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Abstracts are listed in alphabetical order by the last name of the senior author.

Does pollen feeding have a single origin in *Heliconius* butterflies?

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The genus *Heliconius* or passion-vine butterflies and its relatives *Laparus*, *Eueides* and *Neruda* are one of the best known groups of neotropical butterflies. These four genera have undergone rapid speciation and divergence. Additionally, this genus has been considered one of the most remarkable examples of mimicry, and it has been important in understanding ecological processes like coevolution between insects and plants. A well-resolved phylogenetic hypothesis for the group is needed to understand how unique traits such as pollen feeding evolved, and to test the role of mimicry in driving speciation. A recent morphological study suggested that adult pollen feeding has evolved twice, in the genera *Laparus* and *Heliconius*, but mtDNA sequence data places the species *Laparus doris*, and the genus *Neruda*, within *Heliconius*, suggesting a single origin of pollen feeding. Here I address the question: Is *Heliconius* a monophyletic lineage? I sampled 111 individual butterflies, representing 37 *Heliconius*, 1 *Laparus*, 2 *Neruda*, 10 *Eueides*, and 10 outgroup species and sequenced COI and COII mtDNA and a nuclear marker Elongation factor 1∝. Additionally, representative individuals from each genus were sequenced for 16s mtDNA and two further nuclear genes Apterous and Decapentaplegic making a total of 2100bp of mtDNA and 1245bp of nuclear sequence. The results suggested that *Heliconius* is paraphyletic, with *Laparus doris* falling within the genus, demonstrating a single origin for pollen feeding. However different genes do not produce a clear agreement as regards relationships of the genera *Neruda* and *Laparus* within *Heliconius*, suggesting a rapid basal radiation of *Heliconius*.

SAGE-aided transcriptome analysis of the silk gland cells of *Bombyx mori*

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The silk gland of Bombyx mori provides an excellent model to study differential gene expression which lies at the heart of cell specialization in multi-cellular organisms. The current view is that the specification of each category of cell during development is accomplished by employing cooperativity and transcriptional synergy in which a limited repertoire of transcription factors is used to execute a diversity of regulatory decisions. Such a model could apply to the specification of the two categories of secretory cells in the silk gland : the posterior cells that produce fibroin and the middle cells that secrete sericins, the two major components of silk. Silk gland morphogenesis results in the spatial distribution of the fibroin and sericin-secreting cells, in posterior (PSG) and middle (MSG) position, respectively. Accordingly, the very high expressed genes encoding the fibroin subunits (H-fib, L-fib and fhx) and the sericins (Ser-1 and Ser-2) are strictly spatially controlled. Investigations on the mechanisms of silk protein gene regulation have led to identify a series of transcription activators (POUM1, SGFB, BmFA, SGF1/ fork head…) but the manner by which the discrimination of PSG and MSG specific genes is controlled is still not understood. It is likely that the combination of the elements of the transcriptional machinery may play a decisive role in this process. It is also

suggested that chromatin remodeling factors act in controlling the accessibility of the transcription factors to their target sequence in the PSG and the MSG cells. Likely, both qualitative and quantitative differences in regulatory gene expression probably induce the selective activation of the different silk protein gene promoters in the posterior and the middle silk gland cells. To better understand the genetic basis of PSG and MSG cell specialization we analyzed the transcriptome of the two categories of cells, using the SAGE method (Serial Analysis of Gene Expression -Velculescu et al. 1995) which allows to study the redundancy and the diversity of mRNA. The power of this technique is that it allows the identification of rare mRNA that are present at very low cellular concentrations.
Two SAGE libraries have been constructed from mRNA purified from middle silk gland at day 3 of the 5th intermolt. Presently, 2304 clones of the MSG library were sequenced (representing more than 20000 transcripts) but only 96 clones (1700 transcripts) from PSG library have yet been analysed in a pilot study. The analysis of the SAGE data needed the development of adapted softwares. Identitag was designed to create a reference library of annotated mRNA tags from a public cDNA databank, for the comparison of two experimental libraries and for statistical analysis and graphical representation. We extracted 20362 (MSG) and 1712 (PSG) tags that correspond to 4750 and 621 different species, respectively. The reference database has been constructed from 29114 GenBank cDNA sequences. The properties of the MSG mRNA population and a tentative comparison of MSG and PSG mRNA are presented.

Spodoptera frugiperda: genomic tools to study holocentric chromosome structure

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A bacterial artificial chromosome (BAC) library was constructed using *Hind*III partially digested nuclear DNA from eggs of the fall armyworm *Spodoptera frugiperda*. The library contains a total of 36864 clones with an average insert size of 125 kbp, which corresponds to approximately 11.5 genome equivalents. Hybridization screening of the library was performed with 8 single-copy genes, giving an average hit of 9.9 clones per marker gene (ranging from 5 to 16). Contamination of the library with mitochondrial DNA is less than 0.083 %. Genomic regions cloned in BACs were structurally stable. Colinearity between the genome and BACs was checked at the *triose phosphate isomerase* (*tpi*) locus. Probing of the library with a PCR fragment internal to the 18S ribosomal gene allowed an estimation of the rDNA locus size closed to 115 repeats per haploid genome. A new vector (pBAC3.6eGFP) for transient transfection into *Spodoptera frugiperda* cell lines has been constructed. It is based on the BAC vector, pBAC3.6e, in which a gene encoding GFP was inserted under the control of the densovirus P9 promoter (1). This vector has the advantage to accommodate large genomic inserts and to be used in a large lepidopteran host-range. It was used to construct a second BAC library from *Sf*9 cells nuclear DNA, covering potentially 5 genome equivalents, in order to allow comparison between native and cell line genomes. *Spodoptera frugiperda* is the third holocentric organism (2) after *C. elegans* (3) and *B. mori* (4) for which a BAC library is available, which will allow a comparative study of holocentric chromosome structure.

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Functional genomics of silk gland development

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We have used the mulberry silkworm, *Bombyx mori*, as a model to investigate conserved and divergent aspects of embryonic and larval patterning. The *Bombyx* larvae have a pair of silk glands (SGs), divided into three anatomically and functionally distinct regions: anterior (ASG), middle (MSG) and posterior (pSG), which display an immense degree of functional differentiation. The PSGs exclusively synthesize the silk fibre proteins (fibroin H and L chains and fibrohexamerin) while; the MSGs make the glue proteins (sericins). The ASGs serve as ducts to transport the silk proteins in the final secretion process. The silkglands are ectodermal derivatives which arise from the labial segment and thus are considered as evolutionary homologues of the *Drosophila* salivary glands. The silkglands are fully formed by the end of embryogenesis but they grow enormously in size during larval development without further increase in cell number. The DNA replicates without any nuclear or cell divisions, a phenomenon called endomitosis. Using the G1 cyclin, Cyclin E and the mitotic cyclin, Cyclin B as markers, we have shown that the silkglands completely switch over to endomitotic cell cycle by stage 26 of embryogenesis and this correlates with the onset of expression of sericin-2 and fibrohexamerin, the markers of silkgland terminal fate. These data

also shed light on the possible mechanisms governing the attainment of typical silkgland morphology. Not much information is available on the fate specification of the silkgland compartments, although compartment specific expression of various regulatory factors is presumed to be responsible. We investigated the role of segment polarity genes *wnt-1, Cubitus interruptus (Ci)* and *engrailed* in silkgland development. The expression pattern of *wnt-l* and *Ci* were analyzed spanning embryonic to larval stages of silkgland development and was restricted to the MSGs. The two genes were found to be co-expressed in the anterior region of the MSGs. On the other hand, *engrailed* was expressed in the middle and posterior regions ofMSGs. Thus, *wnt-1/Ci* and Engrailed are mutually exclusive in their localization. The expression of different sericin isoforms in MSGs follows a region specific pattern. An overlap of *wnt-1/Ci* expression domain with that of *sericin-2* was clearly seen, while the expression domain of Engrailed matched with that of *sericin-1*. Together, these data suggest the presence of a well defined canonical wnt-l signaling network in the MSGs and may have an important role in defining the MSG subcompartments.

Expression of polydna virus genes in lepidopteran insect cell lines

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Under a general research framework that aims in the identification of potential bioinsecticides derived from insect parasitoids, our specific goal is to express polydnavirus gene products in an expression system similar to their natural hosts. Thus, cDNAs deriving from *Toxoneura nigriceps* bracovirus (TnBV1) and from *Cotesia congregata* bracovirus (CcBVEP1, cystatin CcBVCyst1) were expressed in lepidopteran cells using appropriate vectors previously developed in our laboratory. Vector pEIA enables high-level constitutive expression of cloned ORFs (native proteins) in lepidopteran insect cells, while vector pEIA.hGM-CSF.6xHis–EK enables constitutive expression and secretion of fusion proteins, easy purification by affinity chromatography, and release of the protein of interest after enterokinase cleavage.

