

Genome engineering and parthenocloning in the silkworm, *Bombyx mori*

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Genetic engineering of the silkworm, *Bombyx mori*, opens door to the production of new kinds of silk and to the use of silkworms as proteosynthetic bioreactors. The insertion of foreign genes into silkworm genome and the control of their expression by diverse promoters have become possible but are not yet efficient enough for commercial use. Several methods of gene targeting are being developed to minimize position effect on transgene expression and facilitate cloning. Parthenocloning can be exploited to conserve genetic traits and improve selection and amplification of clones containing genes of interest. Some silkworm clones have been bred for decades as genetically stable female stocks whose unfertilized eggs are induced to develop by heat-shock treatment. Any exclusively female generation contains exact copies of the maternal clone-founder genome. Ovaries transplanted in either direction between the standard and the parthenogenetic genotypes yield eggs capable of parthenocloning. In addition, use of male larvae as ovary recipients eliminates diapause in eggs produced in the implants. Unfertilized eggs of some silkworm clones respond also to the cold-shock treatment by producing homozygous fertile sons; cloned females can be crossed with their parthenogenetic sons to obtain progeny homozygous for the transgene in both sexes. Rational exploitation of available parthenozygous pools and the use of parthenocloning methods enable rapid fixation and maintenance of the desired genotypes.

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1. Introduction

The technology of silkworm (*Bombyx mori*) rearing and silk harvesting from the cocoons was discovered in China about 5000 years ago. Sericulture spread to many other countries and a handful of other moth species became exploited in the silk industry but *B. mori* has remained the major producer of commercial silk. The economic importance of sericulture declined after the discovery of the man-made polymers in early forties of the last century. Nylon and other synthetic materials rapidly replaced silk in most applications and their cost has been decreasing, while the cost of silk has increased because of the gradual rise of the labor cost. However, in spite of the relatively high cost, the excellent silk properties and the tradition of silk

dresses in some countries create sufficient demand for the maintenance of traditional sericulture and associated textile industry. Silk exploitation in medicine and cosmetics is of a lesser but nevertheless persisting commercial importance.

The profitability of sericulture can be enhanced with the aid of genetic engineering that can improve silk properties or employ silkworm as a bioreactor for the production of diverse commercially attractive proteins other than silk. This short review describes the principles of methods used to produce transgenic silkworms and to establish them as genetically stable lines. It must be mentioned that silkworms (and some other insects) can be used as bioreactors without interventions with their genotype. Instead, genes encoding desired proteins are introduced into microbial vectors that express them in the

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infected insects or in the infested insect cells grown *in vitro*. The technique was first published by Maeda *et al.* (1985) who demonstrated production of human α -interferon in *B. mori* infected with the genetically engineered nucleopolyhedrovirus (BmNPV). Parallel investigations in the alfalfa looper *Autographa lifornica* and associated AcMNPV virus led to the development of commercial baculovirus expression vector system employing the cell line Sf9 derived from the fall armyworm, *Spodoptera frugiperda* (Roth *et al.* 1993). This expression system is now widely used for the production of diverse proteins in many laboratories. Exploitation of AcMNPV in *B. mori* has been unsuccessful for many years because the tested strains of *B. mori* resisted infection by this type of baculovirus (reviewed by Kato *et al.* 2010). Only the recent study by Wöltje *et al.* (2014) described AcMNPV expression in several tissues of certain *B. mori* strains. The authors also demonstrated baculovirus penetration into the silk glands. We can expect commercial exploitation of this finding because purification of desired proteins from the glands or from their silky secretion is easier than the extraction from haemolymph or tissue culture media.

The baculovirus expression systems are suitable for the production of specific proteins in cultured cells, less so in living insects. Transgene insertion into the insect genome is preferable to the viral infections because it permits establishment of stable silkworm lines that can be further modified, for example by crosses with other genotypes. The production of strains with defined and inheritable genome modifications requires (a) intervention with the genome such as transgene insertion and (b) subsequent breeding of transgenic insects for stable transgene expression and eventually homozygosity in the affected locus. In the following text we show that the techniques of both steps have been sufficiently elaborated for research purposes, while commercial applications seem to be hindered by fear of a chancy escape of genetically engineered silkworms into the environment. The same concern applies to the use of genetically engineered baculoviruses and other insect pathogens. We believe that these fears are not justified because the risk of silkworm escape into the wild is small (*B. mori* cannot survive without human care) and pathogens can be selected for high virulence in *B. mori* and low in other Lepidoptera. The risks are outweighed by the perspective that the deployment of genetically engineered silkworms could secure jobs to millions of people in the developing countries. Previous investments (mulberry plantations, facilities for silkworm rearing, training of farmers) into the declining traditional sericulture would be redeemed.

