Biotechnology in sericulture

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Genetic engineering may lead to better varieties of silkworm that produce more silk and are more resistant to diseases and environmental fluctuations. The silkworm is also a convenient model for studies of the molecular basis of development and gene expression using the tools of molecular biology.

THE recent advances in recombinant-DNA techniques have revolutionized biology and given a fresh impetus to biotechnology. Techniques of genetic manipulation have found a place in all spheres of modern biology, both basic and applied. Commercial production of biomolecules relies heavily on genetic engineering. Sericulture appears to be one area where the impact of these techniques has not been felt. A major reason for this, perhaps, is that most of the industrially advanced countries where the biotechnology industry has made rapid progress, except Japan, do not have a significant sericulture industry. Even in Japan, silk production is showing a downward trend, and the country has been pushed to third place behind China and India. India's position as the second largest producer of silk and the fact that nearly six million of its people are involved in sericulture are ample reason for development of sericulture by exploitation of biotechnology.

Silk, considered as the queen of fibres, is proteinaceous in nature. The bulk of commercial silk is produced from the mulberry silkworm *Bombyx mori*, of which there are many strains ranging from the exotic high-yielding bivoltines to the more sturdy native multivoltines. The other silkworms commercially exploited for silk production are eri (*Philosamia* species), and tasar and muga (*Antheraea* species) (Figure 1). India is unique in producing all these varieties of silk.

Silkworms are lepidopteran insects. The larvae are caterpillars, which, at the end of the larval stage, spin a cocoon of silk, and transform into pupae and finally into adult moths. The silk proteins are synthesized in the silk glands. Larvae possess a pair of silk glands, which are long tubular structures located parallel to the gut, extending nearly throughout the entire length of the larvae, but much longer, being coiled in the posterior part. Anatomically and functionally the silk glands are divided into anterior, middle and posterior silk glands. The silk fibre protein, fibroin, is synthesized in cells of the posterior silk glands, and secreted into the lumen and transported to the middle silk gland for storage. Glue proteins called sericins are synthesized and secreted in the middle silk glands, and coat the fibroin accumulating there. The two anterior silk glands, which serve as ducts, converge near the oral cavity, forming a scleroid structure, the spinneret, through which the silk is extruded as fibre[1] (see cover picture). 'Spinning' of the cocoon is accomplished by movement of the head. The cocoon is completed in a few days, at the end of which changes taking place within the larva culminate in the transformation to a pupa. The cocoon is thus one long silk fibre, from about 600 metres in the native races to about 1000 metres in high-yielding bivoltine races. The cocoons are the silk industry's raw material—they are boiled to loosen the fibroin fibre, which is then reeled out.

Better races of silkworm

The quality and yield of silk depend on availability of healthy silkworms, which itself depends on high-quality feed and disease-free stocks. With the spread of sericulture to different parts of the country, it has become necessary to adapt the silkworm to local rearing conditions. Traditionally, strain improvement has used breeding methods for production of new, high-yielding strains with such desirable traits as disease resistance, and heat tolerance. Where intensive breeding studies have been carried out for long periods of time, the traditional approaches appear to be reaching the natural biological limits of the silkworm with regard to its growth rate, silk yield and fecundity. Thus it is becoming increasingly important to turn to the new approaches of genetic engineering to produce better strains.

Transferring 'sturdiness' genes

The Indian native races of silkworm (e.g. the Pure Mysore strain), though poor yielders of silk, are much more tolerant to high temperatures as well as fluctuations in temperature compared to the high-yielding bivoltine strains. They are also capable of tolerating poor quality of mulberry leaves used as feed, and appear to be more resistant to the locally prevalent

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Figure 1. Fourth-instar larvae of mulberry silkworm *Bombyx mori* (*a*), final-instar larva of tasar silkworm *Antheraea mylitta* (*b*), and second-instar larvae of eri silkworm *Philosamia ricini* (*c*). The food habits, physiology and silk fibroin genes of these species are different.

diseases. If the genes responsible for any of these traits in the native races can be identified and isolated, they may be transferred to the high-yielders.

