Perspectives in silkworm (*Bombyx mori*) transgenesis

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The development and improvement of the current protocol of silkworm transgenesis open new areas of applications both for fundamental research and for applied fields. In particular we plan to make use of transformed silkworms for the study of the contribution of chromatin structure in the regulation of silk encoding genes. Transformed silkworms could also be used to study the secretion of foreign fibrous proteins as for example the spider silk with the aim of developing new textile fibres. We can also transform silkworms in order to improve sericultural strains. More particularly, it would be very beneficial to produce strains resistant to baculovirus infections. This has been initiated by fighting against viral functions through RNA interference and by attempting to increase host tolerance functions against the virus.

THE recent success of transgenesis of the silkworm *Bombyx mori*[1] opens new prospects on this insect species, exploited both as a powerful biological model system and as a silk producer in industry. Our aim here is to discuss some of the perspectives that this new opportunity offers to all those involved in basic and applied researches on the silkworm.

In basic research, the examples of *Drosophila melanogaster* and the mouse show how mastered transgenesis can be extremely helpful in understanding basic biological functions related to gene expression and regulation. Similar achievements in the silkworm can now be foreseen to decipher basic physiological functions of the insect that, in turn, might sustain further biotechnological and sericultural applications.

The interest in transforming the silkworm rose more than ten years ago, but reaching the objective turned out to be a difficult task. Random integration of DNA following injection of silkworm eggs was attempted years ago[2] in the line of positive results obtained in mouse. This procedure was found non-operative in spite of the possibility to integrate foreign DNA without the help of any vector. This led to an intensive but still unsuccessful search for adapted vectors such as endogenous transposons[3], pseudo-typed mammalian retroviruses[4] or modified densoviruses[5]. At the same time, *B. mori* genes were expressed in insect cell lines[6], in somatic cells of embryos[7,8], in biolisticated silk glands[9] and in transgenic *D. melanogaster*[10,11]. Finally the transposon *piggyBac* which had been isolated from the moth *Trichoplusia ni*[12] was found efficient for making transgenic *B. mori* as well as other species (see below) opening the way to transgenesis in a wide spectrum of insects and possibly other Arthropods.

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Silkworm transgenesis

A brief historical account on insect transgenesis

In *D. melanogaster* – the first ever made transgenic insect and in related species, the use of P-element as a gene vector proved a powerful strategy[13]. P-element mobilization unfortunately appeared to be restricted to drosophilids and attempts to extend its application to transforming other species were unsuccessful, most probably because transposition of P-element requires host-specific factors, and not only ubiquitous DNA repair enzymes.

After a decade of intense efforts, the transformation of a number of non-drosophilid insects was finally achieved. Success was reached with methodologies and techniques inspired by those in *Drosophila*, whereby novel transposable elements substituted for the P-element. Four families of class II transposons were found functional.

Using the Tc transposon family member *Minos* isolated from *D. hydei*, *Ceratitis capitata* was the first non-drosophilid species to be transformed[14]. The transposons *hobo* from *D. melanogaster* and *Hermes* from *Musca domestica,* both members of the hAT family, were efficiently used as gene vectors to transform *D. virilis*[15] and the mosquito *Aedes aegypti*[16], respectively. A mariner element isolated from *D. mauritiana* was shown capable to transfer genes in *D. virilis*[17], in *A. aegypti*[18] and in *M. domestica* germ line[19]. The three above-mentioned transposons isolated from dipterans were also found efficient in *D. melanogaster*[20–23].

More recently, *piggyBac*, another type II transposable element isolated from a cell culture of the cabbage looper moth, *Trichoplusia ni*[12], was shown to be able to transform insects from different orders. It is one of the most extensively utilized and promising gene vectors. To date,

piggyBac derived vectors led to transform five dipterans (the Mediterranean[24], Caribbean[25] and Oriental[26] fruit flies, *D. melanogaster*[27] and *M. domestica*[28]), a coleopteran (*Tribolium castaneum*[23]) and two lepidopterans (*B. mori*[1] and the pink bollworm *Pectinophora gossipiella*[29]).

