA clones resulted in unknown sequences. These clones may encode unidentified proteins that could be unique to lepidopteran, poorly conserved domains of known proteins, or non coding regions.

The subtraction efficiency of the SSH procedure is measured by the enrichment of

cDNAs encoding differentially expressed transcripts and the normalization of the high abundant transcripts within the subtracted library. In evaluation of their SSH method, Diatechnko et al. (1996) mixed various amount of bacteriophage X147 DNA, to be used as a target for subtraction, with human skeletal muscle ds cDNA. The same amount of human skeletal ds cDNAs without the viral DNA was used as a driver. After subtraction the viral DNA fragments were enriched by 100-, 1000, and 5000-fold compared to samples containing 0.1%, 0.01% and 0.001% of viral DNA, respectively. Gaines et al. (2002) analyzed the expressed sequence tags (ESTs) from subtracted and unsubtracted cDNA libraries of the hindgut and Malpighian tubules (HMT) of *Ctenocephalides felis*. They examined the subtraction efficiency of the library in two ways. The cDNAs encoding proteins involved with inorganic ion transport, organic ion transport and vesicle-associated proteins, which mainly have a critical function in the HMT, are all significantly enriched in the subtracted library compared with the unsubtracted library. Moreover, the actin transcript, expressed in both HMT and carcass issues, was decreased by nearly five fold in the subtracted library versus the unsubtracted library. SSH has proven its capability as a powerful technique to identify and clone differentially expressed genes not only in different tissues and stages, but also for differentially regulated genes. Kung et al. (1998) compared gene expression in estrogen receptor (ER)-positive and ER-negative breast cancer cell lines by differential screening and SSH. Differential screening failed to identify new genes and furthermore, known genes differentially expressed at low levels in an on/off fashion were also not isolated. In comparison, 123 sequences were isolated by SSH, representing three known genes and one novel gene that is a member of the G protein-coupled receptor superfamily and associated with ER expression in breast cancer cell lines and primary tumors. Yang et al. 1999 examined the cDNA clones generated by SSH using cDNA microarrays. On the genome level for prokaryotes, SSH was successfully used to identify sequence differences between *E. coli* and *Salmonella typhimutium* (Bogush et al. 1999). In an insect study, seven genes differentially expressed by workers of the honey bee, *Apis mellifera*, and queen destined larvae have been isolated based on the SSH method

(Evans and Wheeler 1999). While evaluation of the subtraction efficiency on the midgut subtracted library of PTM was not calculated, a number of low abundant transcripts belonging to the midgut environment have been isolated and furthermore, high abundant sequences such as housekeeping genes were significantly reduced in this subtracted library. The putative microvilli membrane protein, V-ATPase and allergin were represented by single clones (1.4% of the total sequenced cDNA clones). Each one of these cDNA clones likely plays a role in midgut function. In contrast, highly abundant house keeping genes, for example the ribosomal putative transcript, is also only represent by 1.4% of the 69 cDNA clones. Thus, at least at this level of analysis, common cDNAs are decreased and the differentially expressed cDNAs are enriched in the PTM midgut subtracted library. It is unclear why the defensin cDNAs appear to have been disproportionally amplified during the subtraction process. The high representation of trypsin in the library compared to other digestive enzymes is also unclear.

From 637 SSH clones, 69 clones were randomly selected and sequenced. Determination of the sequence of more cDNA clones may reveal genes related to additional differentially expressed genes. Moreover, the prolongation of exposure time of the membrane to X-ray film may reveal more clones, in addition to the current 637 clones. Forward and reverse subtracted probes may also be used to screen the library which may reveal more clones. Therefore, further analysis of the current library is required to discover more cDNAs that could be deployed in control strategies for the PTM.

CHAPTER VI
EFFECT OF MICROORGANISMS ON THE EXPRESSION LEVEL OF IMMUNE PEPTIDE TRANSCRIPTS IN THE POTATO TUBER MOTH MIDGUT

6.1. Introduction

The first line of defense for an insect is the cuticle, which represents a physical barrier against microorganisms. Another physical barrier is present in the insect midgut. The peritrophic matrix plays a role in protecting the insect from the invasion of microorganisms during feeding. While insects lack an acquired immune system, they have a well-developed innate immune response. Insect innate immunity includes; 1) cellular immune reaction; consisting primarily of phagocytosis and encapsulation of invading microorganisms by cells circulating in the hemolymph, and 2) innate humoral defense based on the synthesis of cationic (positively charged) antibacterial and antifungal peptides/polypeptides. Such antimicrobial molecules are typically produced by the insect fat body and then released into the hemolymph, thus imitating liver function in the mammalian immune system (Takehana et al. 2002). These two types of defense mechanisms are not entirely independent, there is some overlap between humoral and cellular defenses in the recognition of intruders (Lavine and Strand, 2002).

Each antimicrobial peptide is active against one or more of the following pathogens: gram negative, gram positive bacteria and fungi. These peptides are classified according to their structure and in turn their function; cyclic and linear within which they are further classified; cecropin, cysteine-rich peptides, praline-rich molecules and glycine-rich peptides/polypeptides.

Cyclic antimicrobial peptides are cysteine-rich peptides, in which the cysteine residues form disulfide bridges, with molecular masses of 2 to 6 kDa. Defensin, the most common member of this group, is 4 kDa (36-46 residues) with 6 cysteine residues forming 3 disulfide bridges and is active against gram positive bacteria. Defensin is the most prominent immune molecule among insects and is produced by a wide variety of

insect species, scorpions, molluscus and even mammals (see review Boman, 1995). A one minute exposure to an insect defensin molecule is enough to kill bacteria (Bulet et al. 1992). Gram positive bacteria have multiple layers of peptidoglycan with an acidic character which binds to the positively charged antibacterial peptides. The peptide disrupts the permeability of the bacterial cytoplasmic membrane, resulting in loss of cytoplasmic potassium, partial depolarization of the inner membrane, a decrease in cytoplasmic ATP, and finally inhibition of respiration.

Another peptide belonging to this group is drosomycin, from *Drosophila melanogaster*, which has antifungal activity and is inactive against bacteria (Bulet et al. 1999). The lepidopteran antifungal peptide, heliomicin, was isolated from *Heliothis virescens* and is also a member of this family of immune peptides (Lamberty et al. 1999).

The second group of immune-inducible peptides is the linear peptides and these are further divided into 3 families; cecropin (the first inducible antibacterial peptide to be isolated), proline-rich peptides and glycine-rich polypeptides.

Cecropin was identified in 1981 from the silkworm *Hylophora cecropia* (Steiner et al.1981). It is a linear 4 kDa cationic peptide (~39 residues) and consists of 2 α helices linked by a short Ala-Gly-Pro hinge. The C- terminus of cecropins is amidated (has an NH_2 group). They kill both gram positive and gram negative bacteria. Cecropin interacts with membrane lipids by inserting the long axis of the α-helix into the plane of the membrane, resulting in a general disintegration of membrane structure and lysis of bacterial cells. Cecropin shows no toxicity to mammalian cells, even at concentrations as high as 50μM.

Proline-rich peptides vary from 15 to 39 residues, with proline constituting more than 25% of the peptide. Most of the short proline rich peptides are active against gram negative bacteria.

Glycine-rich polypeptides, from 9 to 30 kDa, include glycine residues at an average composition of 10 -22%. They are mostly active against gram negative bacteria. Attacin (20kDa) has been isolated from both lepidopteran and dipteran insects. Its mode of

action is not well understood. Carlsson et al. (1998) speculated on the mechanism of attacin activity. They postulated that interaction of attacin with gram negative bacteria depends on the electrostatic binding of the positive charges on the attacin molecule, to the negative charges on the inner core and lipid region of the lipopolysacharides (LPS) on the bacterial surface, followed by a hydrophobic interaction with the lipid, resulting in partial integration of attacin into the outer membrane. This integration is thought to block physiologically important reaction sites for outer membrane proteins to elicit signaling events. In turn, this leads to inhibition of outer membrane protein synthesis. Attacin is thus thought to have a unique ability to interfere with the synthesis of outer membrane proteins, without entering the inner membrane or cytoplasm.

Gloverin is a glycine-rich polypeptide family and only three molecules have been isolated to date. Gloverin appears limited to Lepidoptera; *Hyalophora gloveri*, *Helicoverpa armigera* and *T. ni* (Axén et al. 1997; Mackintosh et al. 1998; Lundström et al. 2002).

The challenge experiments were performed by feeding PTM larvae on sliced tubers contaminated with either bacterial or viral particles. The expression levels of antimicrobial peptide transcripts were determined by RT-PCR and Northern blot. The results of the challenge experiments presented in this chapter demonstrate the specific up-regulation of the transcription of PTM antimicrobial peptides as a result of bacterial or viral infection.

6.2. Materials and methods

6.2.1. Challenge of PTM larvae with bacterial and viral particles

Larvae were forced to feed on either bacterial or virus particles by introducing the neonate larvae to contaminated potato tubers.

6.2.1.1. Preparation of microorganism-contaminated tubers

Three different treatments were applied to the tubers. *E. coli*, and *B. subtilus*, represent gram negative and gram positive bacteria, respectively, and the *Autographa californica* nuclear polyhedrosis virus was used for a viral challenge.

A) Bacterial preparation

The bacterial strains were prepared by inoculation of 250ml 2YT media and growing overnight at 37°C in a shaking incubator. The bacterial pellet was collected by centrifugation at 6000 rpm in a high speed centrifuge model J2-21, using a JA-14 rotor (Beckman Coulter) for 5 min at 4°C. The pellet was washed by 200ml of 1X phosphate buffer (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.7H_2O$, 1.4 mM K_2HPO_4) and re-suspended in the same buffer and volume.

B) Viral preparation

The AcNPV was provided by Dr. Linda Guarino (Texas A&M University) at a concentration of 1.14 X 10^7 PFU. The viral concentration was diluted to 1.14 X 10^5 in 200ml of 1x phosphate buffer.

C) Tuber preparation

Washed and cleaned tubers were sliced and bricked with a sharp knife and allowed to dry on paper towels at room temperature. The tuber slices were coated with the treated buffers with shaking for at least 30min at room temperature. The treated tuber slices were picked up and allowed to dry on paper towels at room temperature. For each treatment, the potato tuber slices were collected together in a small container. Neonate larvae were added to each treatment. The larvae were allowed to fed and develop to the fourth instar at 26±2°C. The larvae were selected from the treatment and dissected to isolate the midguts.

D) Preparation of the control tubers

The control tubers were treated exactly as for the challenge treatments except that the buffer lacked the presence of microorganisms.

6.2.1.2. RNA extraction

The larvae were grouped into 4 separate pools; control and treatments with gram negative bacteria, gram positive bacteria and virus. Each pool was isolated and the RNA extracted independently, with care taken to avoid contamination between treatments. The midguts were dissected and both total RNA and mRNA were extracted and purified as described in chapter 5, section 5.2.1. The total RNA was treated with RNase-free DNase to remove any PTM genomic DNA associated with the RNA during purification.

6.2.2. PCR analysis

First strand cDNA was prepared from experimental and control larvae as described in chapter 6 section 6.2.3. The 1st strand cDNA was used as a template for RT-PCR amplification. The amount of cDNA used in the reactions was variable depending on the previously detailed expression level for the molecule of interest as follows; 750ng cDNA per reaction for XI-C8 (gloverin), 150ng for X-M12 (attacin), 250ng for IX-K21 (Hyphancin), 100ng for IXP2 (defensin) and 1.5ug for VI-L3 (lipase-1). The amplification conditions started with a preheating period of 2min at 95°C, followed by 25 cycles of 94°C for 15 sec, 60°C for 15 sec and 70°C for 30 sec, ending with an extension at 72°C for 4min and finishing 4°C.

The primers used in the reaction were the same as used in chapter VI, section 6.2.3. For defensin and hyphancin, cDNA from both gram negative and positive experiments were used as templates. For gloverin, only the cDNA from the gram negative experiment was used. The cDNA from the gram positive experiment was used for the attacin RT-PCR amplification. For lipase-1 RT-PCR amplification, only cDNA from the virus experiment was used as a template.

6.2.3. Northern blot analysis

Northern blot analysis was performed as described in chapter VI, section 6.2.1. Ten to twelve micrograms of total RNA from the control was run against a similar amount from treated samples on a 1% denaturing formaldehyde agarose gel. Total RNA from treated sample was used in the northern blot as follows; total RNA from the gram negative experiment was used for the gloverin probe, the gram positive experimental samples was used for attacin and samples from both experiments were used for defensin and hyphancin. The Northern blot for Lipase-1 only used total RNA from the viral treatment. Control samples were included in all cases.