Native expression of TnBV1 after transfection of Bm5 and Hi5 cells with the construct pEIA.TnBV1, led to growth arrest and high mortality compared with mock-transfected cells. The same phenomenon was observed after TnBV1 expression in Sf21 cells (J. Olszewski, Imperial College, London, personal communication), indicating that native TnBV1 protein might have a toxic effect on lepidopteran cells. In an effort to overcome possible toxic effects of polydnavirus gene products on the cells used for expression, we employed an expression strategy based on secretion of the protein of interest in fusion with hGM-CSF. Stably transformed clonal cell lines, expressing and secreting fusions of TnBV1 (~8 μg / 10^6 cells) and CcBVEP1 (~1 μg / 10^6 cells) were constructed. No toxicity was observed for these lines. Different purification strategies were developed for each of the proteins of interest. Culture media containing the secreted hGMCSF-TnBV1 fusion protein were first subjected to 50% ammonium sulfate precipitation in order to remove excess of contaminating (serum) proteins. Protein pellet containing the fusion protein was resuspended in PBS and dialyzed against binding buffer for subsequent affinity chromatography with Ni-NTA agarose (Qiagen) under strong denaturing conditions. On the other hand, culture media containing the secreted hGMCSF-CcBVEP1 fusion protein were dialyzed against binding buffer for subsequent affinity chromatography with Ni-NTA agarose under native conditions. Optimization of the purification protocols is still in progress. Finally, CcBVCyst1, a CcBV gene resembling significant similarity with a family of cystein protease inhibitors known as cystatins (J.M. Drezen, University of Tours, personal communication), was subcloned in pEIA and the construct was used for constitutive expression of the native, secreted protein in stably transformed serum-free HighFive cell lines. Using an enzymatic (papain) assay, culture media from these lines exhibited high inhibition in comparison with medium from non-transformed cells. This is a strong indication that the CcBV ORF is correctly expressed, the protein is secreted into the medium and is active as a cystein protease inhibitor.

Cloning of BmCactus cDNA from *Bombyx mori* and effect of BmCactus on promoter activities of antibacterial peptide genes.

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Insects have developed unique and powerful innate immune systems and adapt themselves to various environments. Antibacterial peptides are known as one of main arms of insect humoral defense reactions against invading pathogens and parasites. They are synthesized primarily in the fat body and haemocytes and rapidly secreted into haemolymph upon bacterial infections. In *Drosophila*, NF-κB binding site-like motif is found in promoter region of antimicrobial peptide gene. And so far, two signal transduction pathways have been reported to induce antimicrobial peptide gene expression; IMD pathway and Toll pathway. *Drosophila* IκB protein homologue, Cactus, is one of the components involved in Toll pathway and inhibits antibacterial peptide gene expresssion by binding NF-κB (Rel) family protein in cytoplasm. NF-κB binding site-like motif is also found in promoter regions of antibacterial peptide genes from *B. mori*. And *Bombyx* NF-κB family proteins, BmRelA and B, have been cloned. In this presentation, we report a novel IκB family protein (BmCactus) gene from the *B. mori* expressed-sequence tag (EST) database. The deduced amino acid sequence from the nucleotide sequence had 25% homology with *Drosophila* IκB protein, Cactus, and there are five ankyrin repeat domains conserved in all IκB family proteins. The BmCactus gene was constitutively expressed at high level in the fat body and haemocytes, and at low level in the midgut and Malpighian tubule but not in the silk gland. BmCactus gene expresssion was increased

transiently by LPS injection. NISES-BoMo-DZ is a LPS-responsible cell-line of the *B. mori*. Transfection experiments using this cell-line showed that BmCactus does not affect LPS-responsive promoter activities of antibacterial peptide genes. However, co-transfection experiments revealed that BmCactus can suppress up-regulated promoter activities by overexpression of both of Rel family proteins of *B. mori*, BmRelA and B. Moreover, the yeast two hybrid experiment confirmed that BmCactus could interacted with rel homology domain of BmRels directly. These results suggest that BmCactus plays a role in the signal transduction pathway which is not activated by LPS.

Linkage mapping reveals a diversity of mechanisms of resistance to *Bacillus thuringiensis* toxins in Lepidoptera

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Insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely utilized in foliar sprays and transgenic plants to control Lepidopteran pests of agricultural crops, but the development of resistance poses a threat to their sustainable use. The most common type of resistance to Bt toxins ("Mode 1") is characterized by recessive inheritance, a high level of resistance to (and reduced midgut binding by) at least one Cry1A toxin, and negligible cross-resistance to Cry1C (Tabashnik et al 1998, Phil Trans Roy Soc B 353: 1751). A 12-domain member of the cadherin superfamily is expressed on larval midgut epithelial membranes and is involved with Cry1A mode of action in Lepidoptera. Mode 1 resistance is caused by mutations in this cadherin in two Lepidopteran species, *Heliothis virescens* (Gahan et al. 2001 Science 293: 857) and *Pectinophora gossypiella* (Morin et al. 2003 PNAS USA 100:5004), making this gene a prime target for developing DNA-based screening methods. However, using a linkage-mapping approach, we have been able to show that Mode 1 resistance in a third species, diamondback moth *Plutella xylostella*, has a different genetic basis. This indicates that screening for mutations in the cadherin gene, while useful in specific cases, may not provide a universal method for detecting Bt resistance in the field. Moreover, non-Mode-1 resistance in the cotton bollworm, *Helicoverpa armigera*, as well as *Heliothis virescens*, is caused by a different mechanism altogether. We discuss the implications of this complex genetic basis of resistance for the durability of Bt as a pest control strategy, and for our understanding of Bt mode of action.

Resources For Comparative Linkage Mapping In Lepidoptera.

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We are developing tools for comparative linkage mapping in Lepidoptera. Specifically, we want to assess the extent of synteny, or gene linkage, and microsynteny, or fine structure, in lepidopteran chromosomes, information that can facilitate gene tracking, positional cloning, finding transgene landing sites, population and ecological studies, and phylogenetic reconstruction. Lepidopteran chromosomes are holocentric, with many microtubule attachment points, in contrast to those of most Metazoa, which are monocentric. This distinctive architecture suggests the possibility that chromosome fragments might persist through many cell divisions, leading to a high level of karyotypic change by chromosome fragmentation or rearrangement. This, in turn, may have contributed to the explosive radiation of lepidopteran lineages (>170,000 extant species today) that occurred between 50 and 100 million years ago. As a reference for comparative studies, we have expanded established genetic linkage maps (Nagaraju & Goldsmith, 2002) for the domesticated silkworm, *Bombyx mori*, to >100 well-conserved codominant molecular markers, based on RFLPs, cloned RAPD sequences, and newly mapped cDNAs and STSs from highly conserved cDNAs in SilkBase (http://www.ab.a.u-tokyo.ac.jp/silkbase) and other public databases. We have also begun testing the feasibility of using two-color chromosomal Fluorescence In Situ Hybridization to assess linkage in species that are not readily used for inheritance studies. We detected the large rDNA cluster (~240 copies) on chromosome 11 in 4th–5th instar larval testes spreads with ~2 kb plasmids, but saw few reliable signals with cDNAs up to 5 kb using indirect labeling systems (biotin:avidin-Texas red-anti-avidin and digoxygenin:FITC-anti-dig). Larger genomic fragments from BAC libraries (~150 kb) and use of larval ovarian tissue for chromosome spreads yielded more consistent results, and we are now optimizing protocols using model chromosomes with linked markers whose recombination distances are unknown. To serve as chromosomal landmarks and as a source of additional markers to examine detailed gene organization and microsynteny, we have constructed contigs covering >30 genetically mapped ESTs by DNA fingerprinting using two new silkworm BAC libraries (>10X coverage using *Eco* RI and *Hind* III partial digests with ave. insert size of 151 and 166 kb, respectively). For use in initial comparative studies we have also constructed *Eco* RI and *Bam* HI BAC libraries with 10.7X coverage for the tobacco hornworm, *Manduca sexta*, which is in the same superfamily as *B. mori* (Bombycoidea), and 13X coverage for *Heliothis virescens*, which is in a different superfamily (Noctuoidea). Average insert sizes for these libraries are 150 kb, 165 kb, 145 kb, and 145 kb, respectively. Comparisons will be facilitated by similar chromosome numbers (N=28, 28, and 31 for the silkworm, the hornworm, and the budworm, respectively) and genome sizes (~530 Mb *vs* 500 Mb,

for *Bombyx vs Manduca* and *Heliothis*), and high levels of sequence conservation in protein-coding genes will aid identification of orthologous genes.