The piggyBac transposon

piggyBac was first discovered as the responsible agent of the so-called *few polyhedra* (FP) mutations in baculoviruses that were passed through the *T. ni* cell line TN-368 (ref. 12). It was subsequently demonstrated that *piggyBac*, the insertion of which into the 25 K gene of the virus causes the FP phenotypes, originated from the genome of the insect cells itself[30].

The element is 2.4 kb in length and terminates in 13-bp perfect inverted repeats carrying 5′CCC...GGG3′ terminal trinucleotide sequences. An additional internal 19-bp inverted repeat is located asymmetrically with respect to both ends[30]. The transposon sequence can be read as a single ORF encoding a functional transposase with a predicted size of 64 kDa.

In the baculovirus genome, inserted elements were systematically found flanked at both ends by the tetranucleotide TTAA, identifying a single chromosome target sequence. Sequences adjacent to the termini of genomic representatives of *piggyBac* were also TTAA, confirming that specificity for the target site is an endogenous property of the transposon. Excision of *piggyBac* from baculovirus insertion sites is precise, regenerating a single TTAA sequence, thereby leaving no footprint behind[31]. The specificity of the target sequence and the precision of excision implicate the occurrence of a site-specific recombination mechanism and moreover suggest that TTAA is part of the sequence recognized by the *piggyBac* transposase. These are remarkable and unique features among type II-transposons.

piggyBac has only been found in certain strains of *T. ni,* suggesting that it recently invaded the genome of this species. A nearly identical element was found in the genome of the distant dipteran, *Bactrocera dorsalis*[26] while no other tested insect species were shown to harbour *piggyBac* elements. Moreover, the experimentally derived vectors are mobile in both species showing that no strong endogenous repression of transposition has developed in the two natural hosts. It is not understood how such a highly mobile element, able to transpose in many diverse species, is not widespread among insects, unless it is a very recent evolutionary event.

The lack of host specificity in *piggyBac* mobilization and the absence of established negative control of transposition create a favourable context for the potential occurrence of horizontal gene transfer. As a practical consequence, *piggyBac* usage as a transformation vector should be restricted to species that are not aimed at being released in the wild.

Transformation methodology in Bombyx mori

For transforming *B. mori*, the *piggyBac*-based vectors described in Tamura *et al.*[1] were derived, as shown in Figure 1, from the cloned wild type element p3E1.2 (ref. 30). As a reporter system, a gene encoding the Green Fluorescent Protein (GFP) was chosen because it provides a neutral dominant marker that is absent in the endogenous genome. The promoter of the cytoplasmic actin gene A3 was used to drive GFP expression as it is active in most of the insect cells and tissues. The non-autonomous helper (pHA3PIG) that expresses the *piggyBac* transposase was controlled by the same cytoplasmic actin gene promoter[32].

The obtained results showed that around 1 to 2 per cent of the injected fertile G_0 insects give transformed progeny. This efficiency of transformation is within the range obtained in *Drosophila* transgenesis experiments. It means that gene insertion in the germ line is not a highly frequent event. However, the proportion of G_1 larvae, which from a single brood exhibit a transformed phenotype, varies from less than 1% to more that 25%. This means that the number of transformed gametes in the GO animals is highly variable, most probably because integration can occur at different stages of the germ line development. Genomic DNA analysis showed that the same unique hybridizing fragment is observed in all GFP positive animals, showing that the transgenes are integrated without detectable modifications.

The transgenes are stably integrated into the genome and inherited in mendelian proportions over more than 20 generations. In parallel, no extinction of the transgene expression has been observed.