6.2.4. 5' and 3' rapid amplification of cDNA ends (RACE)

Five genes were selected for this step; gloverin, hyphancin, lipase-1, attacin and defensin. The synthesis of full length cDNAs with complete 5' ends relied on a procedure used for cDNA library construction. Half a microgram of mRNA from the midgut of the PTM larvae was reverse transcribed to prepare ds cDNA using the SMART® cDNA library construction kit (Clontech), according to the manufactures' protocol. One primer set provided by the kit was used to prepare the full length 1st stand cDNA as follows;

SMART IV™ Oligonucleotide:

5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3'

CDSIII/3' PCR Primer:

5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}N_{-1}N$-3'

Two PCR amplification rounds were used to amplify either the 5' or 3'end of the desired transcript. The ds cDNA was used as a template. The amplification conditions for the first PCR round was 94°C for 3 min, followed by 25 cycles of 94°C for 15 sec, 70°C for 15 sec and 72°C for 2 min, followed by 4 min at 72°C. The second round (nested) PCR amplification used the same conditions as the previous round except the

annealing temperature was reduced to 68°C. To amplify the 5’ end, a combination of nested primer corresponding to the SMART IV™ Oligonucleotide (5’-AAGCAGTGGTATCAACGCAGAGT-3’) with a gene specific primer was used as follows;

NestDefIX-P2 (3'); 5'-TGTCACTCGAGCCTGAC-3’

NestAttX-M12 (3'); 5'-TGAGGAAGTAGGGCTCT-3’

NestGlovXI-C8 (5'); 5'-AAAACCCTGGTGCCGGT-3’

NestHyphIX-K21 (5'); 5'-AGGACCAGCTTTGATGA-3’

NestLipVI-L3 (5'); 5'-TCGTGACGGATCGTGCA-3’

Amplification of the 3’ end followed a similar principle to the 5’ end amplification using a different combination of primer sets, nested CDSIII/3’ PCR Primer (5’-ATTCTAGAGGCCGAGGCGGCCGA-3’) with a gene specific primer as follows;

NestDefIX-P2 (5'); 5'-GAATACGAGCTTAGC-3’

NestAttX-M12 (5'); 5'-CGGGAAAGTTGGAGCA-3’

NestGlovXI-C8 (3'); 5'-GCGGCTACGAACA/CCAAC-3’

NestHyphIX-K21 (3'); 5'-AAGGCTGGTCGCAGAGT-3’

NestLipVI-L3 (3'); 5'-CCCAAGTAGCCGACGG-3’

6.3. Results

PTM larvae were challenged with microorganisms (bacteria and viruses) by feeding to determine whether the expression level of previously identified antimicrobial molecules is up-regulated due to the presence of pathogens. RT-PCR analysis was performed on cDNA prepared from midgut tissues only. The PCR strategy was based on a reduction of both the amount of cDNA template and the number of amplification cycles as a "semi-quantitative method". This strategy enables the demonstration of the difference in PCR product abundance between challenged and unchallenged midguts and hence, the expression level of the corresponding genes. All RT-PCR products were of the expected size (Fig. 6.1). Gloverin and lipase1 show faint bands at 100 and 440bp, respectively, using the challenged midguts compared to non-challenged samples. The minimum amount of cDNA template that could be successfully used in the assay was 750ηg and 1.5μg for gloverin and lipase, respectively, indicating the low expression level of these antimicrobial molecules in the PTM midgut, even in response to microorganism challenge. Conversely, attacin, hyphancin, and defensin resulted in positive amplifications (180, 110 and 200bp) in both challenged and naïve larvae, indicating either constitutive expression of these molecules in the PTM midgut or presence of microorganisms on the normal tubers. However, the differences of PCR band intensities reveals that these molecules are up-regulated due to the experimental challenges. Cecropin and defensin molecules are known to be active against both gram negative and gram positive bacteria. In this experiment, higher band intensities were obtained from midguts challenged with *B. subtilus*, compared to those challenged with *E. coli*, indicating that infection with gram positive bacteria may have a grater induction effect on the expression of these molecules in the PTM midgut.

The RT-PCR results were confirmed by performing northern blot analysis on the total RNA samples extracted from the midgut of challenged and naïve larvae. Equal amounts of total RNA from both midgut samples, 10μg each, were run parallel to each other on the same gel (Fig. 6.2) and then transferred onto a membrane to be used for

northern blot analysis. The probes prepared from cDNA clones related to immune sequences, were allowed to hybridize using the standard conditions described in the previous chapter. For each probe, the difference in signal intensity between challenged and naïve was detected visually on X-ray film (Fig. 6.3). All treatment samples show a relatively stronger signal than for naïve larvae samples.

The 5' and 3' cDNA ends for these genes of interest were amplified using a combination of PCR primer sets designed from the SSH fragments and primers designed from the sequences of the SMART IV™ Oligonucleotide and CDSIII/3' PCR Primers from Clontech. The 5'-and 3'-ends were amplified for three transcripts; X-P2, X-M12 and IX-C8 representing defensin, attacin and gloverin, respectively. The full length cDNA sequences are shown in figures 6.4 through 6.6. Only the 3' end was successfully amplified for the IX-K21 transcript (Figure 6.7). Fig. 6.8 shows the partial nucleotide sequence of VI-L3 including the 5' end.

The full length of the defensin cDNA is 521bp and the coding region is 315bp. The initiation and termination codons are at positions 79 and 394, respectively. The deduced protein is 105 aa and has 7 cysteine residues at positions; 3, 68, 81, 85, 95, 101 and 103. The full length cDNA for the attacin transcript is 763bp and the coding region is 584bp. The initiation and termination codons are at positions 55 and 640, respectively. The deduced protein is 195 aa and includes 24 glycine residues. The full length cDNA of the gloverin transcript is 877bp and the coding region is 507bp. The initiation and termination codons are at positions 79 and 586, respectively. The deduced protein is 169 aa and has 25 glycine residues. The 3' end of hyphancin was successfully amplified by 3'RACE. The length of the hyphancin molecule, excluding the 5'end (untranslated region and part of the coding sequence), is 406bp. The current hyphancin sequence lacks the initiation codon. The deduced aa of the available sequence is 56 residues. The 5' end was successfully amplified by 5'RACE for lipase-1. The length of the lipase-1 molecule excluding the 3'end (untranslated region and part of the coding sequence) is 697bp. The current lipase-1 sequence lacks the termination codon.

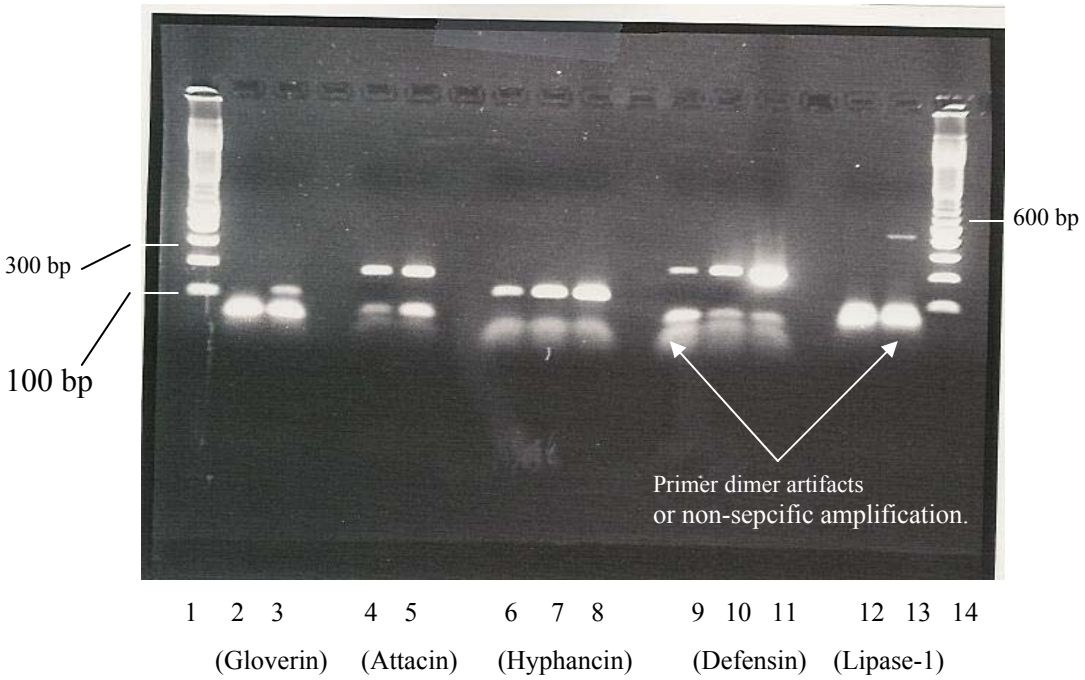


Fig 6.1: RT-PCR comparison of cDNA of naïve vs challenged larvae.Lanes 1 & 14 are DNA markers (100bp). Lanes 2, 4, 6, 9 and 12 are RT-PCR amplification from naïve cDNA templates using gloverin, attacin, hypancin, defensin and lipase-1 specific primers, respectively. Lane3: faint DNA band at 100bp results from RT-PCR amplification using cDNA template from *E. coli*-challenged larvae and gloverin specific primers. Lane5: DNA band of 180bp results from RT-PCR amplification using cDNA template from *E. coli*-challenged larvae and attacin specific primers. Lane7&8: DNA bands of 110bp results from RT-PCR amplification using cDNA template from *E. coli*- and *B. subtilus* challenged larvae, respectively, and hyphancin specific primers. Lane10 & 11: DNA bands of 200bp results from RT-PCR amplification using cDNA template from *E. coli*- and *B. subtilus* challenged larvae, respectively, and defensin specific primers. Lane13: DNA band of 440bp results from RT-PCR amplification using cDNA template from Baculovirus-challenged larvae and lipase-1 specific primers. The bands at 100bp or lower are primer dimer artifacts or non-sepcific amplification.

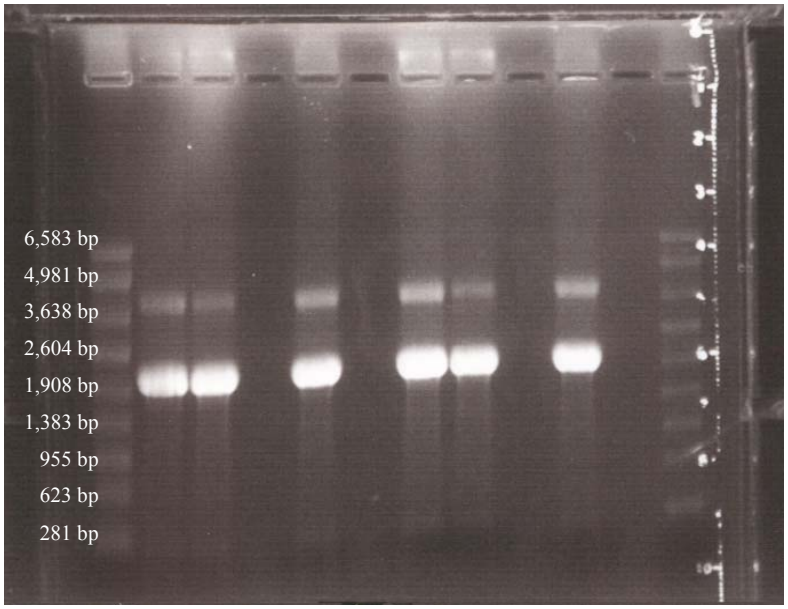


Fig 6.2: Total RNA from naive and challenged larvae. The RNAs were electrophorsed on 1% formaldehyde agarose gel (lanes 2, 3, 4, 5, 6 and 7) against Promega RNA ladder (lanes 1&8).

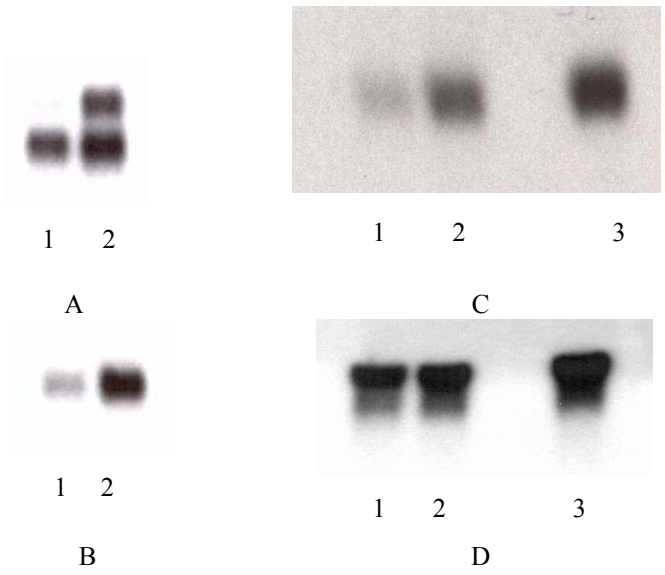


Fig 6.3: Northern blot hybridizations from the challenge experiment. Ten micrograms of total RNA from naïve and challenged larvae were electrophoresed on a 1% formaldehyde agarose gels and then transferred onto nylon membranes. The hybridizations were performed against probes designed from the SSH-cDNA clones. A) Total RNA from naive larvae (1) and from *E. coli*-challenged larvae (2) hybridized with gloverin based probe. B) Total RNA from naive (1) and from *E. coli*-challenged larvae (2) hybridized with attacin based probe. C) Total RNA from naive (1), *E. coli*- (2) *B. subtilus* and (3) challenged larvae hybridized with hyphancin based probe. D) Total RNA from naive (1), *E. coli*- (2) and *B. subtilus* (3) challenged larvae hybridized with defensin based probe.