2. Transgenesis, mutations and gene silencing in *Bombyx mori*

Drosophila melanogaster was the first insect species in which transgenesis (insertion of a foreign gene into the

genome) was accomplished by Rubin and Spradling (1982), who used DNA construct containing a vector plasmid with the mobile P element fused to a marker gene under control of a suitable promoter (for example promoter driving expression specifically in eyes). The construct was injected into early embryos along with the helper plasmid DNA that supplied P element transposase. This enzyme mobilized the plasmid construct and its insertion into *Drosophila* genome. Occasional insertions into the genome of the germ line cells carried the construct to the next fly generation. Transgenic insects expressing the marker gene could be recognized and collected. The routine use of this method contributed substantially to our knowledge of *Drosophila* development.

Numerous attempts to use this method in other insects failed because the P element transposon could not be mobilized and inserted into the genome of the non-drosophilid species. This became possible only after the discovery of transposons whose activity was not restricted to *Drosophila*. The first successful transgenesis of a non-drosophilid insect was reported for the medfly *Ceratitis capitata* injected with the transposon vector *minos* (Loukeris *et al.* 1995). Transgenic mosquito was subsequently obtained with the transposons *Hermes* and *Mariner* (Jasinskiene *et al.* 1998; Coates *et al.* 1998). Transgenic silkworm was obtained with the transposon *piggyBac* as a vector and Enhanced Green Fluorescent Protein (EGFP) as a marker under the control of silkworm cytoplasmic actin gene promoter (Tamura *et al.* 2000) or of *Drosophila* heat shock promoter *hsp70* (Uhlířová *et al.* 2002). The principle of transgenesis is similar to that of *Drosophila*: plasmid DNA with the transposon containing a passenger gene and a marker gene is injected into the embryos simultaneously with a helper plasmid providing the transposase. Transgenic insects are detected in the next silkworm generation (figure 1).

The method of silkworm transgenesis proved efficient and reliable, including stable genomic location of the inserted gene for a number of generations. The method was successfully applied in diverse studies of gene interactions and was standardized for the commercial use. The GAL4/UAS system, which had been developed for *Drosophila* (Brand and Perrimon 1993), was later employed for monitoring gene expression also in the silkworm tissues (Imamura *et al.* 2003). The system has two components, both from the yeast: (1) Genetic strains are prepared in which the *GAL4* transcription activator gene is placed randomly under the control of native gene promoters (driver genes) that stimulate *GAL4* production according to the time and spatial patterns of the driver gene expression. (2) Genetic lines containing the Upstream Activator Sequence (UAS) next to chosen genes is the other component of the system. Hybrids between the *GAL4* and the UAS lines express *GAL4* in response to appropriate cellular regulators. No change is noted in cells missing either *GAL4* or UAS. In the cells expressing *GAL4*,

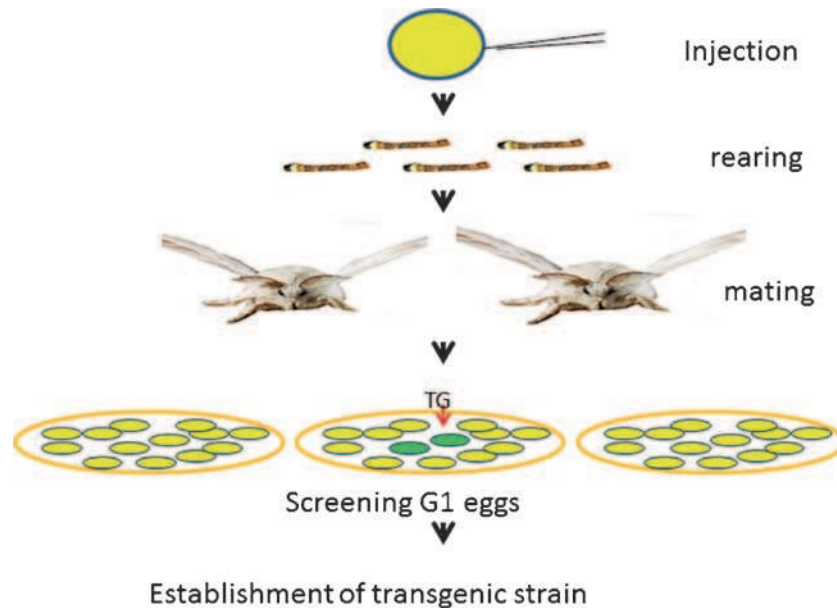


Figure 1. Construction of transgenic silkworms. Plasmid DNA containing *piggyback* transposon with inserted transgene(s) is injected into preblastodermal embryos along with the helper plasmid DNA encoding the *piggyBac* transposase. Transgenic silkworms expressing a marker gene are collected in the next generation.