One remarkable property of the system is that in spite of the low frequency of integration, multiple independent insertions occur in the G_0 transformed parents. The presence of multiple independent inserts in several G_1 larvae indicates that their G_0 parent harboured several inserts in a single gamete and that different gametes carried different inserts. However, if most of these inserts were present in the same gonocytes, the distribution of the inserts in the gametes and consequently in the G_1 animals would be in favour of a larger number of inserts in each animal. This indicates that prior to meiosis each gonocyte carries only a few transgenes and therefore, that gonocytes are different. This may arise by two distinct mechanisms. One is the late occurrence of integration events during development of the germ-line. Another is the existence of successive rounds of transposition taking place after a single or a limited number of initial insertion events. Such a hypothesis would explain why, in spite of infrequent insertions in the parental population, the number of inserts is high in the few transformed animals. Successive transpositions could be driven by the stability of the transposase encoded by the helper vector or by the exis-

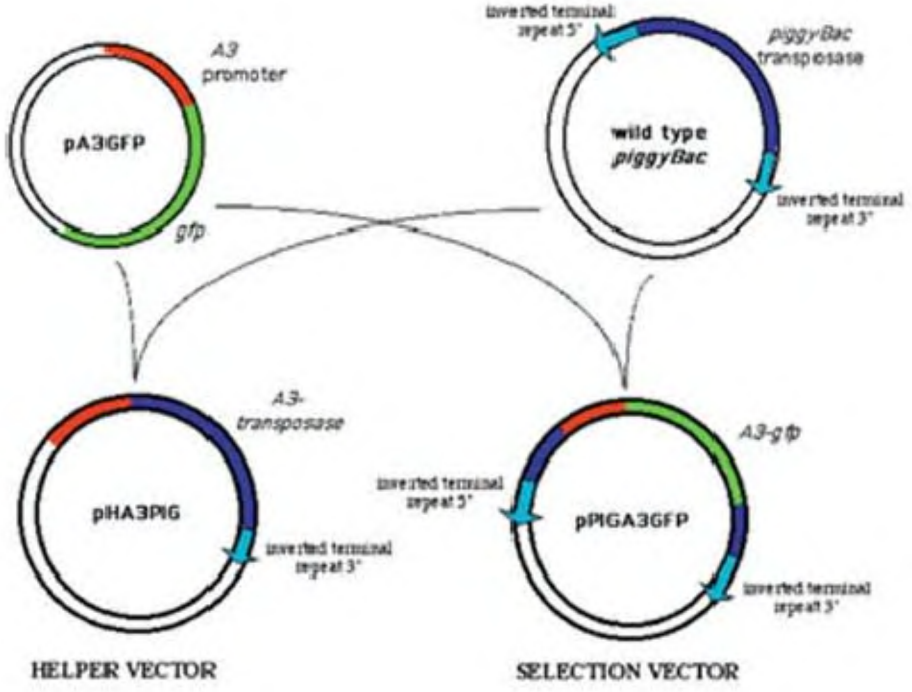

Figure 1. Construction of *piggyBac*-derived selection and helper vectors. The selection vector pPIGA3GFP contains both *piggyBac* terminal repeats whereas the helper pHA3PIG is devoid of the transposon 5′ terminal repeat (fragments are not to scale).

tence of an endogenous cross-mobilizing activity. As the chromosomal insertions are highly stable during the next generations, the second possibility can be ruled out. A similar situation of an unexpected high number of insertions was also observed in transgenic *C. capitata* and it was also attributed to secondary mobilizations after an initial single insertion event[24]. Similar results have been recently obtained in *piggyBac*-transformed *M. domestica*[29] and *B. dorsalis*[26] suggesting that it is a property of the element itself. One can suggest that successive rounds of transposition are not deleterious because of the precise excision of the element.

This property makes the system highly efficient and provides the possibility of deriving numerous independent transgenic lines from a few initially transformed individuals

How to improve the transformation procedure?

Silkworm transgenesis is now a routine method leading to a satisfactory yield of transformed animals and to a reliable expression of transgenes during multiple successive generations though the protocol is perfectible and several parameters wait for optimization. It is likely that the site of injection into the egg, the DNA concentration, the helper to selection vector ratio, the DNA delivery procedure could affect gene transfer efficiency. In all probability, the insert size is not strictly limited but this parameter has not been precisely defined and needs further studies.