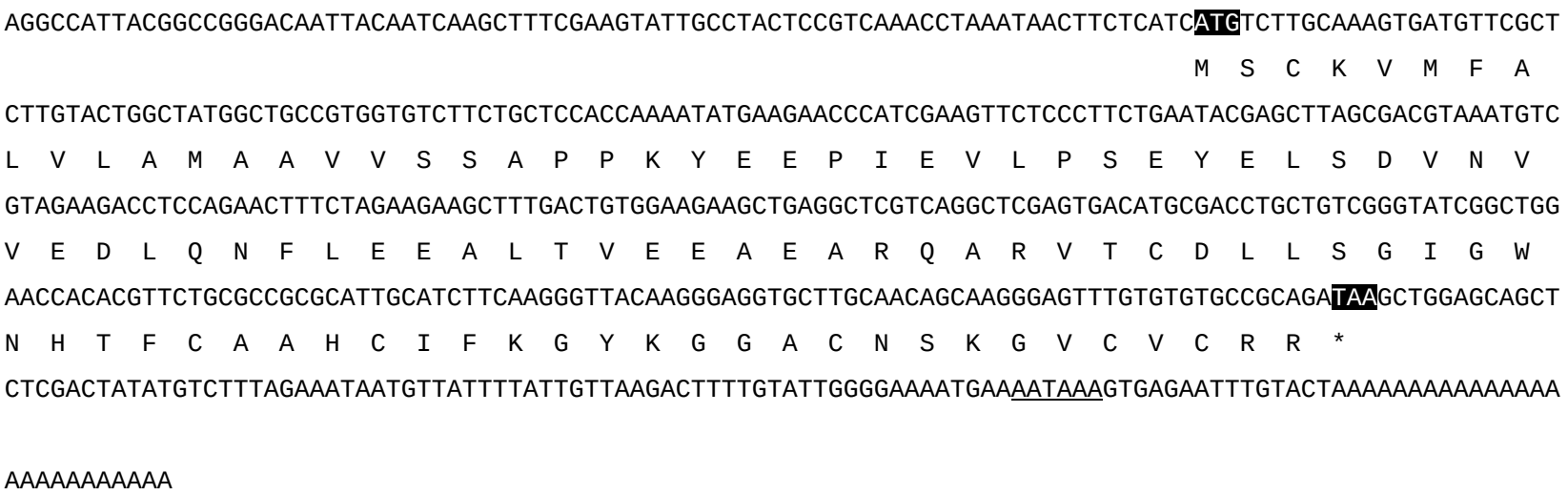


Fig. 6.4: Full length cDNA of the defensin transcript (X-P2) and its deduced amino acid sequence. The initiation and termination codons are shown in black boxes and the putative polyA signal is underlined. The total length of the transcript is 521bp. and the coding region is between 79-394.

```
ATACAAAACAATCGTTGAACACGCGTAGTCGTTTCATTCAACAGTTTCATTAAAATGAGTTCTTTTGGAAATATCACCAACAACAACGATGGGTCTAAAAAC
                                                        M  S  S  F  G  N  I  T  N  N  N  D  G  S  K  N
CTCACTATCGGGAAGGTGAAGGAAGATGGAGCCAATACGACAACTGCGAGTTTGTTTGTCAACAACCAGAGAGACCGCGATTCCCACACCAACATAAGGAAA
L  T  I  G  K  V  K  E  D  G  A  N  T  T  T  A  S  L  F  V  N  N  Q  R  D  R  D  S  H  T  N  I  R  K
AATTCGTACGGTGCCGCAGGAGGAAATTTGAGCTTCCAACATGGAGGAAACCCCAGTGCCACAGGAAGCGTGACTCTGCAGCATATACCGAAGATGGGCAAC
N  S  Y  G  A  A  G  G  N  L  S  F  Q  H  G  G  N  P  S  A  T  G  S  V  T  L  Q  H  I  P  K  M  G  N
CAAGCGTCAGCAGCAGCCGGCATCGGCGTGGGCAGCGAGACGCATCGGGTTGGCGTTGATGCCTTCGCGACGCGTACGATGCCTCATCATCATCCCAACTTC
Q  A  S  A  A  A  G  I  G  V  G  S  E  T  H  R  V  G  V  D  A  F  A  T  R  T  M  P  H  H  H  P  N  F
ACTACTGTTGGTGCTAGTGTCAACGCTCAAGCCGGGAAAGTTGGAGCACATGCTGGGATAACCCACACCCCGATGTTCAAACAGACACAGCATTCGGTAGGG
T  T  V  G  A  S  V  N  A  Q  A  G  K  V  G  A  H  A  G  I  T  H  T  P  M  F  K  Q  T  Q  H  S  V  G
GCAAGTCTAAACCTGCACAAGAGCCCTACTTCCTCAGTTGATATGAACATTGGATCCAGTAGAATGACAACGCCGCACCATAGGGGTGACTGGAACCATGGA
A  S  L  N  L  H  K  S  P  T  S  S  V  D  M  N  I  G  S  S  R  M  T  T  P  H  H  R  G  D  W  N  H  G
GTCAACTTTGGAGTGAAGAAACATTTCTAAGTCTTCACCGTACAACAAAAGAAGCATGACAAATACCTACCTATTTAATCAATTATCATTATTTATACTAAT
V  N  F  G  V  K  K  H  F  *
AAAAACATACTGATTTTGTAGAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 6.5: Full length cDNA of the attacin transcript (X-M12) and its deduced amino acid sequence. The initiation and termination codons are shown in black boxes and the putative polyA signal is underlined. The total length of the transcript is 763bp and the coding region is between 56-640.

```
GGATCATTCAGTTTTTGAGTCTTCAGTGAGAGCAGTGAAAGATTCTAGTTTCGACAAGTTACTTTAAAACTGTTCAAAATGAAATTCCTGTGCATCTTCGCT
                                                                    M  K  F  L  C  I  F  A
GCCGTGCTGGTTGCAGTCGCTGCTGAAAACGAAGAAAGCTACTACGGCTACCCAGACGTGGAATACGTCCCAAACCCAAGGTTCCGTCGTGACGTCTCTCTG
A  V  L  V  A  V  A  A  E  N  E  E  S  Y  Y  G  Y  P  D  V  E  Y  V  P  N  P  R  F  R  R  D  V  S  L
GATCATAAAGTTGGCCAGAATGGAAAGGTCTTCGGCACCCTCGGATCCAACGACAATGGACTTTTTGGCAAGGGTGGCTACGAGCACAACATCTTCAACGAC
D  H  K  V  G  Q  N  G  K  V  F  G  T  L  G  S  N  D  N  G  L  F  G  K  G  G  Y  E  H  N  I  F  N  D
CACCGAGGCAACCTGAACGGGCAGGTCACCGGCACCAGGGTTTTGGGGCCCTACGGAGACTCGAGTCACGTCGGCGGGGCCCTGAACTGGAAGAACGACAGG
H  R  G  N  L  N  G  Q  V  T  G  T  R  V  L  G  P  Y  G  D  S  S  H  V  G  G  A  L  N  W  K  N  D  R
GCCACGGCGTCTTTGGAGGCTAGCAAGATGATCCATGGACCTGCTTCTGTCAATGCAGCGGCCGGCGGCAGATGGCCCGTAGGCAAGAACGGTGACCTCAGC
A  T  A  S  L  E  A  S  K  M  I  H  G  P  A  S  V  N  A  A  A  G  G  R  W  P  V  G  K  N  G  D  L  S
GCGCAGGGCTTCTACAGTCAAACCCAGGGCCTCGGCCGCGACTACGGTGGCAGACTCAACTACAACCACCGTTGGTAGATAGTGATGAAACCGCTCGTCGCA
A  Q  G  F  Y  S  Q  T  Q  G  L  G  R  D  Y  G  G  R  L  N  Y  N  H  R  W  *
AGTGTATAAGAAAAAACGCCAATCCGCGCGGACGCAAATAATTCGATGACTACCTATTTTTTCTTGTAAACTAGCGCCTTTTTGGCGGCGGCCGGCGGAAGA
ATATAAATTTGATATGCCAGTAATGTTGGTATGTTTTGGCTGTGAATTTAAGATTAAATTATATATTTTTTTTATTAATTTTTGGTCAGGGATTGGATTAAG
TATTTTAGAAATTAGAGTATTAAAAGATTTTCCAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 6.6: Full length cDNA of the gloverin transcript (IX-C8) and its deduced amino acid sequence. The initiation and termination codons are shown in black boxes and the putative polyA signal is underlined. The total length of the transcript is 877bp and the coding region is between 79-586.

GGCCGAGGACTTTCTTGCTTTTTGGCACTTAGTGTTGTGTCAGCCAATCCACGATGGAACCCATTCAAAAAATTGGAAAAGGCTGGTCGCAGAGTCAGAGAT

G R G L S C F L A L S V V S A N P R W N P F K K L E K A G R R V R D

GGGCTCATCAAAGCTGGTCCTGCCGTGCAAGTAGTTGGACAAGCCTCAACCATCTACAAACAGGGATAGATATCCCTGGACGAGACACTCAGCGCCATCTAG

G L I K A G P A V Q V V G Q A S T I Y K Q G *

CTGCAAACTACGCAAACTTATGGAACTGTATCCTGCAAAAATTTAAATAAGTAGTCAATTTCTGAATTGTGTACTTAAAAATAGCTGTTATAGTTATAATAA

GTGTAGAGCATACATAACTTACTTATAATTACATTTATCTACTTAAGATATTGCCTTGTTTTAATAAATTTTCGTTAACCACCAAAAAAAAAAAAAAAAAAA

AAAAAAA

Fig. 6.7: Partial cDNA sequence (406bp) of the hyphancin transcript (IX-K21) with the 3'end and its deduced amino acids. The termination codon is shown in black box and the putative polyA signal is underlined.

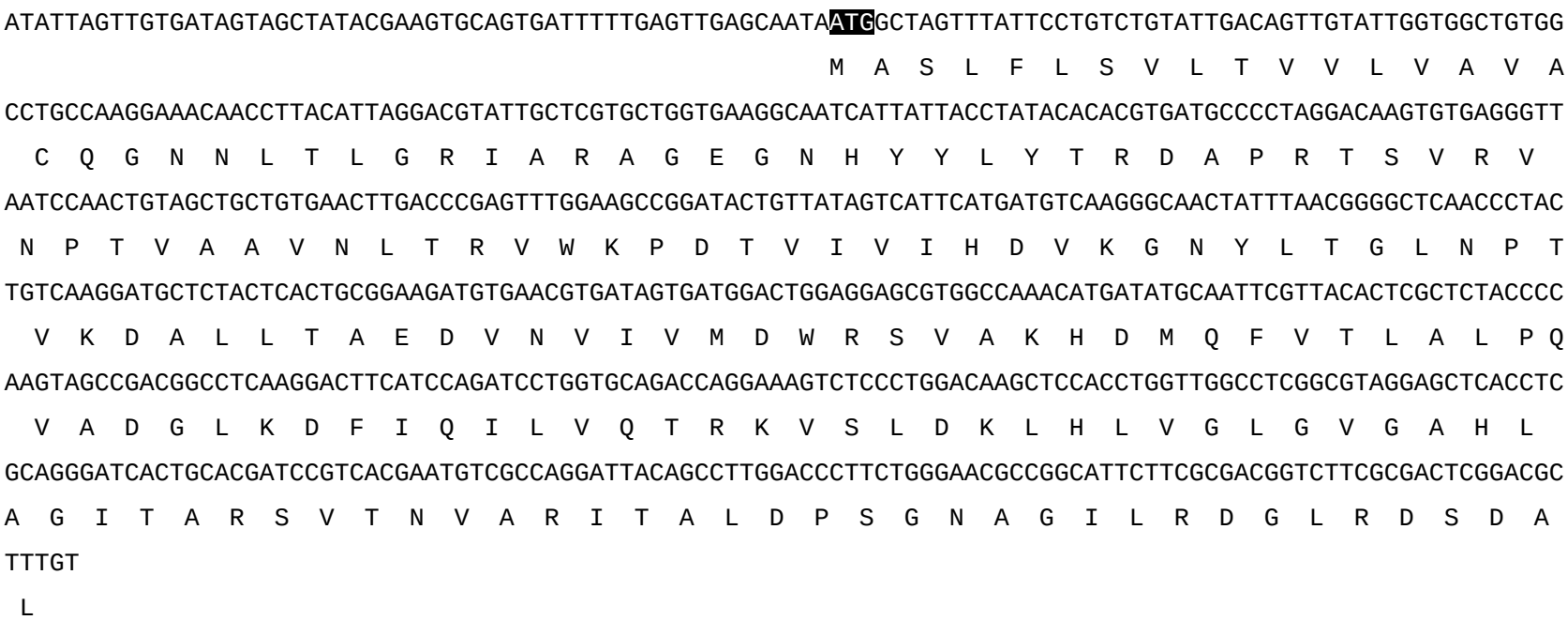


Fig. 6.8: Partial cDNA sequence (697bp) of the lipase-1 transcript (VI-L3) with partial deduced amino acid sequence. The initiation codon is shown in black box.