Transferring fibroin genes

Currently available methods permit isolation and molecular analysis of the silk fibroin genes and related genes. The fibroin heavy-chain (fibroin H) gene from *B. mori* is very large, including an intron of 1.2 kilobase pairs towards the upstream end of the gene, and codes for a messenger RNA of 15 kilobases[2]. A fibroin light-chain (P_{25}) gene has also been isolated, which codes for the small subunit light chain (relative molecular mass 25,000). The heavy (M_r 350,000) and light chains are linked together by disulphide linkage in fibroin, which is highly polymerized and nearly insoluble. If the fibroin genes from a 'high-quality' strain prove to be superior in terms of the quality of silk fibre, transferring such genes to the more sturdy native races may be a convenient alternative to identifying and isolating the less tangible genes for sturdiness. It seems worthwhile to characterize fibroin genes from a variety of silkworms, including the nonmulberry species (eri, muga, tasar). At present, however, there is very little information on fibroin genes from silkworms other than the bivoltine strains of *B. mori*. Preliminary studies have shown that there is no detectable homology between the fibroin genes of mulberry and nonmulberry silkworms.

From the published literature from my laboratory[3–5] as well as from others, there is clear indication that the increased expression of fibroin genes by mature larvae is directly proportional to the increased gene dosage at this stage. Multiple copies of this single-copy gene (fibroin) are provided by the chromosomal endoduplication phenomenon. Therefore increasing the fibroin gene copy number to two or more per haploid set of chromosomes in the germ line by introduction of cloned genomic DNA, if feasible, could result in substantially increased production of fibroin in the genetically engineered silkworms.