Three promoters have already been incorporated into *piggyBac* vectors: the transposon promoter itself[24], the *D. melanogaster* hsp70 promoter[25] and the cytoplasmic actin A3 promoter from *B. mori*[1]. All of them are active in most animal tissues. For further applications tissue-specific promoters are highly desirable. Among many, we are currently assaying the well-characterized silk gland-specific promoters of the silk proteins-encoding genes. For the transposase expressing helper plasmid, the use of a germ line-specific gene promoter active in the very early stages of germ cell precursor multiplication would be very helpful. This will also necessitate basic research on these specialized embryonic cells in *B. mori*.

New selection markers would also be very valuable particularly for inserting different transgenes in the same individual. A recent report described an artificial promoter that was specifically designed to be of universal use[23]. This promoter comprises three binding sites for the *Drosophila* Pax-6 protein, a factor that triggers eye morphogenesis. The conservation of the factor makes this promoter functional in photoreceptor cells of distantly related insect species. Driving the expression of GFP, this promoter proved efficient in the silkworm (Chavancy, pers. commun.). Other new markers should be devised and this also will require fundamental studies.

The versatility of the system could be also enhanced by constructing *Bombyx* lines that would constitutively produce the transposase in order to remobilize transgenes in already established lines. Secondary mobilization, via

appropriate crossings, of inserted *piggyBac* sequences could also be used to detect novel genes by enhancer trapping approaches.

Such a transposase-producing line cannot be easily constructed with the *piggyBac* vector itself because it would be highly unstable. Thus, the availability of new vectors remains a desirable goal. The strategy would be to insert the *piggyBac* transposase coding sequence into another vector and to derive a stable line to be crossed with *piggyBac* carrying lines. Such a new vector will also help to produce *Bombyx* lines with multiple inserts.

Similarly, we consider the construction of a *Bombyx* line producing the yeast GAL4 protein. This transcriptional activator is produced in a variety of *Drosophila* transgenic lines, where it does not induce any developmental defect. Placing the DNA target sequence recognized by GAL4 in front of transgenes of interest will permit their expression after appropriate crossings. On this basis, various developments are conceivable such as studying dominant negative effect of truncated proteins, the effect of overproduction of protein of interest, of identifying anonymous enhancer sequences. The CRE recombinase specifically recognizes the lox sequence. Creating a *Bombyx* line that expresses this enzyme will be helpful to remove foreign sequences placed in between two inverted lox sites. This strategy is largely and successfully used in mouse transformation studies.

The recent report that homologous recombination can be developed in *B. mori* in order to knock out genes is very promising[33]. The method is not currently a routine procedure due to its very low yield, but its improvement would be of prime interest.

The field of *B. mori* transgenesis is largely open to improvements and developments that will require new basic studies on the system. An international collaboration is needed in order to optimize and exploit the potential of this model both in fundamental research and applied fields.

Applications of silkworm transgenesis

We have recently discussed the feasibility of certain biotechnological applications such as the massive production of proteins of interest[34]. In the following part, we shall consider a few other basic and applied applications that could be of interest for the improvement of sericulture and of the development of silkworm as a biological model.

Study of the regulation of silk encoding genes

As shown in Figure 2, the silk gland, the specialized salivary glands that produce silk is composed of two secretory parts, the posterior silk gland (PSG) where fibroin is synthesized and the middle silk gland (MSG) that produces the sericins[35]. Fibroin is a three-subunit complex constituted by the heavy chain (H-Fib), the light chain (L-Fib) and fibrohexamerin (formerly referred to as P25). H-Fib is a large protein (4700 amino acid residues) which accounts for 85% of the silk mass. L-Fib is a 26 kDa polypeptide linked to H-Fib by a disulfide bond[36]. Fibrohexamerin, a 25 kDa peptide interacts with L-Fib/H-Fib complex through hydrophobic interactions[37]. Both small peptides serve as molecular chaperones during the intracellular transport and secretion of the H-chain and are part of the silk thread. Sericins are a family of peptides encoded by two genes *Ser-1* and *Ser-2*, which glue the silk threads[38,39]. Previous studies on the regulation of the silk-encoding genes suggest the existence of two intricate levels of transcriptional regulation.