6.4. Discussion

The insect midgut is in contact with the external environment during insect feeding and is thus vulnerable to microbial attack. The peitrophic matrix in lepidopterans is thought to provide some protection.

Humoral insect immunity is thought to be primarily focused on the fat body expression and secretion of peptides into the haemolymph. Previous studies revealed the absence of antimicrobial peptides in the lepidopteran midgut (Lundström et al. 2002). In contrast, the results presented in this chapter highlight the expression of a variety of antimicrobial peptides transcripts in the PTM midgut. Moreover, the expression of these genes is up-regulated following meal containing microorgaisms. Recent research has revealed the expression of antimicrobial peptides in the midgut of haematophagous insects. Four defensins have been isolated from the midgut of the blood feeding fly, *Stomoxys calcitrans* (Lehane et al. 1997; Munks et al. 2001). The *Stomxys* midgut defensin (Smd) family is exclusively expressed in the anterior midgut (reservoir), not in the posterior midgut in which the blood meal digestion occurs. Several immune-related peptides have been reported in the gut of other blood-sucking insects including; *An. gambiae* (Vizioli et al. 2001), and *Ae. aegypti* (Lowengerger et al. 1999). In the midgut of the soft tick, *Ornithodoros moubata*, four defensin isoforms have been identified (Nakajima et al. 2001; Nakajima et al. 2002). All isoforms are constitutively expressed in the midgut, fat body and reproductive tract. However, three of these defensins were highly expressed in the midgut compared to the fat body. The up-regulated expression of defensin in response to blood feeding was demonstrated in both *S. calcitrans* and *O. moubata* (Munks et al. 2001; Nakajima et al. 2002). Moreover, up-regulation was not detected when *S. calcitrans* fed on a sugar meal. Therefore, it is speculated that the expression of Smd in the anterior midgut is to protect reserved blood (Lehane et al. 1997).

In phytophagous insects as the PTM, immune-related peptides in the midgut would likely have a different function and are more likely to be part of a defense mechanism to

protect the insect gut from microbial invasion. An innate immune response is also observed in the PTM midgut. Expression of some immune genes within the naïve larvae midgut indicates that these genes are constitutively expressed in the PTM midgut or presence of microorganisms in the tubers that stimulate genes expression. The RT-PCR results (Fig. 6.1) indicate constitutive expression of defensin, cecropin and attacin compared to the inducible expression of gloverin and lipase-1. Similarly, northern blot analysis also revealed the transcriptional up-regulation of immune system genes due to microorganism feeding. However, both assays (RT-PCR and northern blot) show discrepancies in the amount of transcripts within the tissue. The signal intensity on the X-ray film may not be accurately related to the expression levels of the transcripts. The RT-PCR analysis is more sensitive for this assessment and reveals that the transcript levels of these genes are as follows; defensin>hypancin>attacin> lipase-1>gloverin.

The expression of defensin and cecropin is affected by the bacterial species used in the challenge experiment. We speculate that gram-positive bacteria stimulate the expression of some immune-peptides more than gram negative. However, more investigations are required to assess this hypothesis and to determine at which level these molecules are regulated. Moreover, septic injury could also be applied to the PTM larvae to compare the whole body immune response, to the midgut response.

The 5’ and 3’ RACE procedures were performed for five of the cDNA clones, and full length cDNAs were obtained for 3 genes; defensin, attacin and gloverin. The aa composition features of the antibacterial peptides isolated from the PTM midgut agree with the general characteristic features of the groups to which the corresponding peptides belong. The deduced aa sequence for defensin indicates the presence of 7 cysteine residues. Insect defensins are characterized by 6 cysteines forming 3 disulfide bridges. The three bridge pattern has the same cysteine pairing among insect defensins; Cys1-Cys4, Cys2-Cys5, and Cys3-Cys6 (Bulet et al. 1999). Both attacin and gloverin belong to a family of glycine-rich antibacterial polypeptides. These polypeptides contain between 10-22% glycine residues of the aa composition (Hoffmann et al. 1996). The deduced aa sequence of attacin and gloverin indicates the presence of 24 and 25 glycine

residues, respectively, in their aa composition. Therefore, the glycine ratio of attacin and gloverin is 12% and 15%, respectively, within the range of other family members. The hyphancin gene shows homology with cecropin. Cecropin consists of 2 α helices linked by a short Ala-Gly-Pro hinge (Boman 1995). The same feature (Ala-Gly-Pro) exists in the deduced aa sequence of the available sequence for the PTM hyphancin molecule.

Comparative experiments between the immune peptides expressed by the midgut and the fat body are required to determine the similarities and differences between . *In vitro* hybridization could be used for each induction experiment and applied for both tissues. There are, approximately, 40 insect defensins characterized from different insect species. However, there is no report for defensin from lepidopteran species. Characterization of this peptide from PTM will represent the first lepidopteran defensin.

CHAPTER VII
GENERAL DISCUSSION

7.1. General discussion

The potato tuber moth is the primary pest of potato production in a number of regions around the world, mostly in developing countries. The larvae mine the foliage and the tubers. The larvae penetrate the skin of the tuber and start feeding within the tuber till pupation. The nature of PTM infestation increases the difficulty of controlling this species. The larvae mine both the leaves and tubers, feeding on the internal tissues of the plant and therefore, are away from direct contact with chemical control agents. This behavior protects the pest by providing a safe environment and minimizing the time span through which the larvae are facing any control strategy. Despite its worldwide distribution and high cost of damage, there is a lack of basic fundamental biological, physiological, and biochemical research on the PTM. From this research, preliminary data is provided that could be deployed for more advanced research on PTM along two scientific lines of inquiry.

Germline transformation experiments for insects began with the successful transformation of *Drosophila melanogaster* more than twenty years ago (Rubin and Spradling 1982). Since that time the same transformation strategy has been applied for *Drosophila* in multiple labs worldwide. The same technique has also been deployed for non-drosophilids and has become routine for some dipteran species. Genetic transformation in insects provides a set of tools by which specific insect species can be more closely studied at the molecular level. Once the genetic transformation system was established for *Drosophila melanogaster*, a large number of insect-specific genes, promoters and other related sequences were identified and later used in the field of insect molecular genetics to serve different branches of the insect sciences. Advanced methods were developed to identify specific gene and regulatory sequences such as sequence tagging and enhancer trapping (Bellen et al. 1989; Sentry and Kaiser, 1992). Insect-

based genes have been isolated and used as visual genetic markers for the rescue of eye color in mutant dipteran species (Lidholm et al. 1993; O'Brochta et al. 1996; Handler and Harrell, 1999). After the first successful development of a transgenic system for mosquitoes, modifying the genetic characteristics of these medically important species became the main target for mosquito research, including the reduction in transmission of parasites that cause human disease. The molecular interactions between the parasite and its microenvironment within internal tissues would be difficult to understand without transformation systems. Hence, interfering with these interactions could lead to blocking the multiplication and transmission of the parasites using transgenic mosquitoes. Replacing the wild type population with the transgenic mosquitoes could eventually lead to the eradication of some human diseases and save millions of people from suffering death each year.

To initiate studies of the PTM at a molecular level, the development of a transformation system is the primary goal. Prior to conducting transformation experiments, a number of critical components important for successful transformation should be investigated within the PTM tissues.

7.1.1. Promoter activity in PTM

Appropriate promoters to drive the expression of marker genes and transposases, are an important component of successful transformation systems. The transcriptional activity of five promoters was determined within the PTM soma by expressing the reporter gene Firefly *luciferase* (*luc*) and the control Renilla *luc*. The five promoters used in this assay were selected according to their history of previous usage for transformation of other insect species. Five experimental plasmids were designed to express the Firefly *luc* under the regulation of each of the following promoters; *hsp70*, *hsp82*, *actin5C*, *hr5-ie1* and *pUb*. The expression activities of the *hsp70*, *hsp82* and *actin5C* promoters were relatively low and *pUb* showed a significantly higher activity compared to these three promoters. The highest level of activity was demonstrated for

the *hr5-ie1* enhancer-promoter regulatory DNA sequences.

All promoters, excluding *hr5-ie1*, were originally cloned from drosophilid insects, which are distantly related to lepidopterans. In addition, the drosophilid promoters in this assay lack enhancer sequences. The *hr5-ie1* enhancer-pomoter is cloned from the *Autographa californica* nuclear polyhedrosis virus (AcNPV) which is infectious to lepidopteran insects and utilizes the host genetic machinery to replicate its genomic DNA. The immediate early genes are transcribed by host factors (Guarino and Summers 1986; 1987), utilizing the host genetic machinery for viral replication. The *hr* elements function as enhancer elements for the early viral genes (Rodems and Friesen 1993).

7.1.2. Marker gene for PTM

The expression of enhanced green fluorescence protein (*EGFP*) gene has been demonstrated within the PTM embryonic soma to be a promising candidate for a genetic marker for PTM transformation. Egg fluorescence was detected in more than 50% of the embryos injected with a plasmid carrying the *EGFP* gene. The intensity of fluorescence was variable among the embryos, indicating different expression levels of the plasmids according to the injection conditions for each individual embryo.

7.1.3. Transpositional activity of *Hermes*, *Mos*1 and *piggyBac* within the PTM soma

The mobility of the transposable elements was compared in the PTM embryonic soma using an interplasmid transposition assay. The assay is based on the mobility of a transposable element between the injected plasmids, rather than into the insect genome. In this study, three helper plasmids were constructed to express each of these transposases under the regulation of the *hr5-ie1* as determined by the results of the promoter assay. The helper plasmid expresses transposase enzyme which excises the corresponding transposable element from the donor plasmid and insert it into pGDV1 target plasmid. The transposable element flanks the ColE1 origin of replication and

kanamycin resistant gene between its inverted terminal repeats. Insertion of this transposable element enables the target plasmid to replicate within *E. coli* cells and to be selected in the presence of kanamycin, in addition to chloramphenicol antibiotics. The mobility of transposable element is determined by the transposition frequency. The each transposition frequency is calculated by the number of transposed elements (target plasmid received the DNA fragment from the donor) relative to the number of recovered donor plasmids. The *piggyBac* transposon showed active mobility within the PTM soma, compared to no activity detected for *Mos*1 and *Hermes*. The transposition frequency of *piggyBac* within the PTM soma is 4.2×10^{-5}. The transposition frequency of *piggyBac* in the PTM was improved by incorporating a fourth plasmid for injection into the PTM embryos. The fourth plasmid, a "transactivator", expresses the IE protein that elevates expression from the *hr5-ie1* regulatory element on the helper plasmid and hence, increases the expression of the *piggyBac* transposase. Five different ratios of the transactivator relative to the helper (1, 0.1, 0.02, 0.01 and 0.005) were examined. Addition of the transactivator resulted in improved mobility of *piggyBac* within the PTM at all ratios examined. However, the two middle concentrations showed the highest *piggyBac* activity, indicating that the amount of the transposase, expressed under the regulation of *hr5-ie1* in combination with the transactivator protein IE1, is a critical factor in determining the activity of the transposon. Incorporation of the transactivator plasmid within the transposition assay has not been applied previously in insect species. Previous studies had applied the use of either a purified transposase protein to elevate the amount of transposase within the host (Coates et al. 2000).

Sequence analysis of the insertion sites reveals the duplication of TTAA target sequences, confirming that the integration of the donor-DNA fragment into the pGDV1 target is due to the active transposition of the *piggyBac* element.

The PTM genome was screened for the existence of *mariner* and *piggyBac* element using PCR analysis and Southern blot hybridization. Degenerate PCR followed by DNA sequencing of the PCR products has shown the presence of a *mariner*-related element within the PTM genome. No evidence of *piggyBac*-like elements within the PTM

genome was found.