Gene-transfer methods

It is clearly essential that methods for transfer of foreign

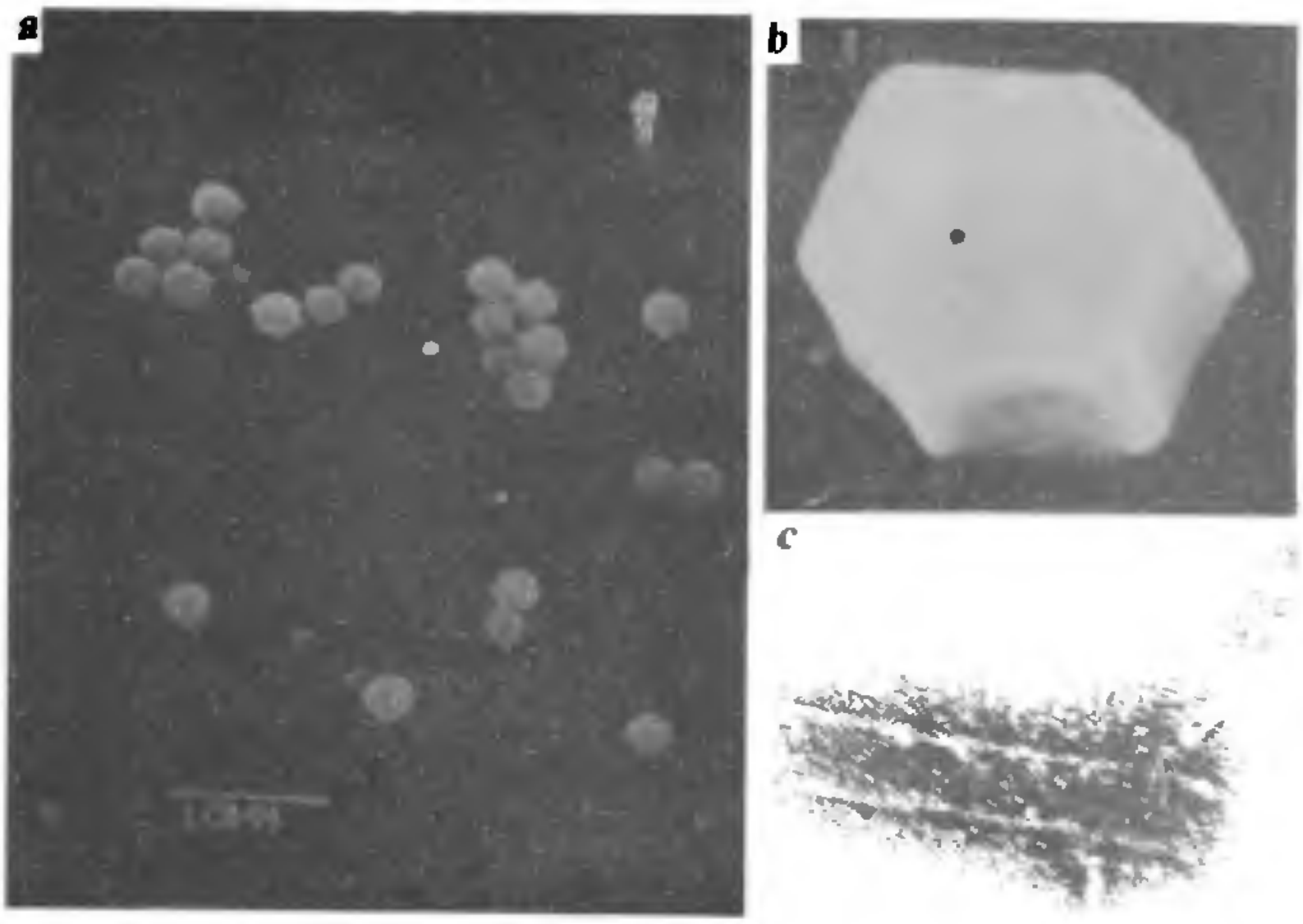


Figure 2. Scanning electron micrographs showing *Bombyx mori* nuclear polyhedrosis virus polyhedra (***b*** is 10 into ***a***) Each polyhedral body which is chiefly composed of viral polyhedral protein, contains many virus particles (***c*** is a transmission electron micrograph showing a single virus particle, into 65,000) The polyhedra are highly infective when ingested by silkworm larvae The polyhedrin dissolves in the alkaline gut juices and the virus infects the cells of the midgut The fact that the polyhedrin gene is a nonessential one in the virus' multiplication, and that the protein is synthesized in large quantities (because of the presence of a strong promoter in the gene) have marked the polyhedrin gene as a convenient site for cloning foreign genes for high-level expression

genes into the germ line of silkworm be established. At the moment, there are two ways of doing this. However, these are yet to be perfected in silkworm.

Microinjections. Cloned genes (say fibroin) are directly introduced into eggs or early embryos by microinjection techniques. If such embryos accept and integrate the microinjected DNA into their genome, they should develop into 'transgenic' animals, harbouring additional or alternative copies of the gene. Electroporation and other methods of direct introduction of DNA into dechorionated eggs may also be useful for arriving at the same result. The discovery[6] of transposon-like sequences in silkworm DNA (analogous to the P element of *Drosophila*) raises the possibility of exploitation of these sequences to target and integrate injected foreign DNA.

Virus-mediated gene transfer. The nuclear polyhedrosis virus (NPV), which is pathogenic to silkworm, can be exploited as a vector for introduction of foreign genes. Experiments that use insect-derived cultured cells have already made use of this method[7].

Other applications

Silkworms are generally susceptible to a variety of infections (viral, bacterial, fungal and protozoal). Methods for early diagnosis of such infections or their effective control can be developed by application of immunological and recombinant-DNA techniques.

The molecular biology of nonmulberry silkworms is nearly uninvestigated For instance, understanding of the molecular mechanisms of pupal diapause (to avoid erratic moth emergence) and reproductive biology (to improve fecundity) is important in tasar silkworms. This could ultimately contribute to better silk yields.

Although there are a number of races and strains of mulberry and nonmulberry silkworms employed for silk production in India, at present there is no inventory of these. A computer-based data base for the different silkworms that includes genetic and molecular characters would be useful.

Silkworms as bioreactors

Silkworms may also be exploited to produce biologically important molecules other than silk fibre. Introduction of foreign genes, such as α-interferon and interleukin-3 and other growth factors, into silkworm with genetically manipulated NPV genomes, and expression of these products in substantial amounts in the haemolymph of larvae have already been reported[7–9]. For the NPV, polyhedrin, is a nonessential protein for its multiplication, but is synthesized in substantial quantities towards the latter part of the virus' life cycle. Completed virus particles are embedded within a polyhedrin matrix to form characteristic 'polyhedral' bodies (Figure 2), which serve as a protective mechanism for survival of the virus in the environment. On ingestion of polyhedral bodies by a larva – the most common mode of infection -- the polyhedrin dissolves in the alkaline gut juices, releasing the virus particles and facilitating infection The polyhedrin gene is a convenient target for genetic manipulation to introduce foreign genes for their high-level expression

without interference in viral replication. In practice, the desired genes are inserted into plasmid vectors harbouring the viral polyhedrin gene along with its promoter and other control elements. After transfection *in cell culture, recombination is allowed to take place* between such hybrid constructs and infecting normal viral DNA. The recombination takes place within the homologous polyhedrin-flanking sequences. Recombinant viruses arising from successful genomic exchanges and now carrying the cloned gene(s) can then be picked up with the aid of convenient 'reporter' genes. Genetically engineered viruses harbouring foreign genes under the control of the strong polyhedrin gene promoter, which permit high levels of expression of the cloned genes, are already available. Gene products expressed thus in the larva are expected to be biologically active. (See box)

With the availability of sophisticated computer-controlled rearing methods such as those used in countries like Japan, the exploitation of silkworms for production of important biomolecules appears to be a highly promising area. As an alternative, cultured insect cell lines (*B. mori*-derived or related) may be exploited for such purposes using *B. mori* NPV as the preferred virus expression vector.