Analysis and functional annotation of genes expressed in the midgut of the cotton bollworm, *Helicoverpa armigera*.

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Lepidopteran insects include important pests of agriculture, like *Helicoverpa armigera*, the cotton bollworm. Many control strategies for these pests involve orally-transmitted control agents that target the larval midgut. A functional genomics study of the larval midgut has the potential to identify new targets for pest control, and to increase understanding of the molecular biology of these important animals. We have commenced an EST project focussed on genes expressed in midguts of younger larvae to identify genes important for midgut growth and development and gene/enzyme pathways involved in processes such as insect-pathogen and insect-plant interactions. Sequence analysis of midgut cDNA libraries now covers ca. 4,900 ESTs from 2nd and early 3rd instar and over 2100 from 5th instar larvae. Clustering using Stackpack yielded a unigene set of about 2,500 contigs. Searching against Swissprot-TrEMBL allowed confident annotation (probability better than 1E-8) of ca. 55% of the contigs. Functional classification using GO terms identified a role in a biological process for 40% of the contigs, a molecular function for 50%, and identified a quarter as cellular components. 45% of the contigs have confident orthologs in the *Drosophila* proteome; a similar number had good matches against the human proteome. For comparison of lepidopteran genomes, the 35,000 silkworm ESTs at Silkbase have been subjected to a similar analysis. Clustering using Stackpack yielded 12,500 non-redundant sequences. About half the *H. armigera* contigs yielded orthologs among the silkworm contigs. The *H. armigera* set also includes a significant number of well-annotated sequences with no match in the silkworm set, likely reflecting the midgut-specific origin of the bollworm ESTs.

Regulation of baculovirus late and very late gene expression.

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Baculovirus expression vectors are widely used due to their high level expression of foreign genes under the control of the strong polyhedrin promoter. Transcription of the very late polyhedrin gene and the standard late genes is mediated by a viral-encoded RNA polymerase. This is a multi-enzyme complex composed of four viral proteins. The purified complex has the ability to recognize viral promoters, transcribe linked genes, cap the 5' ends of mRNA transcripts, terminate transcription, and polyadenylates the 3' ends. Although the four-subunit complex is multi-functional, additional viral proteins are known to be essential for late transcription in infected cells. One of these proteins, called LEF-5, has a zinc ribbon that is homologous to a domain in the eukaryotic transcription elongation factor SII. To determine whether LEF-5 is an elongation factor, it was added to purified baculovirus RNA polymerase. LEF-5 increased transcription from both late and very late viral promoters, and acidic residues within the zinc ribbon were essential for stimulation. Unlike SII, however, LEF-5 did not appear to enable RNA polymerase to escape from intrinsic pause sites. Initiation and elongation assays suggested that LEF-5 functions as an initiation factor, not as an elongation factor as expected. The late expression factor LEF-6 also increased the activity of purified RNA polymerase, and also appeared to act at the level of initiation. The effects of LEF-5 and LEF-6 were additive. The viral protein ORF-69 has an S-adenosyl-methionine (AdoMet)-dependent methyltransferase signature motif. More significantly, it shows high conservation at residues diagnostic for (nucleoside 2'-O)-methyltransferase activity. Photo cross-linking experiments showed that MTase1 bound AdoMet, and functional assays demonstrated cap 0-dependent methyltransferase activity. The core baculovirus RNA polymerase specifically recognizes and transcribes viral genes, indicating that at least one of the subunits has promoter recognition activity. But it transcribes late and very late genes with equal efficiency, while they are differentially regulated in vivo. In an infected cell, the late genes are expressed first and at lower levels than the very late genes, which are maximally transcribed after the production of progeny virions. This suggests that the core RNA polymerase recognizes a feature common to both classes of late genes, and cannot discriminate between late and very late promoters. Addition of purified VLF-1 (very late factor-1) to RNA polymerase resulted in a concentration-dependent increase in transcription from the polyhedrin promoter but had no effect on the late 39k promoter. This indicates that VLF-1 is a discociable transcription initiation factor that is responsible for promoter specificity.

Amyloid fibrillogenesis of silkmoth chorion protein peptide-analogues via a liquid crystalline intermediate phase

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Chorion, the major component of silkmoth eggshell, consists of the A and B classes of low-molecular weight structural proteins. Chorion protects the oocyte and the developing embryo from environmental hazards and this is due to the extraordinary physical and chemical properties of its constituent proteins. We have shown previously that peptide analogues of the A and B classes of chorion proteins form amyloid fibrils under a variety of conditions, which led us to propose that silkmoth chorion is a natural, protective amyloid. In this work, we present data showing conclusively that, the first main step of amyloid-like fibrillogenesis of chorion

peptides is the formation of nuclei of liquid crystalline nature, which is reminiscent of spider-silk formation. We show that these liquid-crystalline nuclei (spherulites) 'collapse'/deteriorate to form amyloid fibrils in a spectacular manner, important, it seems, for chorion morphogenesis and amyloid fibrillogenesis in general. The molecular 'switch' causing this spectacular transformation is, most probably, a conformational transition to the structure of chorion peptides, from a left-handed parallel β-helix to an antiparallel β-pleated sheet. Apparently, these peptides were suitably designed to play this role, after millions of years of molecular evolution.

Molecular and cDNA microarray analysis of steroid-induced muscle degeneration during insect metamorphosis

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During metamorphosis in the hawkmoth, *Manduca sexta*, segmental homologs of the accessory planta retractor muscle (APRM) undergo programmed cell death (PCD) in a segment-specific pattern. The PCD of APRMs is triggered directly and cell-autonomously by the steroid hormone, 20-hydroxyecdysone (20E; Hazelett & Weeks, 2000 and in prep.). To seek genes involved in intrinsic segmental identity, 20E signaling and muscle degeneration, we performed suppressive subtractive hybridization (SSH) on mRNA from APRMs in abdominal segments A3 and A6; APRM(6)s undergo PCD while APRM(3)s survive during the larval-pupal transformation. mRNA was obtained on the first day of the prepupal peak of 20E. We generated a library of approximately 2000 clones, some of which are redundant. These cDNAs, plus other candidate cDNAs, were spotted on microarrays and hybridizations are in progress to study gene expression in APRMs during prepupal 20E signaling. Approximately 400 of the clones were sequenced and, based on comparison with *Drosophila* and other species, many encode potentially interesting regulators of development. Concurrently, we examined the expression of the Bithorax Complex (Bx-C) gene abdominal-A (abd-A), and ecdysone receptor (EcR) isoforms, using real-time PCR. Bx-C genes regulate axial patterning of the insect abdomen (Lawrence & Morata, 1994). Real-time PCR data suggest that abd-A is expressed in APRMs in a segmental pattern similar to that seen in fly embryos. Furthermore the expression level of abd-A is strongly upregulated during the prepupal peak of 20E. Potentially, the expression of axial patterning genes of the embryonic stage is recapitulated during metamorphosis to direct the segmental fates of individual cells in response to hormonal cues.

Diversity of trypsin genes in the Mediterranean Corn Borer *Sesamia nonagrioides*

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Insect resistant transgenic corn plants expressing Cry toxins from *Bacillus thuringiensis* (Bt) are commercially cultivated since 1996-1998. Due to the entophytic behaviour of corn borers, Bt-corn offers an especially appropriate alternative to conventional insecticides traditionally used for pest control. However, one of the mayor problems predicted for the transgenic technology applied to pest control will be the rapid selection for resistance in pest populations. The study of possible mechanisms of resistance developed by insects may collaborate to design the appropriate strategies for resistance management. Digestive endoproteases may be involved in resistance to Cry toxins. They participate in degradation and they are needed for toxin activation. We are studying the diversity of trypsin endoproteases in the Mediterranean Corn Borer *Sesamia nonagrioides* (Lepidoptera; Noctuidae) by PCR on genomic DNA and by RT-PCR and RACE-PCR performed on mid-gut RNA. Three types of trypsins conserved among other lepidopteran insects, are recognised in our sequences. N-terminal sequences from type I, II and III active trypsins purified from midgut extracts are homologous to predicted proteins from obtained clones. The predicted protein sequences have been use in building three-dimensional models for active enzymes. These models suggest the importance of diverged residues conserved in each trypsin type that could influence on the differential specificity and interaction with inhibitors showed by purified trypsins. Studies on gene expression in different developmental instars confirm previous experimental data (Novillo et al. 1999) suggesting that type I trypsin is functionally important in early developmental stages, being type II trypsin more important in late developmental instars. The interaction between Cry1Ab native protein and purified insect endoproteases is being studied.