7.1.4. Genetic transformation experiment

The transformation experiment was conducted on the basis of the results of the proceeding assays. Seven experiments were performed to produce transgenic PTM and a total of four different plasmids were used in a variety of combinations and concentrations. The total number of injected embryos was 13,153 and the total number of screened G_1 individuals for transgenic insects was 93,307. The results from the seven experiments have not yielded any positive results since neither G_1 larvae nor pupae showed the expression of the *EGFP* marker gene.

Factors that may inhibit insect transformation include physical handling during microinjection, the volume of the injected DNA and interruption of the internal cellular organization, and species-specific factors that might inhibit the genomic transposition of the transposable element (Atkinson et al. 2001). Handling of the potato tuber moth embryos for microinjection is relatively primitive and may lower the embryo survival following injection. The rate of survival may reduce the chances to produce transgenic individuals. The transformation experiments were performed using two different DNA concentrations. The handling of the embryos and the DNA concentration may not be the only factor behind the unsuccessful transformation of the PTM. Host factors may have an effect on the germline transformation, such as position-effect variegation that has been determined to affect eye color marker gene in *Ae*. *aegypti* (Coates et al. 1998) and *D*. *melanogaster* (Henikoff, 1996). The *piggyBac* transposon is automonous and is thought to require no host specific factors (Atkison and James, 2002). However, host factors may affect the ability of *piggyBac* to integrate into the PTM genome. These possibilities require further experimentation to determine their existence and mechanisms of action.

7.1.5. Isolation of differentially expressed cDNAs from the PTM midgut

Differentially expressed cDNAs within the PTM midgut were identified using the suppression subtractive hybridization (SSH) procedure. A subtracted midgut library was produced and transferred onto nylon membranes using the Q-botics system. From a total of 2984 clones from the subtracted library, 637 clones are candidates for being differentially expressed genes within the PTM midgut, according to selectively positive hybridization with a midgut-based probe. Sixty-nine clones were randomly selected and sequenced. Sequence results were analyzed against the GenBank database using the algorithms, BLASTX-nr, BLASTN-est and BLASTN-3'UTR. Digestive enzymes and insect immune system related-cDNAs constitute approximately 70% of the randomly selected clones. cDNA clones matching with midgut-related sequences represent 7.2%. Microvilli protein, V-ATPase, allergen and apolipoprotein are known to play a functional role in the midgut. Others referred to as miscellaneous sequences are 5.8 %; they include 40S ribosomal protein, putative transcription factor, sensory appendage protein and Biphenyl dioxygenase. Unknown sequences, hypothetical proteins, 3' UTR, are as high as 17.4% of the total sequenced clones.

The subtraction efficiency of the SSH procedure is measured by the enrichment of cDNAs encoding differentially expressed transcripts and the normalization of the highly abundant transcripts within the subtracted library (Diatechnko et al. 1996; Gaines et al. 2002). Evaluation of the subtraction efficiency on the subtracted midgut library of PTM was not calculated. However, the subtracted library includes a number of low abundant transcripts belonging to the midgut environment, such as a putative microvilli membrane protein and allergin. The high abundant sequences such as housekeeping genes were significantly reduced in this subtracted library. The high representation of trypsin and defensin in the library compared to other sequences suggest that the normalizationis not complete.

Twelve cDNAs representing each cDNA category were randomly selected for further molecular analysis. Both Northern bolt hybridization and RT-PCR analysis

produced consistent results. All of the cDNAs were shown to be expressed in the PTM midgut, except for the chemosensory binding protein clone which could not be detected in the midgut or carcass.

7.1.6. Effect of microorganisms on the expression level of immune peptide transcripts in PTM midgut

The cDNA clones showing homology with immune molecules were further examined by detecting their expression level in the midgut of microbial challenged larvae. The larvae were forced to feed on potato slices contaminated with bacteria or a virus. All clones were shown to be up-regulated transcripts within the PTM midgut in challenged larvae, compared to naïve larvae. The expression profiles were demonstrated by RT-PCR and Northern blot hybridization analysis. Both types of analysis revealed up-regulation of the transcript levels of the antimicrobial genes due to feeding on bacteria or virus. The RT-PCR analysis is more sensitive for this assessment and reveals that the relative transcript levels of these genes were as follows; defensin>hypancin>attacin> lipase-1>gloverin.

The full length cDNAs of three transcripts were identified by rapid amplification of cDNA ends (RACE). The deduced aa sequence for PTM defensin indicates the presence of 7 cysteine residues. Insect defensins are characterized by 6 cysteines forming 3 disulfide bridges (Bulet et al. 1999). Both attacin and gloverin belong to a family of glycine-rich antibacterial polypeptides. These polypeptides contain between 10-22% glycine residues of the aa composition The deduced aa sequence of attacin and gloverin indicates the presence of 24 and 25 glycine residues, respectively, in their aa composition. The glycine ratio of attacin and gloverin is 12% and 15%, respectively, within the range of glycine rich peptides (Hoffmann et al. 1996).

7.2. Conclusion

The enhancer-promoter cassette *hr5-ie1* has a proven high transcriptional activity by expressing a high level of the reporter gene (firefly *luc*) within the PTM embryonic soma. It is assumed that the high transcriptional activity of this regulatory DNA in PTM is related to the organism from which *hr5-ie1* was isolated. Baculoviruses infect lepidopteran insect species and utilize their genetic machinery for viral replication. The immediate early genes (*ie*) are the first genes to be activated upon viral infection and use the host transcriptional factors for viral DNA replication. The usage of a marker gene other than *EGFP* in PTM is limited. False positives due to selective pressure are highly probable, in the case of deploying a chemical resistance gene, in addition to the environmental hazards during the screening of transgenics. Lack of information about a suitable mutant strain and allele to be used as a marker system for PTM, limit the ability to use this kind of genetic marker. The *EGFP* has proved its efficacy as a genetic marker in wide range of insect species. In this study, *EGFP* yielded the expected results and the fluorescent protein has been detected in the PTM embryonic soma.

The Interplasmid transposition assay has revealed promising results based on the mobility of the *piggyBac* element within the PTM and is a candidate for use in transformation experiments. The idea of increasing the transposase level within the host soma using another plasmid has improved the transposition frequency of *piggyBac* within the PTM soma. The transposition frequency was increased approximately10 times by including a transactivator plasmid in a 1% ratio with the helper plasmid.

The unsuccessful transformation results for the PTM are likely to be due to either an internal activity factor interfering with the integration of the transposon into the genome, or perhaps the transposase activity or age of the injected embryos or lack of *ie*1 promoter activity in pole cells.

The SSH procedure has proven its efficiency for the enrichment of differentially expressed and low abundant sequences within a wide range of cDNA populations. The subtracted library of the PTM midgut comprises a number of cDNA clones that are

expressed in the midgut. Some of theses clones are exclusively expressed in the midgut and others are expected to be expressed in the body tissues as well as midgut. Digestive enzymes and immune peptides represent the most abundant transcripts in this library. The cDNA encoding proteins involved ion transport, microvilli-associated proteins and proteins associated with lipid absorption that have a critical function in the midgut, are also represented in the subtracted library. Other cDNAs encoding a putative transcription factor and Biphenyl dioxygenase may be expressed in the PTM midgut. The cDNA clones encoding hypothetical proteins and those of unknown sequences are also represented in the library. These cDNAs may represent novel genes that have not been identified so far and may play a role in midgut physiology.

The cDNAs encoding immune peptides are constitutively expressed in the midgut as well as being up-regulated due to challenge with either bacteria or viruses.

7.3. Future experiments

The enhancer-promoter regulatory sequence (*hr5-ie1*) that has been used is highly active as demonstrated in the luciferase assay. However, PTM-promoters or those isolated from other lepidopteran species such as the *B. mori* cytoplasmic actin gene *BmA3* promoter, may show higher activity or at least may more compatible with PTM genomic integration. Germline specific promoters would be also valiable.

Other fluorescent genes have been recently identified and applied in other insects such as DsRed. Such genes could be used solely or in addition to the *EGFP* as dual markers for identifying PTM transgenics and determining the tissue specificity of certain promoters or the activity of transgenes.

One of the important issues affecting transformation efficiency is the age of the embryos during injection. In this study, the injection was carried out within 4-hours of egg laying. Minimizing the injection time window of the embryos to as early as 1.5-2 hours may achieve successful transformation esults.

During this study, in some cases, insufficient numbers of wild-type females was

problematic, in addition to some of the females being used in mating with G_0 males being sterile or laying few eggs. Such females are not good candidates for supporting transformation experiments. Increasing the number of wild-type females to 5 for each family is more likely to increase the number of G_1 individuals. Thus, the probability for achieving transgenics individuals will be increased.

Only 69 cDNA clones have been sequenced from 637 that showed a positive hybridization with the midgut-derived probe. More clones are still available to be investigated to identify their role in midgut function and how they could be deployed in control strategies. More characterization is still required for the cDNAs encoding immune peptides. The expression pattern of antimicrobial peptides in the midgut need to be compared to that of the fat body and haemolymph.

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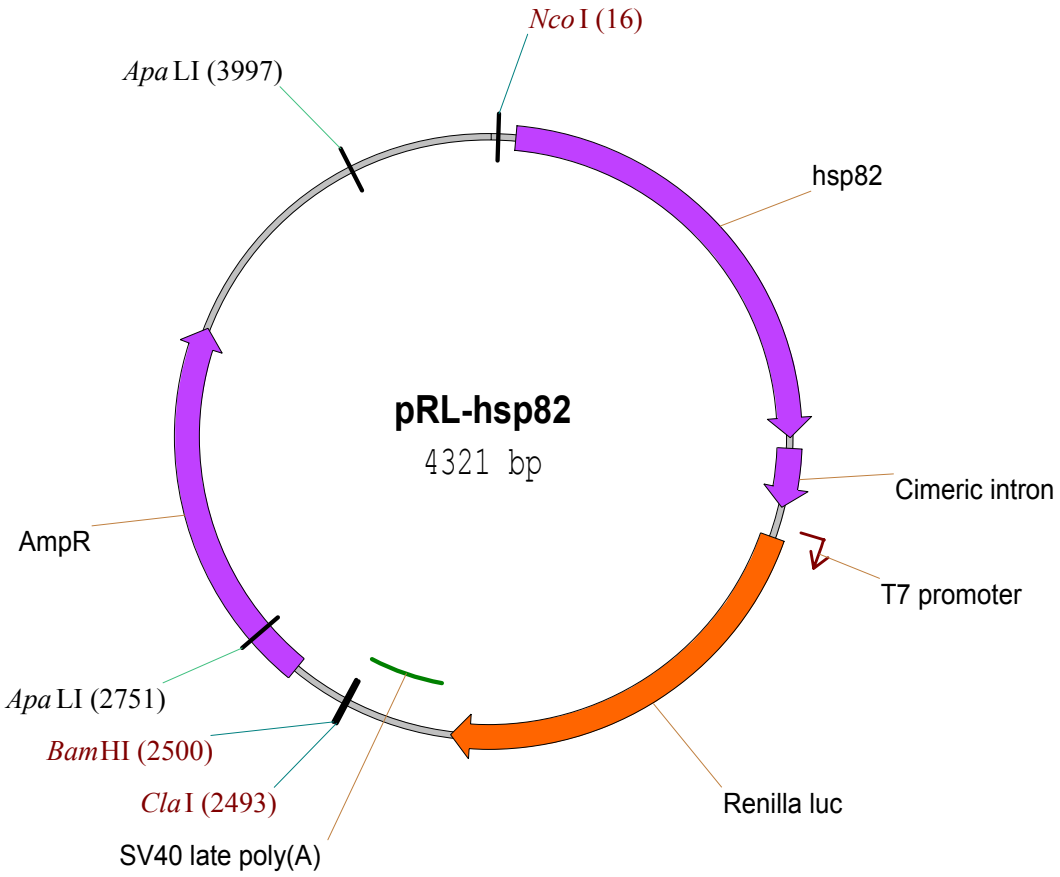
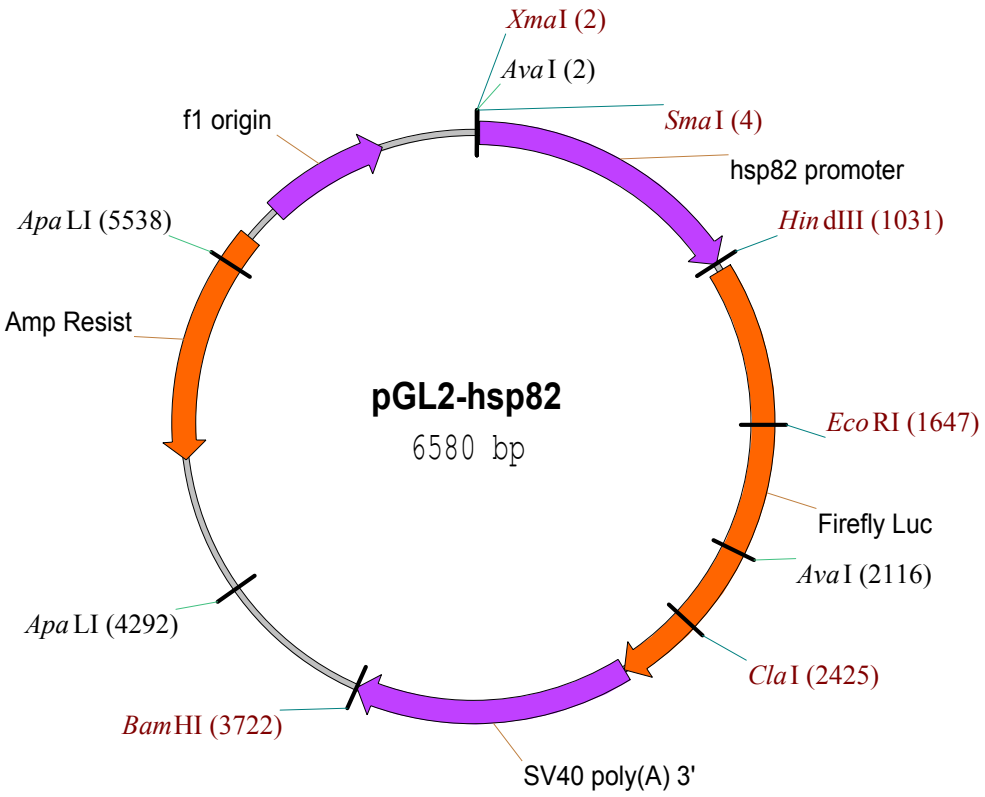
APPENDIX A