One lies in the existence of silk gland-specific and territory-specific transactivators. The factor SGF1, a forkhead-family protein, belongs to the first category and is responsible for the activation of *Ser-1* in MSG cells and of *fhx* in PSG cells. This factor, present in both territories of the silk gland, likely interacts with specific partners able to discriminate between the posterior and the median subparts[40].

The second concerns chromatin accessibility. The organization of chromatin around the *fhx* promoter is correlated to the functioning of the gene and thus is supposed to control the access of transactivators to their target DNA. It was shown that the chromatin structure of *fhx* in the PSG (where the gene is expressed) exhibits two DNase hypersensitive sites and a MNase accessible region that are not present in the MSG. This indicates that an open chromatin configuration characterizes the gene in the PSG, whereas it remains non-accessible in the MSG[41]. This would suggest that during early morphogenesis of the silk gland in the embryo, a mechanism discriminates the *fhx* gene in the two prospective subparts of the gland. The involvement of the same SGF1/forkhead factor at early morphogenesis may suggest that the same factors that regulate transcription in differentiated cells are also involved in this determination process.

Using transgenesis now makes it possible to address new questions on this system. The study of SGF1 expression during silk gland embryonic development will allow elucidation of the role of this factor in morphogenesis, prior to analysing the phenotypes induced by over-expression or inhibition (via RNA interference), or by dominant negative versions of the factor. Transgenesis would also allow the localization of the DNA sequences involved in the recognition of the *fhx* gene to-be-expressed in the future PSG cells, by studying mutated *fhx*-derived transgenes and looking at both their DNA topology and their transcription abilities. It should thus be possible to delineate which sequences are required for defining the tissue specificity of expression in the silk gland.