Aminopeptidases N expression in the midgut of *Spodoptera exigua* larvae.

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The mode of action of the crystal toxins from the entomopathogenic bacterium, *Bacillus thuringiensis* (Bt), is a multiple step process that includes: solubilization of the crystal to release the Cry proteins in their protoxin form, processing of the protoxins by midgut proteases to their active form, binding of the toxin to a midgut receptor, and pore formation. Receptor/toxin-interactions are considered major determinants of specificity of the toxins. Previously identified putative receptors for Cry proteins comprise cadherin-like proteins and aminopeptidase N. In order to study changes in midgut gene expression correlated with exposure to a sublethal dose of toxin, Suppression Subtractive Hybridization was

used to construct cDNA libraries of genes that are up- or down-regulated in the midgut of last instar larvae of the beet armyworm, *Spodoptera exigua,* exposed to Cry1Ca toxin. A similar approach was used to compare gene expression between susceptible insects and larvae of a colony selected for resistance to this toxin, in order to identify gene expression changes associated with toxin resistance. Among the clones of these libraries, fragments of three different aminopeptidases N (APN) cDNA's were obtained. Starting from these fragments, using RACE the complete cDNA sequences of these genes were obtained. A fourth APN encoding cDNA was obtained using degenerated primers based on published sequences of other lepidopteran APNs. Here we show the "*in silico*" analysis of these four proteins and the comparison in the expression levels of these genes between susceptible and resistant insects. Whereas 3 APN genes are expressed equally in sensitive and resistant larvae, a fourth APN is only expressed in sensitive larvae. Combined with the proposed function of APN as toxin receptor, this suggests that at least part of the observed toxin resistance may be caused by lack of expression of this putative receptor.

Study of genomic DNA sequences of the four aminopeptidases-N within *Plutella xylostella*

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Plutella xylostella (diamondback moth) is the most important crop pest lepidopteran of crucifers. Moreover, *P. xylostella* is the only insect species that has developed resistance to insecticidal Cry proteins of *Bacillus thuringiensis* in the open field. Our group is working with the diamondback moth as a model to study the biochemical and genetic basis of resistance to Cry proteins.

The key step in the mode of action of Cry proteins is the binding to specific sites in the epithelial membrane of the midgut. In fact, four aminopeptidases-N (APN-1, APN-2, APN-A and APN-3) involved in this binding have been reported in *P. xylostella*. These APNs are implicated in the digestion of the proteins as physiological function.

Mapping APNs is a first step to determine its genetic linkage to resistance. This study requires the presence of alleles in the studied populations. The reported APN1 of *Manduca sexta* has been used as a pattern genomic sequence for aligning with the four APNs cDNA of *P. xylostella*, and determine intron positions. Length polymorphism in the first intron of three *P. xylostella'*s APNs has been detected. The results of the present study indicate that three APNs (APN-1- APN-2 and APN-A) are not linked. Possible genetic linkage of resistance to one APN gene would mean that only this APN would be involved in the resistance.

Unique features of the structural model of hard cuticle proteins

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Cuticle is a complex, bipartite, composite material made of chitin filaments embedded in a proteinaceous matrix. The interaction of cuticular proteins with chitin and the detailed structure of insect cuticle have not yet been resolved. Sequence studies on cuticle proteins have revealed that certain sequence motifs occur in proteins from even distantly related species and such conserved motifs have common and important roles for the proper function of cuticle. One such motif is the "R&R consensus sequence" first identified by Rebers and Riddiford (1988) in seven cuticular proteins: G-x(8)-G-x(6)-Y-x-A-x-E-x-G-Y-x(7)-P-x(2)-P or a modification of it: G-x(7)-[DEN]-G-x(6)-[FY]-x-A-[DGN]-x(2,3)-G-[FY]-x-[AP]-x(6). An extension of this motif is a stretch of approximately 68 amino acids, which appears to be conserved, the "extended R&R consensus". It was postulated that this motif might be involved in protein/chitin interaction. Recently, we presented secondary structure prediction and experimental data indicating that β-pleated sheet is most probably the underlying molecular conformation of a large part of this extended R&R consensus, especially the part which contains the R&R consensus itself. We also proposed that this conformation is most probably involved in β-sheet-chitin chain interactions of the cuticular proteins with the chitin filaments. This proposal and our recent experimental findings are in agreement with earlier experimental findings and proposals that β-sheet should be involved in chitin–protein interactions. Also, recently, we detected an unexpected distant sequence similarity of soft cuticle proteins with bovine plasma retinol binding protein (RBP). Retinol binding protein has a β-sheet barrel as its basic structural motif. A large part of this β-sheet barrel is the part similar in sequence to the "extended R&R consensus" sequence of "soft" and of "hard" cuticular proteins. Based on the sequence similarity of RBP with a representative member of the "soft" cuticle proteins, HCCP12, we constructed, by homology modelling, a structural model of the "extended R&R consensus". This model has several attractive features to serve as a chitin binding structural motif in cuticle and to provide the basis for elucidating cuticle's overall architecture in detail. The model was subjected to a further test: as such, it should provide for the right positioning of histidine residues in the "hard cuticle" proteins, so that these histidines might play a significant role in cuticle sclerotization. Interestingly, it is seen that histidines occupy "exposed" positions either in turns or at the "edges" of the half-beta barrel or its periphery, in excellent positions to be involved in cuticular sclerotization, readily reacting with activated N-acetyldopamine residues, or being involved it the variations of the water binding capacity of cuticle and the interactions of its constituent proteins, due to the fact that small changes of pH can affect the ionization of their imidazole group. A few (2-3) histidines are also interspecies conserved. These observations are in excellent agreement with the predictions made for the role of histidines from secondary structure prediction and strengthen further the value of the model proposed both for "soft" and "hard" cuticle proteins.

Production of Recombinant Proteins in Posterior Silk Gland of Transgenic *Nd-S* Mutant Silkworm.

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We have shown that recombinant protein can be produced in the silk gland and expelled into the filament of cocoons formed by the transgenic silkworms using fibroin L chain promoter and cDNA (Tomita, et al., 2003). However, the efficiency of the protein production is rather lower because the recombinant proteins compete with the normal L chain in the process of forming disulfide linkages with the fibroin H chain. In most cases, the formation of the disulfide linkages of normal L chain is thought to be faster than the recombinant protein. Therefore the poor production in the transgenic line of the normal silkworm may be due to a lower rate of forming disulfide linkages. To circumvent the problem we investigated the possibility to use the *Nd-sD* mutant, which is secretion deficient of fibroin molecule, for more efficient production of recombinant proteins in the transgenic silkworm. Silk consists of two types proteins, sericin and fibroin. The sericin is synthesized in middle division of silk gland and the fibroin is produced in the posterior division. The fibroin occupies the core of cocoon filament and the sericin cover the surface of the filament. Silk is about 75% fibroin, with the remaining part sericin. The fibroin consists of three polypeptides, a heavy chain, a light chain and fibrohexamerin. The formation of complex of the three peptides in the ratio of 6:6:1 is crucial for the secretion of the molecules from the posterior silk gland cells to the lumen of silk gland (Inoue et al., 2001). The *Nd-sD* mutant is known to produce a sericin cocoon since the part of L chain gene is deleted and the mutant L chain cannot form S-S linkage with the fibroin H chain. The complex does not form in the silk gland cell. As the result, the fibroin does not secrete into the lumen and also into the filament. Therefore the cocoon produced by the mutant contains only sericin. In the present study, a construct was made with the normal L-chain gene fused to GFP (green fluorescent protein) gene to observe the flow of the product and inserted into the *piggyBac* vector plasmid with the selection marker of 3XDsRed. The vector and helper were co-injected into early embryos of the *Nd-sD* mutant after treatment of acid to break the diapause. We injected about 550 eggs and hatched 340 larvae. The sib mating of adults developed from the hatched larvae gave 110 G1 broods and the transgenic larvae were obtained from 20 broods. One line was confirmed by mating the transgenic silkworms and used to investigate the morphological change of the mutant and production of the introduced gene. A high level of gene expression in the posterior silk gland cells of the transgenic silkworm was determined with a Northern Blot. The production of fused protein was investigated by observing the fluorescence of GFP and Western Blotting with L chain and GFP antibodies. The recombinant protein was secreted into the lumen of the gland and expelled as a thread of the cocoon. Interestingly, the shapes of posterior silk gland and cocoon of the mutant were completely recovered by the introduction of the L chain-GFP gene, suggesting that the immature development of the posterior silk gland is caused by the deficient secretion of fibroin. Furthermore the molar ratio of fibroin H chain, recombinant L-GFP peptide and fibrohexamerin in the filament was 6:6:1, indicating the only peptides formed the complex are secreted into the lumen and expelled as a silk. The amount of the recombinant protein in the transgenic mutant was much higher than that of the normal.