PLASMID MAPS

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ClaI (2425)
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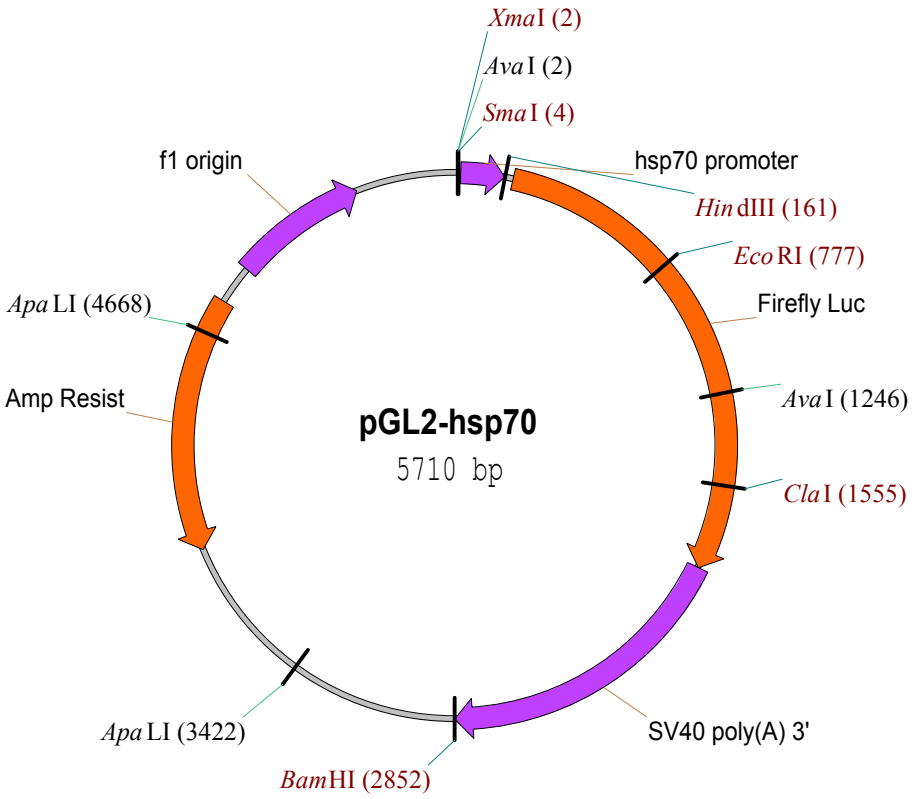
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Amp Resist
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ApaLI (3422)
SV40 poly(A) 3'
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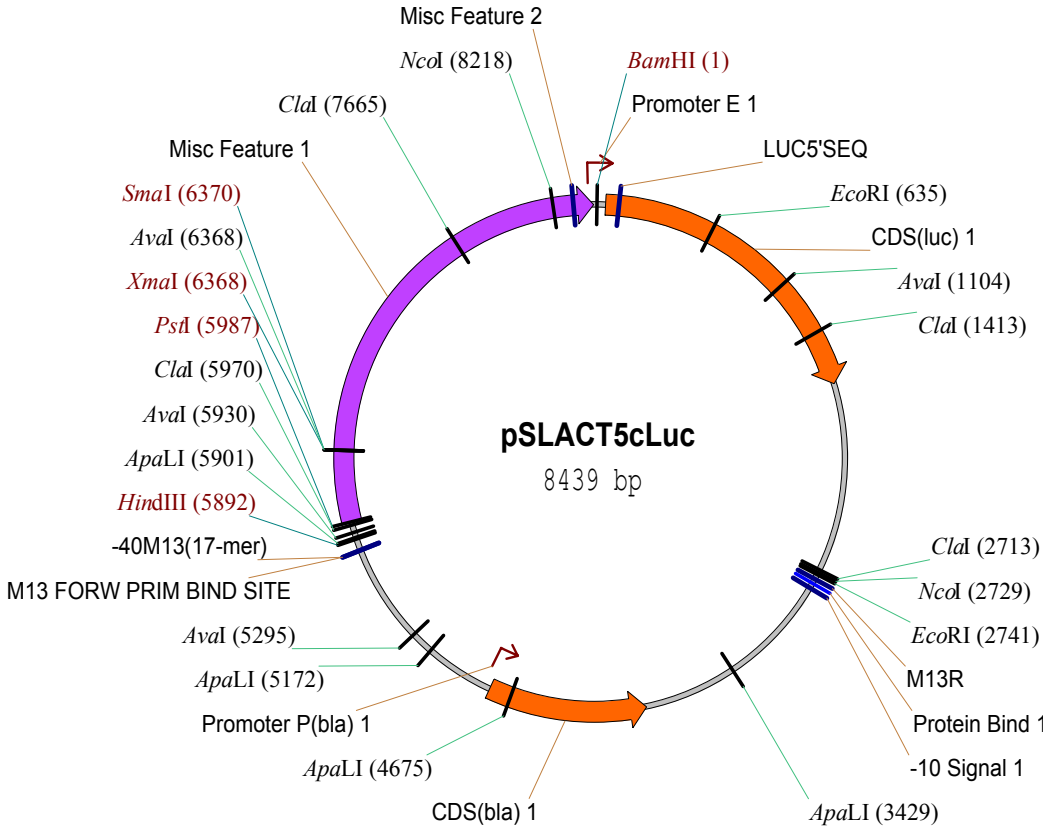
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AvaI (6368)
CDS(luc) 1
XmaI (6368)
AvaI (1104)
PstI (5987)
ClaI (1413)
ClaI (5970)
AvaI (5930)
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8439 bp
ApaLI (5901)
HindIII (5892)
ClaI (2713)
-40M13(17-mer)
NcoI (2729)
M13 FORW PRIM BIND SITE
EcoRI (2741)
AvaI (5295)
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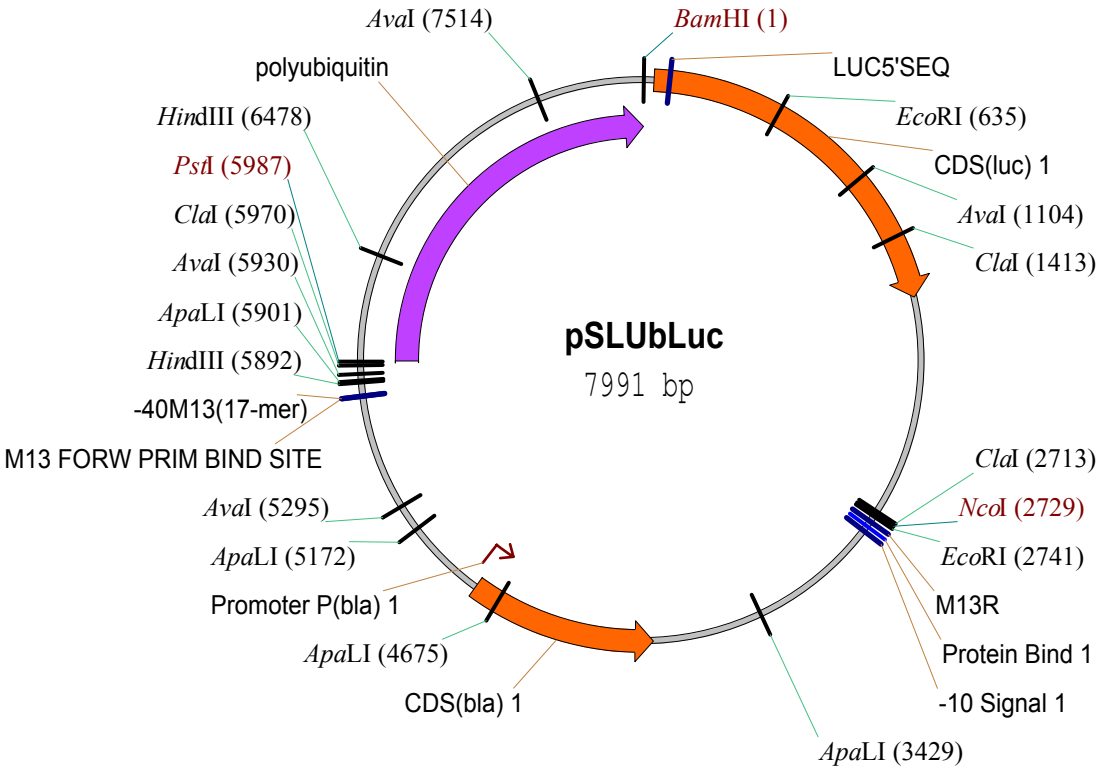
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AvaI (5295)
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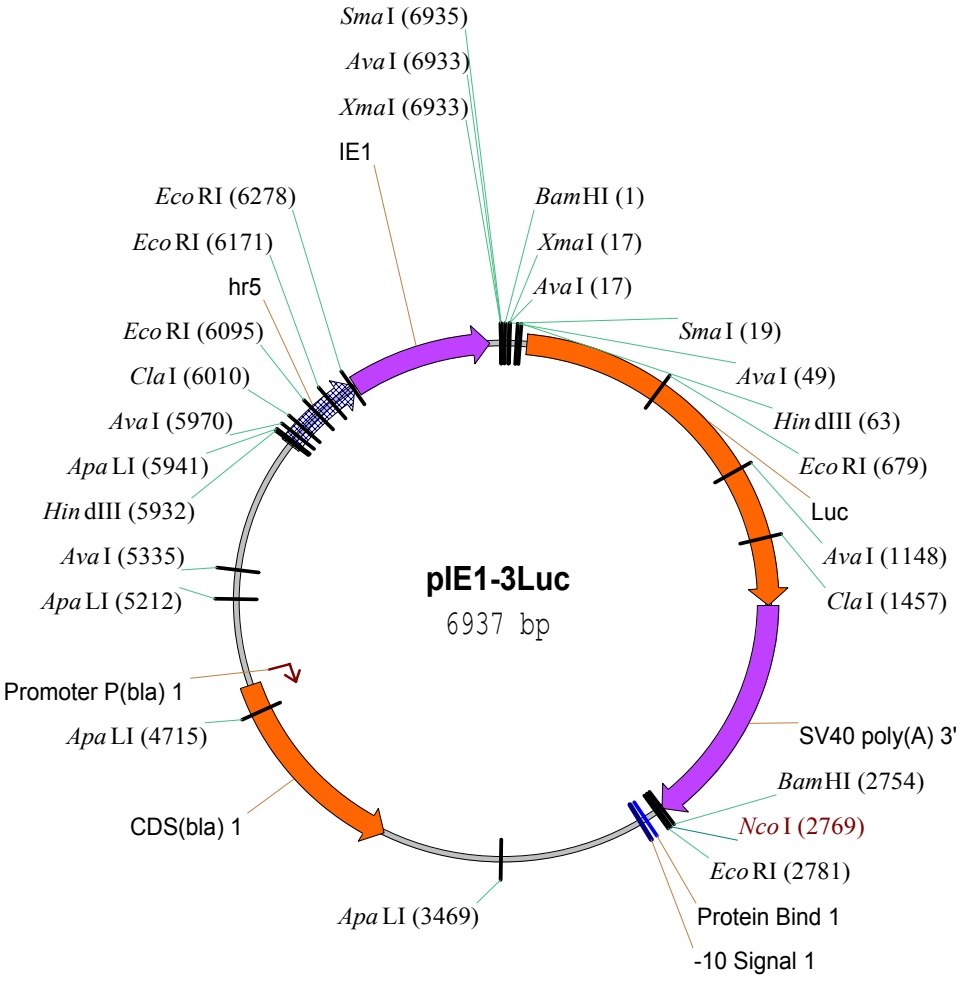
AvaI (7514)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hr5
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ApaLI (5212)