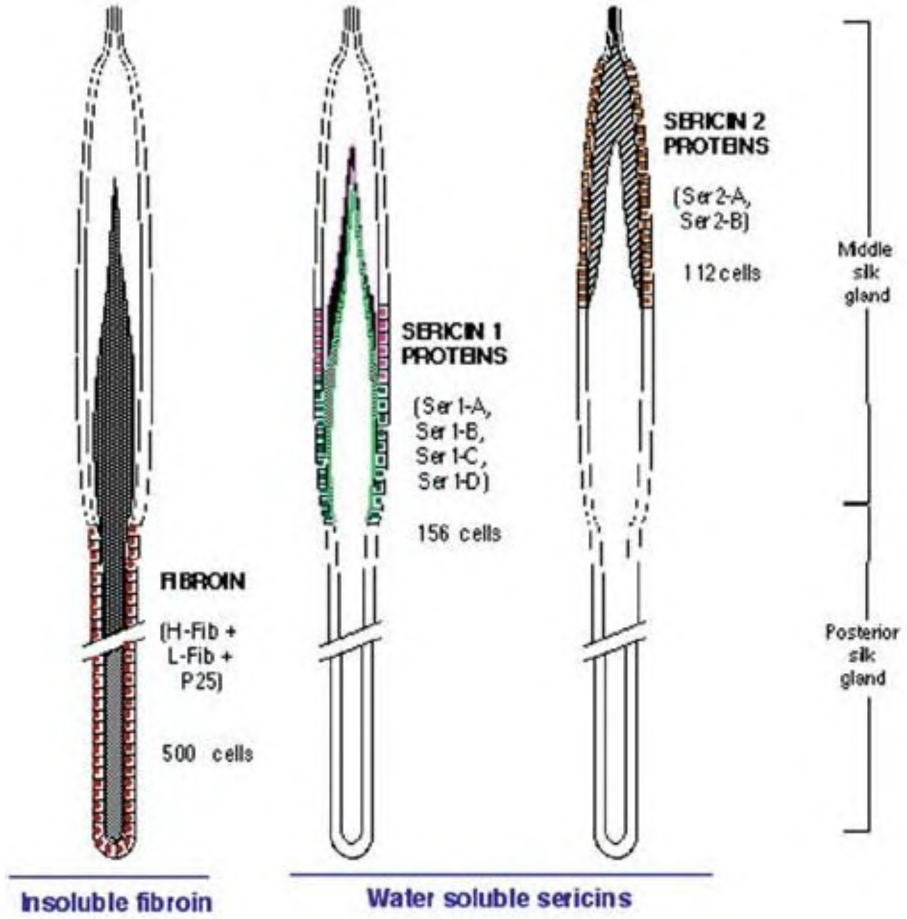

Figure 2. Functional organization of the silk gland. The 500 cells of the posterior silk gland synthesize the three subunits of the fibroin complex, which is secreted into the lumen and accumulates in the middle silk gland where it is covered by successive layers of sericins. The various sericins produced in different subparts of the middle silk gland are encoded by the *Ser-1* and *Ser-2* genes, active in 156 and 112 cells, respectively.

Making new textile fibres

The availability of novel fibres with tailored physico-chemical properties would be of extreme interest in textile industry. The market demand of clothing materials with superior easy care and wear properties as well as the development of innovative textile products are some of the factors stimulating the research of new fibres. Producing proteins of textile interest via the silk protein biosynthetic pathway in transgenic silkworms appears an achievable goal.

The idea is to use a silk gland specific gene promoter in order to deliver the foreign protein in the organ that is naturally able to secrete fibrous proteins. For this, it could be appropriate to first suppress the endogenous secretion. This will necessitate knocking out the fibroin genes by homologous recombination, which is not an easy task as shown by preliminary attempts. As a first step, one can imagine to make transgenic animals able to secrete a mixture of the endogenous proteins and foreign ones. Our knowledge of the promoters of the silk-encoding genes will make this rapidly operational. As fibrous proteins are highly repetitive, and this is the source of their favourable properties, one of the difficulties to be got over is the presence of repeated DNA sequences in the transgene that are not easy to clone in bacteria and this will impede the construction of appropriate vectors.

The next step will be the design of completely artificial protein fibres, the sequence of which could be determined through computer-aided modelling. This, however, would necessitate a better theoretical knowledge of the relationships between the conformation, crystal structure and self-assembly behaviour of protein sequence.

Another prevalent idea is to use transgenic silkworms to produce large amounts of heterologous silk proteins, such as those of spiders. Compared to that of *Bombyx*, spider silks contain less beta-sheeted material. Spider dragline silks, the main structural web silk, which are also used for the spider's lifeline, are exceptionally resistant and extensible, and equate in strength the costly, artificial fibre kevlar[42]. The dragline of *Nephila clavipes* consists of two main threads produced by the major ampullate glands. Both are made of fibroin-like proteins termed spidroins. The cloning of partial coding seque-

nces of some spidroins has led to the expression of portions of the polypeptides in bacteria. Inserting these sequences into a *piggyBac* vector would permit to study how a foreign fibrous protein is synthesized, transported and secreted in the silk gland of the silkworm. The question is open as to whether the endogenous chaperones are capable of interacting with the spider molecules. This obviously is a completely new field that would provide unexpected information on the shaping of natural fibrous protein in the cell.

Improvement of sericultural silkworm strains

In India, sericulture is a major economical resource, which has an important social impact. However, the subtropical climatic conditions that are prevalent in the south of the country are responsible for epizooites that significantly affect silkworm-rearing conditions. Baculoviruses (BmNPV), densoviruses (DNV), cytoplasmic viruses (CPV) as well as bacterial agents are among the most destructive (see the article by Watanabe in this issue, page 439). The idea is to engineer *B. mori* strains that would be more resistant or refractory to pathogens. Recent advances in the biology of pathogens (baculoviruses in particular) and on the immune system could be used to devise new experiments towards the production of genetically resistant silkworms.

An antiviral strategy would include two main approaches designed to modify the host–virus interactions. The first strategy would aim at inhibiting the aggressive functions of the virus and the second one at developing and reinforcing the antiviral functions of the host cells. We are aware that combining both approaches could be the final strategy.