Elements of odor recognition in moths: G proteins and receptors.

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In insects, olfactory receptors (OR) have been found to date only in a few species: *Drosophila* [1,2,3], *Anopheles* [4], and more recently in some lepidopteran species [5,6]. Due to an extreme divergence between species, putative OR sequences could only be identified from genomic data available on these species and not by homology cloning. These receptors are seven transmembrane domain proteins coupled to G-protein-mediated second messenger cascade. In parallel, olfactory-specific proteins (SNMP for Sensory Neuron Membrane Protein) of two transmembrane domains uniquely expressed in olfactory neurons have also been characterized in several moth species [7, 8]. These proteins are homologous with CD36 type receptors, that predominately recognize proteinaceous ligands. One could then not exclude a possible role in olfaction, considering the probable interaction with odorant binding proteins carrying the odorant molecules to the receptor. In this context, we have characterized several olfactory elements in the antennae of the noctuid moth *Mamestra brassicae* and their putative implication in olfaction will be discussed. First, we have identified a lepidopteran G protein α subunit belonging to the Gq family, through immunological detection followed by molecular cloning [9]. It presents high homologies with the Gqα3 from *Drosophila* that has been proposed to be implicated in visual and olfactory response. The expression pattern of the Gq subunit in adult antennae appeared associated with olfactory sensilla. This suggests that this G protein α subunit may be involved in the olfaction transduction process through interaction with G protein-coupled receptors (GPCR). In contrast to the high divergence usually observed in OR sequences, we have cloned one antennal specific putative OR in *M. brassicae,* that shows sequence conservation across insect order. This GPCR belong to the HR2 moth receptor type [6], and *in situ* hybridizations revealed an expression in numerous antennal cells associated with olfactory sensilla, as observed for the other members of the HR2 family. These receptors thus may have a special and important function in chemosensory neurons of insects. In addition, two SNMP homologues were cloned in antennae. One presents all features of SNMPs known to date: 2 transmenbrane domains, a high extracellular loop, specific expression in olfactory neurons. The other one, although very similar in sequence, is not olfactory specific. Diversity and divergence of different elements implicated in odor

detection (elements described here in addition to odorant binding proteins), could have combinatory roles and thus participate to the specificity of odor recognition. The availability of genomic data will now allow to further identify olfactory candidates, in particular olfactory receptors. We now propose to study their role in olfaction through expression or invalidation of the different elements associated with functional studies.

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Divergence population genetics of speciation in *Heliconius* butterflies.

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Reconstructing the phylogenetic relationships of closely related species is complicated by the fact that polymorphisms may be shared between species for some time after speciation, and also by the possibility that genes may be transferred between species through occasional hybridization. Here I present DNA sequence data for four gene regions (one mitochondrial, one sex-linked and two autosomal) for two species, *Heliconius melpomene* and *Heliconius cydno* that are sympatric and still hybridise occasionally. Only one locus, *Tpi*, shows fixed differences between the species. A mitochondrial locus is fixed between the species except for one population in Colombia, suggesting a single introgression event. The two autosomal loci, *Mpi* and *Ci* show polyphyletic relationships suggesting frequent gene flow between the species. Although the results imply that species relationships are difficult to reconstruct and differ across the genome, this information can be used to infer something about population history of the speciation event. They also imply a model of speciation whereby certain regions of the genome are maintained distinct by selection, whilst others are free to introgress between species.

Sequence analysis of BAC contigs covering a densovirus-nonsusceptibility gene, *nsd-2* in *Bombyx mori*.

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Bombyx mori densovirus (BmDNV) multiplies in the columnar cell nuclei of the mid gut epithelia of the silkworm, *Bombyx mori*. It is classified into 2 types, DNV-1 and DNV-2 based on their symptoms, serological characters, genome sizes and conformations. Some silkworm strains were identified as resistant against DNV-1 and/or DNV-2. In such strains the response reflects non-susceptibility rather than resistance because the inoculation dose does not affect their survival rate. So far four non-susceptibility genes have been reported, namely; *nsd-1* (L21-8.3), *Nid-1* (L17-31.1), *nsd-2* (L17-24.5) and *nsd-Z* (L15-?). However, none of them have yet been isolated as cDNA clones or translated products. Recently, we identified four closely linked cDNA markers with *Nid-1* and *nsd-2*. Studies on these genes are useful in understanding the mechanism of non-susceptibility in relation to the viral invasion and multiplication and for possible transformation by introducing such genes. By taking advantage of *Bombyx* genome information, BAC contigs covering these two genes were constructed and we initiated the sequence analysis of these BAC contigs. Forty-three BAC clones were screened by hybridization with the closely linked four markers and subjected to DNA fingerprinting, resulting in three BAC contigs. The longest contig spanning 820 kb contained three out of four cDNA markers and another cDNA marker constructed the second contig of 260 kb. Sequence analysis of these BAC contigs was carried out using shotgun sequencing of several BAC clones consisting these contigs, resulting in the sequence contigs of 593 and 170 kb, respectively. Every thousand nucleotide sequences were applied to homology search in the silkworm database, Silkbase, and public protein databases with BLASTx. The domains that showed strong homologies with 25 kinds of *Bombyx* cDNAs and 13 kinds of other organisms' genes were identified. These were distributed unevenly. Repetitive sequences appeared very frequently and were distributed also unevenly. To identify nonsusceptibility or susceptibility-specific sequences, the PCR products are now compared on the sequence-analyzed areas between nonsusceptible and susceptible strains.

Towards development of baculoviral resistant strains of the silkworm, *Bombyx mori*.

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In an attempt to develop transgenic silkworm refractory to baculovirus *Bombyx mori* Nuclear Polyhedrosis Virus (BrnNPV), we exploited the RNAi mediated viral inhibition in two lepidopteran cell-lines, Sf9 and BrnN. As a first step, double stranded RNA was produced by *in vitro* transcription using T3/T7 RNA Polymerase, against an essential early viral gene immediate early-l gene (*dsie-1*). The *dsie-1* was transfected to Sf9 or BmN cells. The transfected

cells were infected with either *Autographa californica nucleopolyhedrosis virus* (AcNPV for Sf9) or BrnNPV (for BrnN) 24 hours post transfection. Total Protein was isolated from infected cells at different hours post infection (hpi) and western blot was carried out using anti-gp64 antibody. The results indicate that viral proliferation was halved at about 24 hpi in *dsie-1* treated cells. As low as 1 µg of *dsie-1* was sufficient to block viral proliferation by half in $5xl0^5$ Sf9 cells. However, virus proliferation was increased at later stages of infection (48 and 72) and by 96 hpi the virus proliferation was fully recovered even in presence of higher amount of *dsie-l*. Consistent with the anti-viral activity, the lysate from *dsie-1* treated cells cleaved radiolabeled anti-sense *ie-1*. Simultaneous incubation of virus with *dsie-1* did not elicit viral inhibition. A transgenic Sf9 cell-line producing *dsie-1* showed delayed and feeble anti-viral activity. Our result indicates that targeting a single essential viral gene is not sufficient in blocking the viral proliferation completely. Multigene targeting is the next approach to increase the efficiency of RNAi and the studies are underway in our lab in this regard.

Odorant-Degrading Enzymes of the moth *Mamestra brassicae*.