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ApaLI (4715)
CDS(bla) 1
ApaLI (3469)
BamHI (1)
XmaI (17)
AvaI (17)
SmaI (19)
AvaI (49)
HindIII (63)
EcoRI (679)
Luc
AvaI (1148)
ClaI (1457)
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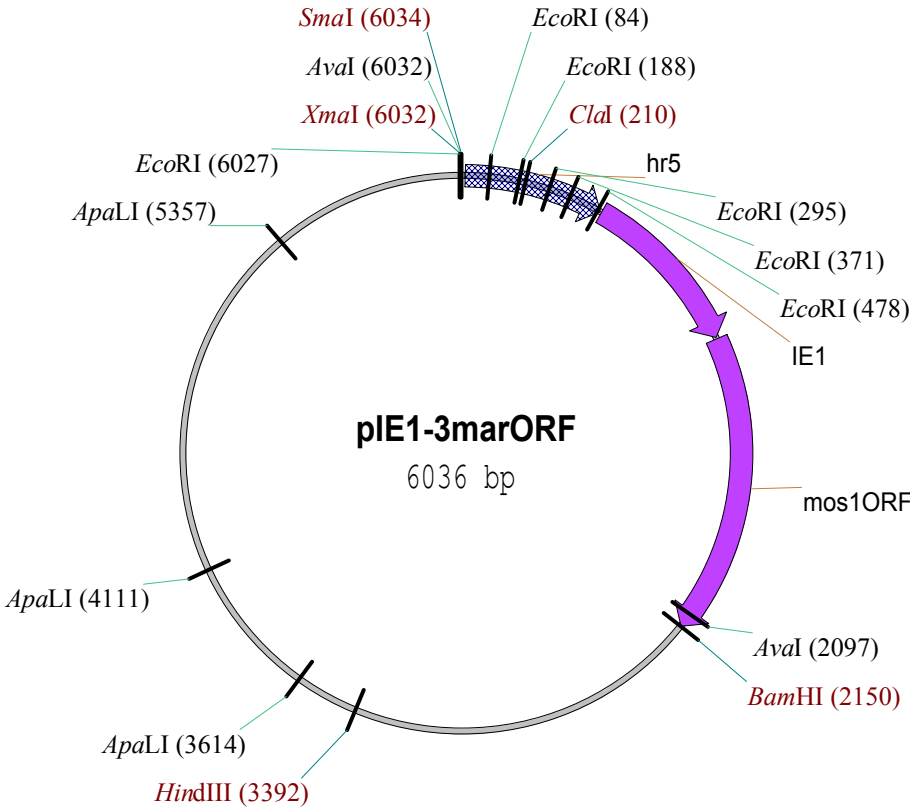
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EcoRI (371)
EcoRI (478)
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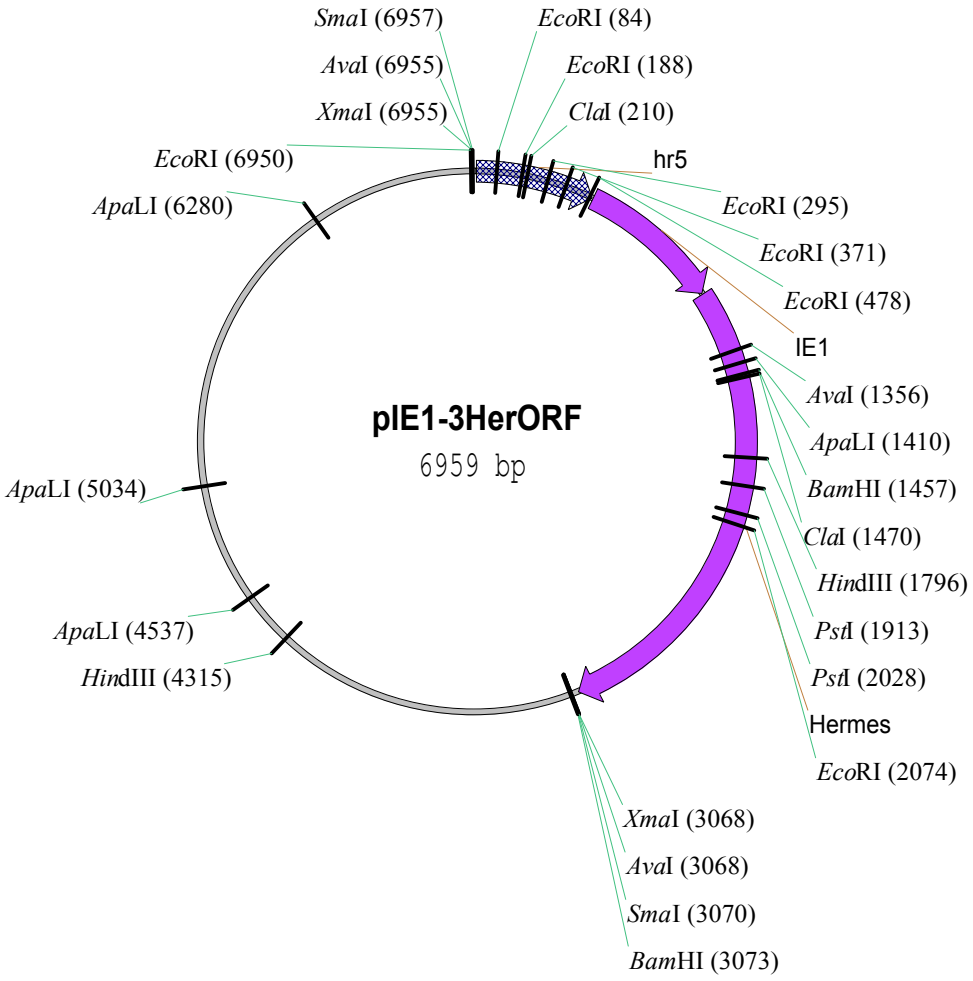
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BamHI (1457)
ClaI (1470)
HindIII (1796)
ApaLI (4537)
PstI (1913)
HindIII (4315)
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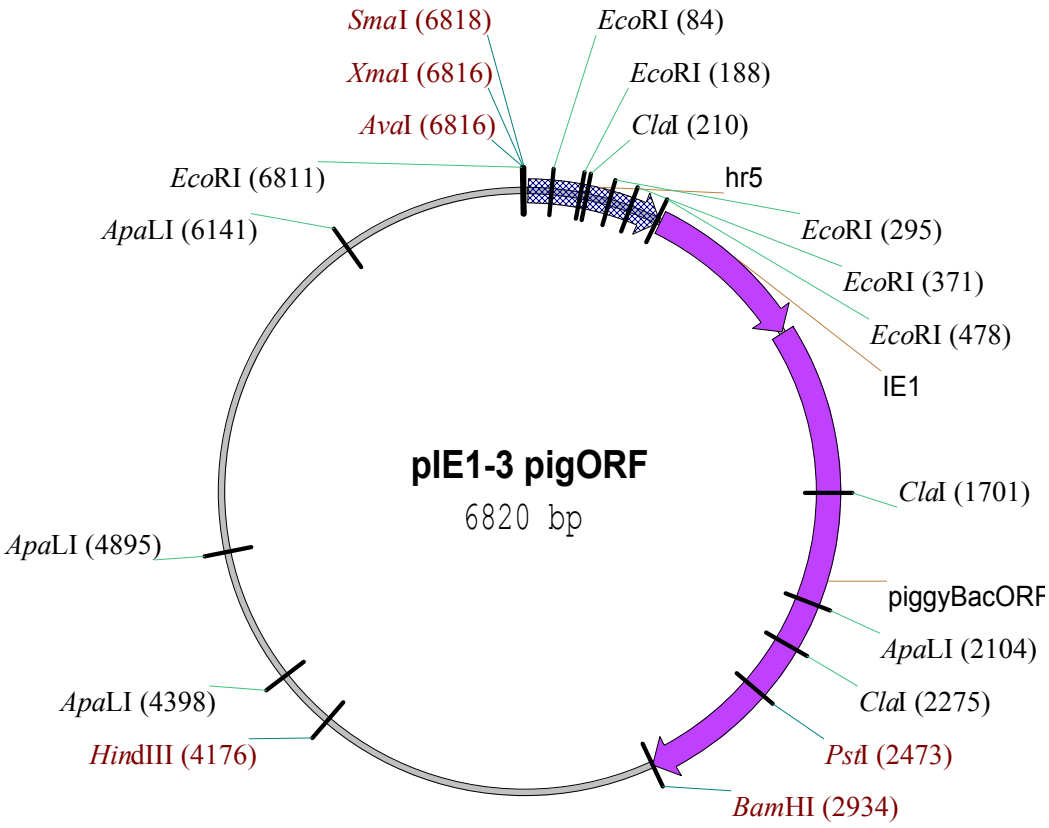
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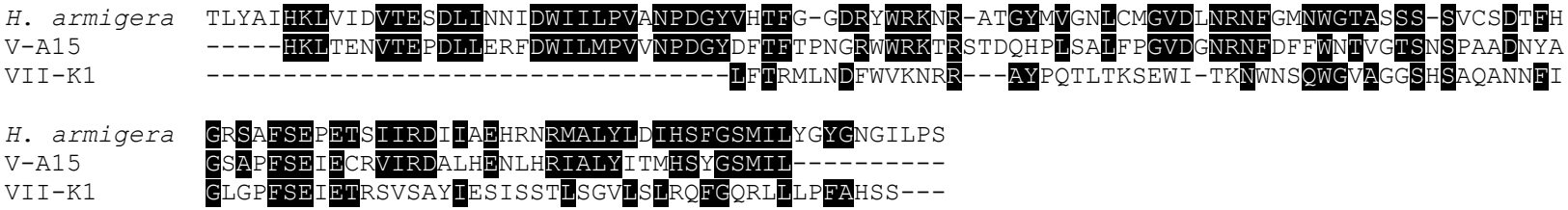
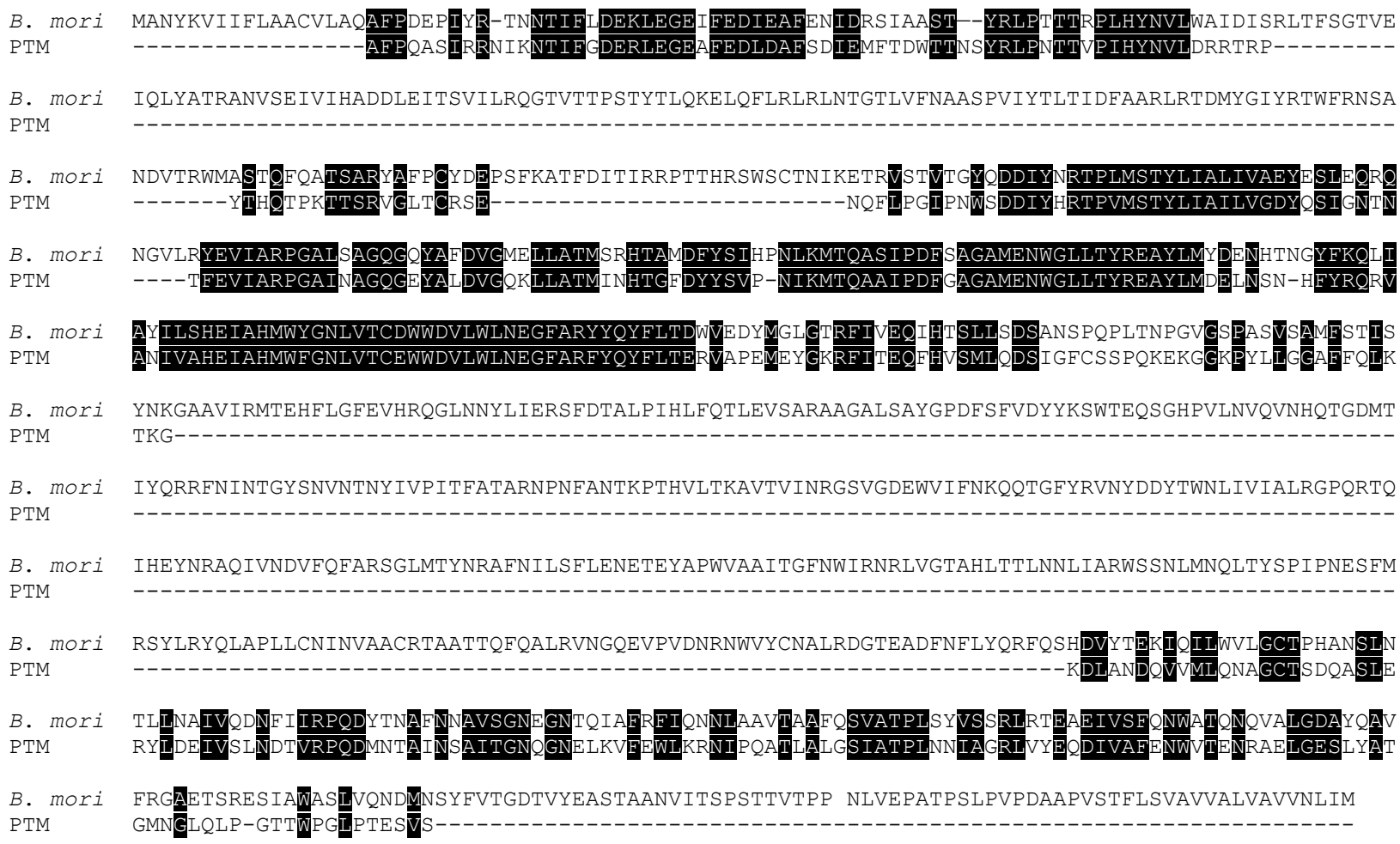
APPENDIX B

AMINO ACID ALIGNMENT



Amino acid alignment. Deduced amino acid of V-A15 and VII-K1 cDNA clones from PTM midgut with carboxypeptidase from *H. arimgera* (Acc. # CAA06419). The shaded areas represent either identical or similar amino acids of both sequences.