Studies in basic biology

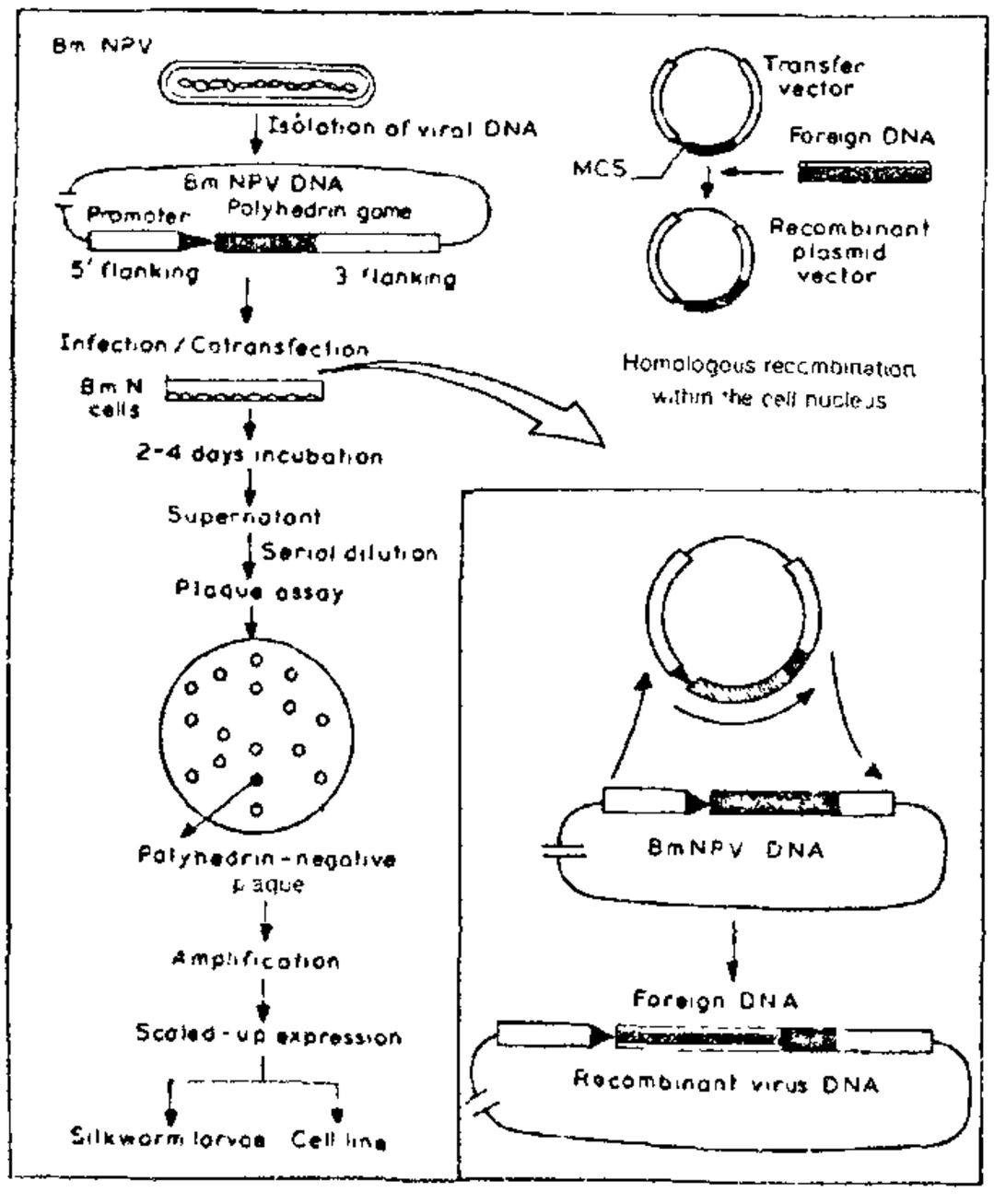
The silkworm has long been used as a model system for studying fundamental problems of biology in such areas as genetics, physiology and development. This has led to improvement of silkworm strains and cultural practices used for commercial production of silk. The synthesis of silk proteins is regulated developmentally and in a tissue-specific manner. Understanding of the *molecular mechanisms governing* these aspects of regulation can potentially contribute to increased production or extended synthesis of silk by the larvae.