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Olfactory systems of terrestrial animals are specialized in the detection of odorant volatile hydrophobic molecules, like pheromones or allelochemical compounds. However, many of these molecules are cytotoxic xenobiotics and the olfactory systems have evolved mechanisms for detoxifying these substances and minimize the signal saturation. Indeed, the sensory neurons of the olfactory system are among the few nervous cells in direct contact with the external environment. In insects, olfactory neurons are present within the antennal sensilla, with their dendrites bathing in the sensillum lymph, which contains Odorant-Binding Proteins (OBP) and extracellular Odorant-Degrading Enzymes (ODE) (1). In Lepidoptera, studies of odorant degradation pathways were focused on enzymes able to transform the functional groups of the sex pheromone components, generally straight chain esters, aldehydes or alcohols. Two types of antennal-specific ODE were first identified, the esterases (1) and the aldehyde-oxidases (2). More recently, an olfactory-specific GST was isolated in the sphinx moth *Manduca sexta* (3), and various ODEs were characterized in the antennae of *Drosophila melanogaster*, among which one cytochrome P450 (4). Looking for P450s involved in odorant metabolism in the cabbage armyworm *Mamestra brassicae*, we have used a PCR-based strategy with degenerate primers to amplify cDNA fragments from antennal extracts. Four cytochrome P450s encoding cDNAs were isolated, 3 belonging to the CYP4 family and one to the CYP9 family. The expression patterns of the CYP4 genes were studied by northern-blot and *in situ* hybridization. One of these cDNA is specifically expressed in the antennae, and its transcript is only associated with olfactory sensilla, tuned to sex pheromones and plant's volatiles detection, which strongly suggests a role in odorant clearance (5). All these P450s present structural features of microsomal P450 and are most probably expressed in the support cells embedding the sensory neurons, which involved that they presumably act on odorants only after their internalization, as for other intracellular ODEs. Moreover, the cDNA encoding the NADP-P450 reductase, which is the redox partner of microsomal P450s, was also isolated from the *M. brassicae* antennae. These results strongly suggest the possible role of a P450-dependant oxidative metabolism in odor degradation in insects, as shown in vertebrates. More recently, we have isolated a cDNA encoding an esterase expressed in the olfactory sensilla of both male and female moths. The major compound of the pheromone blend of *M. brassicae* is the Z11-hexadecenyl acetate, and it could thus be inactivated by this class of enzymes. Such inactivation has been shown in *Antheraea polyphenus* antennae: the sensillar esterase rapidly degrades the pheromone, that allows the male moths to detect minor concentration changes in the sex pheromone plume emitted by the female (6). Genomic data will allow to identify new candidates of antennal ODEs and further studies are also necessary to precise the functional properties of these enzymes, for a best understanding of odor termination in insects.

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***Bombyx* genome information available for functional studies**

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Genome studies provide essential bases of gene structure, gene expression profile information, promoters, and so on for transgenic works. In Lepidoptera, however, the genome information was quite limited so far. Now the situation changed quickly, since *Bombyx* genome project has just been launched extensively and the explosive accumulation of *Bombyx* genome information will be expected. We will present the *Bombyx* genome information obtained so far and

future views for functional studies.

The *Bombyx* genome information now available:
a) EST db: 36,000 ESTs from 35 cDNA libraries are compiled into SilkBase at the web site, http://www.ab.a.u-tokyo.ac.jp/silkbase , which everyone can access.
b) Linkage map: About 2,000 genetic markers were mapped on 28 linkage groups under the efforts of several groups (Yasukochi (NIAS); Sugasaki & Shimada (Tokyo U); Hara et aI. (NIAS); Goldsmith (URI); Tan et al. (Hunan Normal U); Nagaraju (CDFD)).
c) BAC library: Three BAC libraries are available: I) EcoRI partial digests with 44x genome equivalents and 168kb insert size; 2) Hind III partial digests with 9x genome equivalents and 125kb msert size; 3) BamHI and EcoRI partial digests with 13x genome equivalents and 16Skb insert size.
d) EST microarray: EST microarray containing 6,000 non-redundant ESTs was available for functional studies.

Immobilization of diverse foreign proteins in viral polyhedra and potential application for protein microarrays

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We considered that the polyhedra produced by certain insect viruses might be a useful platform for protein immobilization without destruction of biological activity. Polyhedra are stable particles composed of progeny virions occluded within a crystalline array of viral proteins. The major viral protein in polyhedra, which is called polyhedrin, is produced in massive quantities during viral infection. One group of insect viruses that produce polyhedra during infection is the cypoviruses (CPV), members of the family Reoviridae. In this study, we demonstrated that the N-terminus, but not the C-terminus of the virion structural protein, VP3, functions as an "occlusion signal", which can direct the stable incorporation of foreign proteins into polyhedra. A VP3-EGFP fusion protein was shown to be uniformly occluded within the polyhedra, to be detectable at the surface of these particles, and to be highly stabilized against dehydration. A broader survey revealed that the VP3 occlusion signal could direct incorporation of the majority of 50 different human proteins into polyhedra. Immune reactivity and protein-protein interactions were detected on the surface of polyhedra containing occluded foreign proteins when these particles were arrayed on a glass slide. Thus, this approach is well suited for the development of protein microarrays for various biomedical applications.

***Mariner*-like sequences and their possessing insertion sequences classified into the Cecropia-ITR-MLE family in the genome of some invertebrates including lepidopteran insects, grasshopper and a coral inhabiting Asian region**

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Mariner-like elements (MLEs) were amplified by PCR against genomic DNAs from several Japanese lepidopteran species, using the inverted terminal repeats (IR) of the *Hyalophora cecropia* MLE as a primer. Clones thus obtained were of a size of about 1.3 kb, and expected to contain the full-length MLE. A 1.3-kb band was also amplified against genomic DNA from the grasshopper, *Traulia ornata*, and from a coral allocated to the *Fungia* family. All of the 1.3-kb bands were cloned and analyzed for nucleotide sequence. All of the 1.3-kb bands were cloned and analyzed for nucleotide sequence. Multi-alignment analyses of the results indicated that the clones were highly similar to each other and classified into the cecropia subfamily of MLEs. The coral MLE was found to have a complete ORF coding for transposase, a situation similar to that previously found in the Emperor moth, *Attacus atlas*, from the Ryukyu island. These findings, together with the fact that all of the insects and coral species that exhibited a positive signal for the full-length MLE were collected in relatively close regions around Japan, indicated that the horizontal transfer of MLEs has taken place even among phylogenetically remote organisms. The same PCR method against DNA from *Bombyx mandarina* individuals inhabiting different regions of East Asia observed for chromosome number (*n*) was applied, and detected not only full-length but also various lengths of MLEs. Each specimen gave several bands with different sizes. The 1.3-kb band, expected to cover a full-length MLE, was ubiquitous in all of the *B. mandarina* specimens as in *B. mori*. However, in some cases, the bands of *B. mandarina* were inserted with other sequences. The MLE making a complex was found to be situated in the same phylogenetic group as that of the previously analyzed MLE clone (*BmTNML*) from *B. mori*, indicating that the insertion had occurred in the ancestor of *B. mori* before the genomic segregation of *B. mandarina* between the Japanese/Korean types (n = 27) and the Chinese/Korean types (n = 28). One individual from China had a discrete 4.2-kb band containing a *BMC1* retrotransposon, which is a unique fragment detected in almost all of the silkworm races and strains. Thus, the 4.2-kb unit might be

made spontaneously by crossing with *B. mori* after the segregation of *B. mori* genome.

Functional genomics of genes involved in odorant reception from the tortricid moth, *Epiphyas postvittana*.

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Olfaction is the most important sense for insects especially moths. Chemoreception is used to locate a mate (sex pheromones), find sources of food and identify sites on which to oviposit. How moths detect the compounds characteristic for these different sites has become a key question; particularly how such high levels of specificity and amplification are encoded in the reception system. We are studying the elements that make up the peripheral part of the odorant signal transduction system in the tortricid horticultural pest, *Epiphyas postvittana* (light brown apple moth). Using both simple genomic (EST) and proteomic approaches we are isolating genes and proteins from antennal tissues that are likely to be involved in the odorant reception process. Sequences from EST sequencing and from subtracted cDNA libraries have been collected from antennal tissues. 2D-gel electrophoresis is also being used to identify proteins that are more abundantly expressed in male antennae in the hope that these may be involved in pheromone reception. A comprehensive bioinformatics system has been used to analyse these data. Mining of this resource has identified many genes that are likely to be involved in the process including odorant binding proteins, odorant receptors, ion channels and hydrolytic enzymes. Functional genomics tools are being developed to assess function including RNAi, *in situ* hybridisation and over-expression systems. To date the most detailed functional studies have been conducted on one of the Pheromone Binding Protein genes (EposPBP1). *In situ* hybridisation studies have located the sites of expression of Pheromone Binding Protein 1 to two rings per antennal segment coinciding with the bands of trichoid sensilla around the male antennae. Radio-labeling studies with recombinant protein have demonstrated EposPBP1 binds the major component of the pheromone blend (E11-14:OAc). The protein has been compared across 14 species of native tortricid moths that use pheromones that differ in length and double bond position have. This analysis has revealed amino acid sites likely to be under selection that may be involved in selectivity.

