RECENT ADVANCES IN GENE TECHNOLOGY OF SILK SPINNING INSECTS

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ABSTRACT

Among lepidopteran insects, species belonging to the Bombycidae, Saturniidae and a few other genera have been traditionally used for the silk production. However, recently developed knowledge on the basic molecular biology of these silk spinning insects, especially *Bombyx mori*, has guided us towards advanced gene technology for their novel beneficial applications. Establishment of a baculovirus expression vector (BEV) system using *B. mori* larvae provided an excellent *in vivo* production system of recombinant proteins. Subsequently, novel BEV systems using wild silkmoths such as *Hyalophora cecropia and Antheraea pernyi* were constructed and demonstrated that they could offer practical advantages in producing recombinant proteins over the BEV system using *Bombyx mori* larvae *i.e.*, in certain cases recombinant protein levels produced in diapausing pupae were higher than those in larvae and diapausing pupae can be stored in a refrigerator for a considerable time until needed. From great interest in both basic and applied genetics of the silkworm, efforts to generate the transgenic silkworm have been made by several strategies such as microinjection of engineered foreign DNA to the egg, search for effective transposons and infection of non-permissive baculovirus as a gene transfer vector, although any reliable method has not yet been established. Further progress in the genome projects of *B. mori* will provide us comprehensive knowledge on the silkworm genetics and more sophisticated gene technology applicable not only to the mulberry silkworm but also other silk spinning insects.

INTRODUCTION

Among lepidopteran insects, species belonging to the Bombycidae, Saturniidae and a few other genera have been traditionally used for the silk production. Because of practical importance as well as biological interest, intensive studies on the genetics of these silk spinning insects, especially of *Bombyx mori*, have been conducted and led to discovery of a number of mutants, establishment of highly productive and robust strains and construction of the linkage map (Fujii *et al.*,1998). Utilizing these historically accumulated resources, molecular biology of the silkworms has rapidly developed and newly obtained knowledge have guided us towards advanced gene technology for their novel beneficial applications. In this paper, recently developed technologies and their future directions are briefly discussed.

Baculovirus - Mediated protein production by insect factory

Establishment of a baculovirus expression vector (BEV) system using *B. mori* larvae provided an excellent *in vivo* production system of recombinant proteins (Maeda *et al.*, 1984). Expression levels of several recombinant proteins in the silkworm larvae, such as human α-interferon (Maeda *et al.*, 1985), mouse interleukin-3 (Miyajima *et al.*,1987), hepatitis B virus surface antigen (Higashihashi *et al.*, 1991), feline interferon (Sakurai *et al.*,1992), frog α-amidating enzyme (Kobayashi *et al.*, 1992) and monoclonal antibody (Reis *et al.*, 1992), were significantly higher than those in the cell culture. The sophisticated modern technologies as well as facilities established for the silkworm mass rearing can be easily converted to those for the large scale recombinant protein production using alive larvae.Thus, the BEV system paved a new way of the silkworm utilization as an insect factory. In fact, a silkworm strain with the improved baculovirus-mediated protein production was obtained from the naked pupa strain (Nd) (Okazaki *et al.*, 1996) and an automatic rearing machinery was also innovated for the mass rearing of the virus-infected silkworm larvae (Ohura and Peng, 1998). A major drawback of the *in vivo* production system behind the cell culture is the difficulty in down stream processing of the recombinant protein because of much more insect components in the starting materials. Further improvements of purification procedure accompanied with the utilization of modified recombinant viruses containing effective purification tag sequences and/or losing viral cysteine protease (Suzuki *et al.*, 1997) and chitinase genes, both of which are involved in degradation and liquefaction of infected insect tissues, will provide us the best solution to the problem.

The diapausing pupae of the wild silk moths are also considered as alternative hosts for baculovirus-mediated protein production with a practical advantage over the BEV system using *B. mori* larvae, because any research laboratories without insect rearing facilities can be purchased and stored the diapausing pupae in a

refrigerator for a considerable time until needed. Early studies on the construction of novel BEV systems using diapausing pupae of the wild silk moths such as *Hyalophora cecropia* (Hellers and Steiner,1992) and *Antheraea pernyi* (Zhang *et al.*, 1992) demonstrated that expression levels of foreign genes in the diapausing pupae were higher than the cell culture, and for some cases, higher than the larvae. An additional advantage of the pupal production was reported for the *H. cecropia* diapausing pupae that a kind of post- translational modifications such as α-amidation occurred more efficiently in the pupae than the larvae (Heller and Steiner, 1992).