Amino acid alignment. Deduced amino acid of four cDNA clones (VIII-A17, VI-F9, V-E6 and II-C12) from PTM midgut with chymotrypsinogen from Agrotis ipsilon (Acc. # AAF71515). The shaded areas represent identical amino acids between the clones sequence and the cymotrypsinogen sequence.



Alignment of four cDNA contig clones (III-M1, V-B17, V-O7 and V-O3) from PTM midgut with aminopeptidase N from *B. mori* (Acc. # AAL83943). The shaded areas represent either identical or similar amino acids of both sequences.

```
H. cecropia   MFAKLFLVSVLLVGVNSRYVLVEEPGYYDKQYEEQPQQWVNSRVRRQAGALTINSDGTSGAVVKVPITGNENHKFSALGSVDLTNQM
PTM           MSSFGNITNNNDGSKNLTIGKVKEDGANTTTASLFVN---NQRDRDSHTNIRKNSYGAAGGNLSFQHGGNP----SATGSVTLQHIP

H. cecropia   KLGAATAGLAYDNVNGHGATLTKTHIPGFGDKMTAAGKVNLFHNDNHDFSAKAFATKNMPNIPQVPNFNTVGAGVDYMFKDKIGASA
PTM           KMGNQASAAAGIGVG----------------------------SETHRVGVDAFATRTMP--HHHPNFTTVGASVNAQAG-KVGAHA

H. cecropia   NAAHTDFINRNDYSLGGKLNLFKTPTTSLDFNAGWKKFDTPFFKSSWEPSTSFSFSKYF
PTM           GITHTPMFKQTQHSVGASLNLHKSPTSSVDMNIGSSRMTTPHHRGDWNHGVNFGVKKHF
```

Amino acid alignment. Deduced amino acid of full length of cDNA clone (X-M12) from PTM midgut with basic attacin from *H. cecropia* (Acc. #CAA44179). The Shaded areas represent either identical or similar amino acids of both sequences.

```
H. cunea     MNFSRILFFMFACFVALASVSAVPEPRWKVFKKIEKVGRHIRDGVIKAGPAITVVGQATALGK--
     PTM     -------GRGLSCFLALSVVSAN--PRWNPFKKLEKAGRRVRDGLIKAGPAVQVVGQASTIYKQG
```

Amino acid alignment. Deduced amino acid of cDNA clone (IX-K21) from PTM midgut with Hyphancin IIIG precursor from *Hyphantria cunea* (Acc. # P50723). The shaded areas represent either identical or similar amino acids of both sequences.

```
T. ni   MQSSILLIFAAFVACTYAQVSLPPGYAQKYPQYKYSKVARHPRDTTWEHNVGR-GKIFGTLGSNDDSVFGRGGYKQDIFNDHRGRLSGQAYGSR
PTM     --MKFLCIFAAVLVAVAAENEES--YYG-YPDVEYVPNPRFRRDVSLDHKVGQNGKVFGTLGSNDNGLFGKGGYEHNIFNDHRGNLNGQVTGTR

T. ni   VINDYGGSSILGGKLDWSNDNARAALDVHKEIGRGSGMKLSGDGVWKLDHNTRFSAGGNLQKNFGHNRPEFGIQGKIEHDF
PTM     VLGPYGDSSHVGGALNWKNDRATASLEASKMIHGPASVNAAAGGRWPVGKNGDLSAQGFYSQTQGLGR-DYGGRLNYNHRW
```

Amino acid alignment. Deduced amino acid of cDNA clone (XI-C8) from PTM midgut with Gloverin from *T. ni* (Acc. # AAG44367). The shaded areas represent either identical or similar amino acids of both sequences.

```
D. melanogaster  MKFFVLVAIAFALLACVAQAQPVSDVDP---------IPEDHVLVHEDAHQEVLQHSRQKRATCDLLSKWNWNHTACAGHCIA
PTM               MFALVLAMAAVVSSAPPKYEEPIEVLPSEYELSDVNVVEDLQNFLEEALTVEEAEARQARVTCDLLSGIGWNHTFCAAHCIF

D. melanogaster  KGFKGGYCNDKAVCVCRN
PTM              KGYKGGACNSKGVCVCRR
```

Amino acid alignment. Deduced amino acid of cDNA clone (IX-P2) from PTM midgut with defensin from *D. melanogaster* (Acc. # NM 078948). The shaded areas represent either identical or similar amino acids of both sequences.

```
B. mori       MPDGEGVPHLVDLEEPAEEDILMSRNGANNQYWLFTRRNQNNHQVITNGNVNSIRNSNYNGNLPLFVIVHGWNSNGNSAVNTM
PTM     MASLFLSVLTVVLVAVACQGNNLTLGRIARAGEGNHYYLYTR-DAPRTSVRVNPTVAAVN-LTRVWKPDTVIVIHDVKGNYLTGLNPT

B. mori      IRPALLAVSDCNVIVVDWRGLANGLYNTAVNGVPSVGQFLGNFLVWLINNGGGNWGRVHLIGFSLGAHVVGNAGRQAGGRPNRVTGLDP
PTM          VKDALLTAEDVNVIVMDWRSVAKHDMQFVTLALPQVADGLKDFIQILVQTRKVSLDKLHLVGLGVGAHLAGITARSVTN-VARITALDP

B. mori      AGPRWGGNNQALNRNAGAYVEAIHTDGGLLGIFDRIAHGDFYPNGGRNPQPGCRVSTCSHSRAYELYASTVRHNRFVGRLCNNLNQAQN
PTM          SG------------NAGILRDGLRDSDAL
```

Amino acid alignment. Deduced amino acid of cDNA clone (VI-L3) from PTM midgut with lipase-1 from *B. mori* (Acc. #AB076385). The shaded areas represent either identical or similar amino acids of both sequences.

```
B. germanica RGVGINGLIDDVIAILPVDELYALFQEKLESSPEFKALYDAIRSPEFQSIVQTLKAMPEYQDLIQRLKDKGVDVDHFIELIKKLFGLS
PTM          -GTNFSAFITDVIG-AAQEKLAALYEEKIAKDEEFRTAMENLQSEEWNTIYNAFLNSEEFKREAEILEQNGIDIEAIFDQVVAIFG-
```

Amino acid alignment. Deduced amino acid of VI-B18 cDNA clone from PTM midgut with major allergen Bla g 1.02 from *B. germanica* (Acc. # AAD13531). The Shaded areas represent either identical or similar amino acids of both sequences.

```
A. aegypti  MKTIFVLAALVAIATASAIPDSRALKDDFQEFVDLVPVDKLVNVALQYLVSDKEFKEFFGYLQGEEFSAVWDQFFALNEVKDVLNYLE
PTM         ----------------------NFSAFITDVIGELPKEKLAALYEEKIAKDEEFRTAMENLQSEEWNTIYNAFLNSEEFKREAEILE

A. aegypti  AADLAVYDALNTVADFLGLHHVKPTVHTLRTGGLTGFFDETVALLPLDKFEALFEEKLKTSPEFKAFFEKLRNLDYQKFVDFHNNSKE
PTM         QDGIDIEAIFDQVVAIFGQNNLTDSFSSR---NTTAFNLSSQASRIFGLIGDIYDIPREMS--YAILFIHLTELHVAVYQHYFVSRIK

A. aegypti  VQGFLQKLR SYGLDVDGFFNLVAGFFGWGKF
PTM         YKLSVPARA
```

Amino acid alignment of cDNA clone (VI-A2) from PTM midgut with microvilli protein from *A. aegypti* (Acc. # AAL05408). The shaded areas represent either identical or similar amino acids of both sequences.

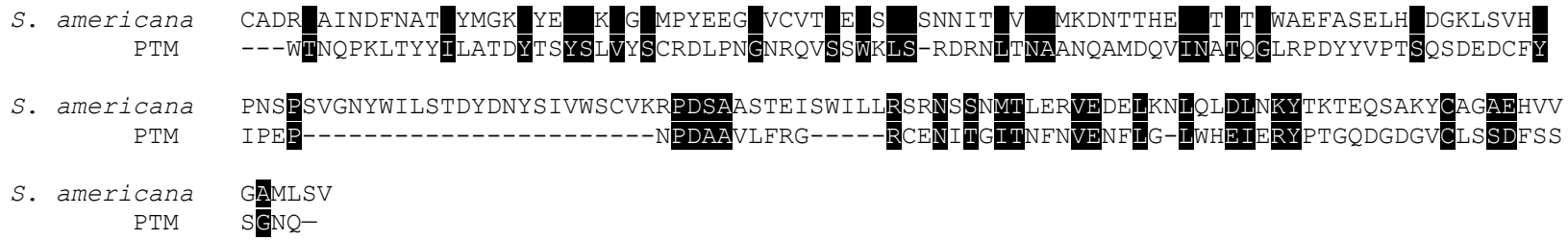
```
Samia cynthia ricini   AGKWYEIKKLPLANEG--KGQCAIATYTLDGDSLKVKNSHVINGVEKYVLGVAKKADDANGSGKLVLTVTVGKFSRV
                PTM    ---WREIETYPKEQAGGQCINHEFSAVATSNNTLQLISNQVFNSETLVSNDGQLVLDSTDGSGRFSLTINVGNSSFT

Samia cynthia ricini   APLWILTTDYTNYAVSYSCKYNEKNNTHRLNIW
                PTM    IPYWVITTDYENYALVYSCMNRDNDTRAS----
```

Amino acid alignment. Deduced amino acid of cDNA clone (VII-B22) from PTM midgut with biliverdin binding protein-I from *Samia cynthia ricini* (Acc. # BAB85482). The shaded areas represent either identical or similar amino acids of sequences.

```
Rhodococcus sp.    --MTSDIVVIGGGVAGVTAAQSLRSEGYDGRLVLIGKERELPYDRTALSKAVLAGDLADPPLLFPADWYDEWQIE
            PTM    PCDSKSVVIIGGGPSGATCAETLRSEGYTGTITLVAREPYLPYDRIKVSKIGTVTDINKLQARNDE---------
```

Amino acid alignment. Deduced amino acid of cDNA clone (VIII-E5) from PTM midgut with biphenyl dioxygenase from *Rhodococcus sp*. RHA1 (Acc. # BAA06871). The shaded areas represent either identical or similar amino acids of both sequences.



Amino acid alignment. Deduced amino acid of cDNA clone (VII-D6) from PTM midgut with lazarillo protein precursor from *Schistocerca americana* (Acc. # P49291). The shaded areas represent either identical or similar amino acids of both sequences.

Amino acid alignment. Deduced amino acid of X-B5 cDNA clone from PTM midgut with 40S ribosomal protein S17 from *An. gambiae* (Acc. # Q9U9L1). The shaded areas represent either identical or similar amino acids of both sequences.

Amino acid alignment. Deduced amino acid of IX-A24 cDNA clone from PTM midgut with sensory appendage protein from *An. gambia*e (Acc. # AAL84186). The shaded areas represent either identical or similar amino acids of both sequences.

VITA

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Publication	

Mohammed, A., Douches, D. S., Pett, W., Grafius, E., Coombs, J., Liswidowati, Li, W., Madkour, M. A. 2000. Evaluation of potato tuber moth (Lepidoptera: Gelechiidae) resistance in tubers of Bt-cry5 transgenic potato lines. J. Econ. Entomol. 93, 472-476