Preliminary Construction of BAC Contigs Covering the Chorion Locus of *Bombyx mori*.

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The chorion gene locus of *Bombyx mori* has been extensively studied morphologically, genetically, biochemically and genomically until 1990 by the Kafatos lab and colleagues. The chorion is composed of more than 100 proteins, which are encoded by six closely related gene families, ErA, ErB, A, B, HcA and HcB, that map in two clusters on chromosome 2. The two clusters are separated by 4.0 centimorgans corresponding to about 2.4 Mb. Recently, our group has been engaged in the analysis of the *Bombyx* genome using modem genomic tools and resources, including an EST database, BAC library, fingerprinting, and so on. In the present study, we are focusing on the construction of BAC contigs covering the chorion gene locus of chromosome 2 by the following strategy: (1) identifying chorion-specific EST markers by EST analysis of a follicle cell cDNA library, (2) screening BAC clones from a *Bombyx* BAC library by chorion- specific EST markers, and (3) constructing BAC contigs by fingerprinting and use of FPC software. We have sequenced 1,774 cDNA clones from a cDNA library prepared from mRNA of follicle cells of day 8 pupa, p50 strain. EST analysis showed that the fraction of transcripts of early chorion gene families was 4.6%, that of middle was 18.3% and that of late was 13.3% in the cDNA library. We chose 37 representative EST markers which were specific to the follicle cell cDNA library and with a high level of transcription to screen for BAC clones derived from the chorion locus by HDR filter hybridization, and obtained more than 200 BACs. Fingerprinting of these BACs and subsequent analysis using the FPC program produced three main BAC contigs: Contig 1 spanning about 710 kb contained late chorion gene markers, Contig 2 (about 500 kb) covered middle and late EST markers, and early gene markers were confined to Contig 3, spanning about 400 kb, We are now trying to connect these BAC contigs by BAC end-walking.

Bombyx morl nucleopolyhedrovirus (BmNPV) based eukaryotic surface display system for recombinant proteins.

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We have developed BmNPV as a "eukaryotic display system" for heterologous proteins on the viral and host cell surfaces. The reporter gene *gfp (green fluorescent protein)* was fused to the *gp64* gene encoding the full length BmNPV envelope protein GP64 or to its 5' region encoding only the N:terminal domain harbouring the signal sequence, and recombinant viruses expressing the corresponding fusion proteins under the strong, viral po/yhedrin promoter were

generated. On infection of the host insect *B. mori* or the host derived BmN cells with the full length GP64-GFP virus, abundant expression of the recombinant proteins and its display on the cell surface were achieved. The fusion protein was also a component of the budded virions. BmNPV based display system, thus provides an alternative to the previously established AcMNPV display system. The recombinant virus expressing GFP has also been used in preliminary pathological investigations on virus infection in *B. mori* and it provides a simple method for screening for antiviral agents. We have also generated recombinant BmNPVs displaying the immunodominant ectodomains of fusion glycoprotein F of *Peste des petits ruminants* virus (PPRV) and the hemagglutinin protein H of Rinderpest virus (RPV), on the budded virions as well as the surface of the infected host cells. The recombinant virus selection in BmNPV was improved by incorporating gfp as selection marker under a separate promoter within the transfer cassette harbouring the desired genes. Following infection of the insect larvae or the host- derived BmN cells with these recombinant BmNPVs, the expressed GP64 fusion proteins were displayed on the host cell surface and the budded virions. The antigenic epitopes of the recombinant proteins were properly displayed on the recombinant virus particles, and induced immune response in mice against PPRV or RPV, generating neutralizing antibodies.

Effect Of Multiple R&R Consensus Domains Upon Chitin Binding

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More than half of those arthropod cuticular proteins for which sequence data is available have a conserved consensus domain of G-x(7)-[DEN]-G-x(6)-[FY]-x-A-[DGN]-x(2,3)-G-[FY]-x-[AP], known as the R&R consensus. This consensus was first reported by Rebers and Riddiford in 1988 (J. Mol. Biol. *203*:411), based on seven cuticular protein sequences, and has since been shown in a wide range of insect cuticular proteins, as well as in cuticular proteins from other arthropods. Rebers and Willis (Insect Biochem. Molec. Biol. *31*:1083) showed that the R&R consensus is necessary and sufficient for chitin binding. A glutathione-S-transferase (GST) fusion protein that included the R&R consensus from an *Anopheles* cuticular protein bound to chitin, whereas GST alone did not bind. Nearly all arthropod cuticular proteins that include the R&R consensus have only a single copy of this motif. The single exception so far characterized is DD5, a cuticular protein from the tailfin of *Penaeus japonicus* , the kuruma prawn (Ikeya et al., 2001, Comp. Biochem. Physiol. B. Biochem. Mol. Biol. *128*:379). This protein is comprised of 14 tandem repeats, each of which includes an R&R consensus. To determine if a cuticular protein containing multiple R&R consensus repeats binds more chitin more tightly than a protein with a single repeat, we plan to make an fusion protein with R&R consensus repeats from DD5 fused to glutathione-S-transferase. PCR has been used to amplify the DD5 gene, which produced a range of products, primarily from ~900 bp to ~2000 bp. These products will be cloned in the GST expression vector pGEX4T1 (Amersham Pharmacia), to produce a fusion protein. The chitin binding properties of this fusion protein will be compared to GST alone and to a protein comprised of GST fused to the R&R consensus region from an *Anopheles* cuticular protein. Results from these experiments should improve our understanding of cuticular protein-chitin interactions in all arthropods, including Lepidoptera.

Yeast two hybrid screens for the identification of follicular cell proteins interacting with the silkmoth transcription factor BmGATAβ: Characterization of cDNAs encoding proteins that interact with BmGATAβ in follicular cells

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The transcription factor BmGATAβ member of the family of GATA transcription factors, which control a variety of developmental processes in a wide range of organisms from fungi to vertebrates, is expressed at specific stages of follicular cell differentiation during the development of the ovarian follicle of the domesticated silkmoth *Bombyx mori*. Previous work has shown that the activity of BmGATAβ in follicular cells is regulated at the level of transcription (stage-specific transcriptional activation), as well as post-transcriptionally (stage-specific splicing) and post-translationally (stage-specific phosphorylation). Furthermore, studies on the isolation of interacting factors that are involved in GATA-dependent transcription of chorion gene promoters, based on yeast two hybrid screens with carboxy-terminal and amino-terminal subdomain baits of the BmGATAβ factor against follicular cell cDNA prey libraries, resulted in the isolation of several cDNA preys, that are currently subjected to detailed analysis. Two of the isolated prey sequences, currently referred to as CTP 15-1 and NTP 1-4, were chosen as the best candidates for further analysis based on their developmental expression patterns. Besides the effort for the recovery of their complete ORFs from existing cDNA libraries, GST-fusions have been created for them and used for antibody production and detection of the presence of complexes with BmGATAβ *in vivo*. The progress of this work will be presented together with results from the first co-immunoprecipitation experiments.

Deletion of densoviral sequences reduces the efficiency of somatic integration of jcdnv-derived plasmids in insects.

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Plasmids containing the genome of the *Junonia coenia* lepidopteran

densovirus (*Jc*DNV) can be used to achieve somatic transformation that is stably maintained by integration into the genome of whole insects following microinjection into syncytial embryos (3) or by transfection into cells lines (2). We assessed the effect of sequence modifications including different expression cassettes on the efficiency of *Jc*DNV plasmid somatic transformation activities in Lepidoptera and Diptera. The plasmid pJDsRed[3xP3EGFP]H, which contains the Densovirus P9 promoter controlling expression of DsRed fluorescent protein and the 3xP3EGFP expression cassette (1), was injected into syncytial embryos of the fruitfly *Drosophila melanogaster*, and the moths *Plodia interpunctella, Ephestia kuehniella* and *Trichoplusia ni* and the beetle, *Tribolium castaneum*. Somatic transformation using pJDsRed[3xP3EGFP]H was observed on the basis of either DsRed and/or GFP fluorescence in G0 embryos and larvae of all species at rates from 40-95%. In *Drosophila*, the expression pattern of GFP was consistent with germline transformed insects. Cloning of 3xP3EGFP in the unique *Pvu*I site outside the *Jc*DNV sequence did not affect the rate of somatic transformation. Removal of the *Jc*DNV coding sequences for some of the nonstructural proteins or the right inverted terminal repeat had no effect on the rate of somatic transformation. Serial removal of JcDNV fragments showed that the somatic transformation activity depended upon a 470 bp region within the 3' terminas of the nonstructural protein coding sequence. These modifications demonstrate that the somatic transformation activity is dependent upon left inverted terminal repeat as well as sequences internal to the densovirus sequence in insects.