In addition to being a beneficial insect of economic importance, the silkworm can serve as a model for a variety of lepidopterans that are agricultural pests. The power of concentrating research efforts on a single organism has been shown by the dramatic progress made in the molecular genetics and molecular biology of the fruit fly *Drosophila*. Recent experience with other organisms, ranging from humans and mice to yeasts, nematodes and maize, has demonstrated the importance of combining classical genetics with modern molecular methods for studying basic biological processes.

A first step towards this end is to develop a molecular gene map of the silkworm. Some of the targets for molecular analysis can be the genes affecting assimilation and utilization of food, rates of growth and development, diapause, fecundity and fertility, sex determination, heat tolerance, disease resistance, and silk quality and productivity. Understanding of the structure, activities and control of the genes involved in these processes opens up the possibility of designing new strategies for influencing their properties and behaviour. Interesting new biological facts related to transcriptional regulation of genes have already emerged from the silkworm system where it has been used as the choice organism for basic studies in molecular biology[10,11].

The genome of the silkworm is 530 million base pairs (bp) long. The organism has 28 chromosomes. This size corresponds to 125 times the average bacterial genome (*E. coli*: 4.2×10^6 bp, one chromosome), four times the fruit fly genome (*Drosophila melanogaster*: 1.4×10^8 bp, three chromosomes), and one-sixth the human genome (3.1×10^9 bp, 23 chromosomes). Only a few markers have been mapped on silkworm chromosomes so far. However, use of recently developed techniques like pulse field electrophoresis permits analysis of extremely large (megabase length) DNA fragments. This method in combination with chromosome walking techniques should permit molecular dissection of large segments of chromosome. Thus, besides the benefit of imporvement of sericulture from knowledge of the basic genetics of the silkworm, the organism can be exploited:

(i) to understand molecular aspects of developmental biology
(ii) to understand the mechanism of hormone action (juvenile hormones and ecdysone) and the phenomenon of diapause
(iii) as a model system for other lepidoptera, in



order to understand the action of herbicides, pesticides and insecticides; this information should be useful in the control of harmful insects.

Biotechnology in 'moriculture'

It is clear that the availability of sturdy silkworms is most important as far as the quality and yield of silk are concerned. An essential prerequisite for sturdiness of silkworms is high-quality mulberry leaves. Considering that nearly 90 per cent of silk produced in India is mulberry silk, attempts to improving the mulberry plant (*Morus alba*) itself by application of biotechnology are certainly desirable. The protein and free-amino-acid contents of mulberry leaves contribute substantially to silk yield. At present, the genetics of pure mulberry species is poorly defined. The methods of plant tissue culture and clonal propagation of 'high-quality' mulberry plants can be undertaken. Host-plant improvement is even more important in the case of nonmulberry silkworms.

Development of alternative food sources and synthetic or semisynthetic diets for mulberry and nonmulberry silkworms is another area that needs attention.

Prospects

When the traditional approaches of breeding silkworm appear to be reaching natural biological limits with regard to growth rate, silk yield and fecundity, advances in gene-transfer technology enhance the scope for creating better races of silkworm with desired characteristics. Transfer of genetic elements determining 'sturdiness' to high producers or, conversely, transfer of 'high-quality' fibroin genes to sturdy races is a distinct possibility. Development of efficient and successful gene-transfer systems for incorporation of cloned genes in the germ line by direct microinjection techniques or by virus-mediated gene transfer is an absolute requirement for this approach. In a parallel development, exploitation of the *Bombyx mori* nuclear polyhedrosis virus-based expression system for high-level expression and commercial production of biological molecules of interest, either through silkworm-derived cell cultures or in live caterpillars, has become a reality. The entire field of molecular biology of nonmulberry silkworms, especially those used for silk production in India, offers immense potential for basic research and for sericulture development be it formulation of alternative or synthetic food sources for more efficient and domesticated rearing independent of seasonal variations and climatic conditions, or improved yield of high-quality fibre to benefit the sericulturist.

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