Recently, we have constructed another BEV system of *A. pernyi* using a cell line NISES AnPe-428 derived from *A. pernyi* embryos (Inoue and Hayasaka, 1995) and confirmed that expression level of β-galactosidase gene in *A. pernyi* pupae was higher than several insect cell lines including Sf9, High Five and BmN4-cells, which were frequently used for other BEV systems. We will further investigate capabilities of post-translational modifications in *A. pernyi* pupae to evaluate properties as the *in vivo* production system. To establish a more practical and productive insect factory using the diapausing pupae than that of *B. mori* larvae in the future, we should further develop the low-cost artificial diet for the wild silkworm mass rearing and the automatic machinery not only for mass rearing but also for virus injection to the diapausing pupae as well as for harvesting and purifying recombinant proteins from infected pupae.

Generating transgenic silkworms

From great interests in both basic and applied genetics of the silkworm, efforts to generate the transgenic silkworm have been made. Nawa *et al.* (1971) reported the recovery of transformants with normal (dark colour) egg colour in the progeny of an egg colour mutant (white egg) strain of *B. mori*, when larvae of the mutant strain were injected with the whole DNA genome of the normal egg colour strain. However, because of the extremely low frequency and non-reproducible incident of transgenesis, their method was neither utilized nor developed further. Alternatively, the microinjection method of foreign genes to silkworm eggs at early embryonic stage was developed. Transient expressions of introduced marker genes such as chloramphenicol acetyltransferase (CAT) and β-galactosidase genes under control of the *Drosophila* heat shock protein or *Bombyx* cytoplasmic actin gene promoters were successfully demonstrated when these DNA constructs were injected to eggs at fertilization stage (Tamura *et al.*, 1990, Coulon-Bublex *et al.*, 1993), although they were rapidly degraded in eggs and seldom integrated in the chromosomal DNA. Thus, the development of transgene vectors with higher integration efficiencies of foreign genes to chromosomal DNA as well as the utilization of effective selective marker genes are required for establishment of reliable method to generate transgenic silkworms. For the vector construction, the utilization of transposable elements discovered in the silkworm genomes as well as in other organisms have been considered as a promising approach and several candidates such as the piggyBac transposon and the pantrophic retrovirus are now tested (Marshall, 1998). In addition, Mori *et al.* (1995) reported that the infection of *Autographa californica* nucleopolyhedrovirus (AcNPV) to the silkworm caused transovarian transmission of luciferase gene as well as a viral gene to their F2 progenies. Luciferase activities were detected in the virus-infected larvae and pupae (F0), and in the newly hatched larvae of the next generation (F1). Although, the transmission of the luciferase gene was confirmed by the PCR amplification and southern blot hybridization analysis, no luciferase activity was detected in F2 progenies, probably because of a gene inactivation. Further improvements of the attenuated baculovirus-mediated gene transfer method may enable us to generate transgenic silkworms at sufficient frequency and target any genes in the silkworm genome. Successful establishment of methods for generating transgenic silkworms will realize the silkworm bioreactors to produce large quantities of heterologous proteins.

Silkworm genome projects

In addition to the classic linkage map of *B. mori* composed of visible mutations and biochemical markers, construction of molecular maps, including the Restriction Fragment Length Polymorphism (RFLP) maps (Shi, *et al.*, 1995), Random amplified poly-morphic DNA (RAPD) maps (Promboon *et al.*, 1995) and physical maps constructed by BAC libraries (Yasukochi *et al.*, 1997), have been recently started. Completion of these molecular maps and their integration will enable us to localize any genes of interest in detailed positions on the maps, perform positional cloning of them and study their expression as well as functions at the molecular level. Such strong power of the molecular maps, together with a recently compiled Expressed Sequence Tag (EST) database (Maeda *et al.*, 1998) composed of several source of the silkworm, including embryos, fat body, midgut, wing disc and BmN cells, will accelerate wide varieties of studies on the silkworm genetics and provide us the comprehensive knowledge on the silkworm genome and its organization. The huge data obtained by these genome projects will also enhance the importance of the silkworm as a genetic resource as well as a model for lepidopteran pests and help us to invent a variety of new genetic manipulation technology applicable not only to *B. mori* but also to other silk spinning insects.

CONCLUSION

Trends in recent progress in molecular genetics and genetic engineering of the silk spinning insects give us a strong impression that these insects could not be used solely for the silk production in the future but for various purposes such as insect factories and/or bioreactors for the recombinant protein production, as genetic resources for basic and applied biology and as models for lepidopteran pests. The comprehensive knowledge on the silkworm genetics, which will be brought by further and intensive progress in the *B. mori* genome projects, are required for the rational development and improvement of these new applications. Beyond these realistic approaches based on the practical gene technologies, we may dream more highly advanced gene technologies which enable us to generate an artificial silk gland or fat body as an insect -mimetic bioreactor for the production of many kinds of recombinant proteins, including genetically engineered silk proteins, vaccines, antibodies and so on.

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