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Chorion gene regulation revisited: involvement of C/EBP-like and GATA factors in stage-specific expression of *Bombyx mori* chorion genes.

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We have analysed the promoter region of five early chorion genes and have identified two ubiquitous *cis* elements; one of them has a consensus sequence, which matches that of the C/EBP DNA binding site. This element interacts with a 70 kDa protein (pX2) present in follicular nuclear extracts. Complex formation exhibits early to middle developmental specificity. Strong evidence suggests that this protein belongs to the C/EBP family of transcription factors. The second one is a GATA element, which forms a complex that is obvious from the early stages but is more prominent in later stages. It is noteworthy that the pX2 (C/EBP) factor binds with the same developmental specificity to a similar sequence of a late chorion gene promoter, which has been previously reported as the binding site for a putative late-specific factor, BCFII. The possibility that pX2 and BCFII are isoforms or modifications of the same protein, which is presumably able to bind to the highly similar sequence elements of both early and late genes, will be discussed. This is the first attempt to identify regulatory elements involved in early choriogenesis of the silkworm *Bombyx mori*. A hypothesis involving protein-protein interactions between C/EBP (pX2/BCFII) and GATA during choriogenesis, in conjunction with particular promoter module patterns will be presented in an attempt to explain the temporal specificity of chorion genes. A cDNA clone and its corresponding chromosomal clone coding for a *Bombyx* putative C/EBP-like protein have been isolated and sequenced. Preliminary experiments for expression of this putative protein in a heterologous system are promising.

Generation of a transformed silkmoth cell line that displays enhanced susceptibilitry to baculovirus (bmnpv) infection and supports the production of high titers of budded virus in serum-free media

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The baculovirus/lepidopteran cell line expression system has found an important biotechnological niche for the large scale production of proteins with biological and pharmaceutical value. More recently, baculoviral vectors were also developed for gene transduction and transformation of mammalian cell lines and tissues *in vivo*, thus opening new perspectives for their potential use as gene therapy vectors. For both types of applications, however, it is essential that production of baculoviruses occurs in media without serum as supplement because serum is a potential source of harmful human pathogens. Here we describe the construction of a transformed silkmoth–derived cell line, adapted to grow in serum-free media, that constitutively produces large quantities of a small secreted factor that is normally synthesized by the silkmoth fat body (the promoting protein or PP) and that shows a 100 to 1,000-fold enhancement of infectivity by *Bombyx mori* nucleopolyhedrosis virus (BmNPV). Thus, the cell line (Bm5-SF/PP(10/1)) can function as an effective host for high level BmNPV-mediated protein production and the generation of high titers of BmNPV-derived transduction vectors in serum-free media. Our results indicate that small secreted factors with an MD-related lipid recognition (ML)-domain such as PP in general can be used to effectively enhance the infectivity by baculoviruses in cell lines that are relatively refractory to virus infection.

cDNA cloning of sex-specific genes from the early embryo of

the silkworm, *Bombyx mori*.

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In Bombyx mori, the W chromosome determines the sex, through a mechanism different from those of D. melanogaster and C. elegans in which X:A ratio does. Accordingly it is hypothesized that a strong female deteminant "Fem" is located on the W chromosome. Cloning of the Fem gene is essential to understand the sex-determining mechanism in B. mori. We have previously reported cloning of a sex-determination gene Bmdsx, and shown that its transcript is spliced in a sex-dependent manner. The sex-specific mRNAs for Bmdsx appeared primarily in eggs at 90 hrs after oviposition. It means that Fem functions by this stage. We performed differential display (DD) with using 0-96 hrs eggs of the "sex-limited black egg" strain to find out the Fem cDNA. We have detected 43 female-specific fragments in 30-times DD screening, and sequenced 5 fragments of them. RT-PCR analyses showed that at least one of the 5 fragments was derived from a female-specific gene. A pair of PCR primers for the gene amplified two bands, and one of them appeared female-specifically in eggs of 60, 72, 84 and 96 hrs, although the other one was not sex-specific and detected in all of searched eggs including male eggs and ovarian eggs. The gene encoded a protein similar to mammalian pre-mRNA cleavage factor Im. Since the human genome contains another gene for a paralog of Im, it is plausible that the two Bombyx Im-like mRNAs are also encoded by different genes, and one of them expresses exclusively in female eggs. It should be clarified whether or not the gene product regulates sex-specific splicing of Bmdsx.

Differential activation of antibacterial peptide genes by two close-related Rel family members from Bombyx mori.

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Rel/NFκB family proteins are known as transcriptional factors that bind to the κB-like motif and activate immune genes in Drosophila. A κB-like motif is also located in the 5'-upstream regions of Bombyx mori antibacterial peptide genes, suggesting an important role in gene activation. Two cDNAs encoding BmRelA and BmRelB, which have Rel homology domains were cloned as candidates for binding factors. BmRelB cDNA had the same nucleotide sequence as that of BmRelA except for a 239 bp nucleotide deletion including a putative translation start codon of BmRelA. The BmRelB had an N-terminal region 52 amino acids shorter than the BmRelA. Interestingly, both BmRelA and BmRelB had a leucine zipper motif, which was not present in the C-terminal region of Drosophila Rel family proteins. Transient luciferase assay using a Drosophila melanogaster cell line, mbn-2 indicated that the attacin gene is strongly activated by BmRelB, but very weakly by BmRelA. On the other hand, lebocin 4 gene is activated more strongly by BmRelA than by BmRelB. Immune gene activation by these factors was κB-like motif-dependent. These results suggest that BmRels differentially activate antibacterial peptide genes through a κB-like motif and the 52 N-terminal amino acids of BmRel A contribute to this differential activation.

Butterfly genes and evolution: genetic variation and measuring fitness in the wild

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Lepidoptera can serve as model systems for studying the action of natural selection in the wild since we know much about their ecology, natural history, physiology, and behavior. They are large enough to study in the field yet small enough to grow easily grow in lab colonies. Here I begin with a case example of pervious work in which genetic variation in a well understood gene was found to have fitness consequences in the wild. Genetic variation gives rise to gene products that may vary in their ability to perform at the molecular, as well as organismal level. Such performance variation might result in differential reproductive success among these genotypes. By working with genes whose function is well understood, we can make predictions about the possible fitness effects of such performance variation. Research into the genetic variation at the phosphoglucose isomerase (PGI) gene in *Colias* butterflies (Pieridae) reveals significant biochemical performance variation among genotypes. PGI is the second step of glycolysis and such variation is expected to affect flight and thereby fitness via differential ATP production. Studies of flight performance and survivorship, male mating success, and female fecundity, all show expected patterns of PGI genotype performance predicted from *in vitro* biochemistry. Our institute is focused on insects that feed on Crucifers (i.e. Arabidopsis and relatives) and *Pieris rapae* is their best understood butterfly herbivore. Wishing to extend our understanding of the fitness consequences of genetic variation, we have decided to assess the variation in candidate genes that are well understood functionally. If we can find genetic variation in such genes, we hope to begin studying this variation in the wild. Using our EST library constructed for *P. rapae* (Pieridae), a series of candidate genes were selected for PCR and cloning. We have sampled ten individuals each from a population in both Germany and Italy. We compare these results with a sample from the sister species, *P. brassicae*, taken from Spain. Comparisons among these samples allow us to examine the standing variation both within and between populations, as well as across species boundaries. Moreover, we wish to compare the patterns of genetic variation in our candidate genes with genes whose function we believe to be very conserved and therefore not a candidate for genetic variation that is under current selection in the wild. To date we have results from 4 candidate genes, cecropin, attacin, lebocin, and defensin, which are involved in the innate immune response to bacterial

infection. These results are compared with those for the 60s ribosomal gene. Work is in progress assessing the biochemical performance of these genes as well as their expression patterns. Here we report only on the preliminary patterns of genetic variation within and among these genes which are discussed in light of the action of purifying selection and the population history of this species.