Fighting against the viral functions

Two new approaches could be tested: the RNA interference against transcripts of the invading virus and the construction of dominant negative transgenes.

It has been demonstrated in *Coenorhabditis elegans* and confirmed in *D. melanogaster* and other species that the introduction of double-stranded specific RNA in the cell (and in the organism) can trigger the destruction of the corresponding endogenous mRNA, a phenomenon known as RNA interference[43]. The idea is to establish the suitability of this method in silkworm cells and to use it to direct the destruction of viral transcripts of infectious baculoviruses. Another possibility would be to constitutively produce in *B. mori* tissues, truncated viral proteins capable of negatively interfering with parasite-encoded proteins in order to induce the abortion of the viral cycle. The first choice would be the immediate early genes, the function of which is required for the development of the invading virus. In a first step, using the *piggyBac* vector, cultured cells could be transformed with transgenes encoding different portions of the immediate early proteins and test their resistance to baculoviruses.

In a second step, the most efficient construct could be inserted in transgenic silkworms and monitor resistance to baculoviruses.

We are aware that the constitutive production of either double-stranded RNA or truncated viral proteins could also be harmful for the silkworm. It may be preferable to deliver these molecules upon viral infection. This could be achieved by using transgenes equipped with viral-induced promoters. This could be attempted by constructing a transgene under the control of a natural viral promoter itself trans-activated by the IE1 protein. A cell line carrying such a construct directing the expression of the GFP could be constructed and tested for its ability to develop fluorescence when infected by the virus. Such a promoter would be used later for the constructs described above.

Improving host cell resistance functions against baculoviral infections

It is truly surprising to note that among the thousands of published articles addressing insect baculoviruses as efficient recombinant protein systems, there are virtually no studies dealing with mechanisms of anti-viral responses elicited by the insect host cells. This means that special attention must be focused on this area to determine the genes and proteins involved in conferring resistance to viral infections in order to modulate their expression in genetically transformed cell lines and silkworms. In this newly-developing field, a cDNA from *B. mori*, which has high similarities in its kinase domain to the vertebrate anti-viral kinase (PKR) was isolated, cloned and sequenced (Paul Brey and Dharma Prasad, pers. commun.). Whether it is playing an active role in antiviral immunity in the silkworm remains to be established. To elucidate the role of the BmPKR-like homologue in viral infections it will be necessary to interfere with the putative PKR pathway, for example, by expressing trans-dominant negative mutants of the BmPKR-like homologue and/or the use of RNA interference against the BmPKR-like homologue.

Very recently it was shown that increased PKR activity in vertebrates induces apoptosis in response to numerous stimuli including viral infection. These observations support the hypothesis whereby PKR limits viral replication, not simply by inhibiting protein synthesis, but by actively inducing apoptosis. In this context, it is well known that baculoviruses synthesize antiapoptotic proteins, such as p35 and inhibitors of apoptosis (IAPs), to prevent cell death induced by the insect cell apoptotic machinery in response to viral infection. There is a possibility that the BmPKR-like homologue may be playing a role in apop-

tosis and may be directly countered by baculoviral proteins such as p35. There is thus a need to establish the possible involvement of the BmPKR-like homologue in apoptosis particularly with respect to cross talk between p35 and the BmPKR-like homologue in terms of physical association, as well as the ability of the BmPKR-like homologue to intercept p35-mediated inhibition of apoptosis.

Conclusion

As we discussed above, the development of transgenesis opens new possibilities for genetic applications to the domain of silkworm research. Moreover, the emergence of silkworm genomics including the sequencing of the genome will make *B. mori* another reference insect model to compare to *D. melanogaster*. Such a comparative approach will be very informative for setting up species-specific strategies of insect pest control. However, the genetics of most of the insect pests is poorly known. *B. mori* which is both a useful insect and a laboratory model insect could thus become a very good model for transferring our biological knowledge from the lab to the various application domains.

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