this transcription activator binds to the UAS sequence and thereby stimulates the adjacent target gene. With the aid of marker genes generating visible markers like GFP (Green Fluorescent Protein), the expression pattern of the driver genes can be determined. Optimized binary system composed of a GAL4–Ser1 promoter construct and an UAS construct attached to the TATA box region of *Drosophila* *hsp70* gene and containing the signal peptide and introns, yielded large amounts of recombinant proteins (Tatematsu *et al.* 2010). Various lepidopteran genes have been identified and characterized with the aid of this system: genes controlling the color of egg, eye, larva and cocoon, respectively, genes involved in the hormonal regulation of molting and metamorphosis, genes important for insect resistance to the insecticides, etc. (Tan *et al.* 2005; Ito *et al.* 2008; Fujii *et al.* 2010; Atsumi *et al.* 2012; Daimon *et al.* 2012; Tsubota *et al.* 2014).

The current technique of transgenic silkworm production with the aid of a transposon is useful but the yield of recombinant proteins is rather low because the activity of transgene promoters is always much weaker than that of the promoters of endogenous genes with stable location in the genome (Tatematsu *et al.* 2010). This drawback may be to some extent eliminated with the use of more efficient promoters from different organisms. For example, over-expression of the *Ras1^{CA}* gene in the posterior silk gland region increases the yield of silk proteins by 60% (Ma *et al.* 2011). The random transgene insertion into the host genome is often associated with a strong positional effect that makes

transgene expression unpredictable and unstable. Inferior transgene position in the genome and strong expression of some endogenous genes reduce expression of the transgene(s). The impact of these factors can be reduced by targeting gene mutation or insertion to a desired position. This requires site-specific breaks of double stranded DNA followed by transgene insertion into the created nick. Controlled DNA cuts are made with nucleases that are engineered to recognize specific DNA sequences. Three types of such nucleases have been used for genome editing in *B. mori*. Zinc finger nucleases (ZFN) consist of a pair of fusion proteins, each of which is composed of a zinc finger DNA recognition domain and the FokI nuclease domain. The zinc finger domain is designed to recognize specific DNA sequence that is then cut by the nuclease. Homologous recombination with the target gene, which is followed by its insertion into the silkworm genome, occurs during the process of the cut site repair. The applicability of ZFN technique to the silkworm was demonstrated by injecting ZFN mRNA into the embryos followed by detection of targeted mutagenesis in the chosen gene (Takasu *et al.* 2010). However, genome editing using ZFN is extremely laborious because of poor activity of the vast majority of designed ZFN.

Genome editing is more efficient with the recently described Transcription Activator-Like Effector Nucleases (TALENs) that use a TAL effector domain instead of the zinc finger domain to recognize the target DNA sequence. Encouraging results were obtained in the tests of a TALEN

in the silkworm (Ma *et al.* 2012; Sajwan *et al.* 2013). The contingency to construct efficient TALEN is much higher than that of constructing a ZFN of similar potential. Based on a study of the efficiencies of an array of TALENs to cut the target site, it was possible to construct a very efficient TALE backbone plasmid adapted for the silkworm (Takasu *et al.* 2013). Protocol was developed for a very efficient genome editing system that yielded reliable results in the silkworm as well as in *Drosophila* (Takasu *et al.* 2014). Additional techniques of genome editing may emerge, such as the Clustered Regularly Interspaced Palindromic Repeats/CRISPR-associated proteins (CRISPR/Cas) system, which was tested in the silkworm with encouraging results (Wang *et al.* 2013).

DNA cuts may either be allowed to repair with endogenous mechanisms that often give rise to non-homologous recombination between available DNA ends. Homologous recombination can occur between cuts in the genomic DNA and the matching DNA sequences in provided transgene construct. It has been shown that such homologous recombination is mediated by the enzyme integrase from the bacteriophage ϕ C31. The original integrase function is to stimulate recombination between two 34 base pair sequences, one in the phage and the other in the genome of its bacterial host. This recombination activity of ϕ C31 integrase is retained in diverse organisms, including *B. mori* (Yonemura *et al.* 2012a, b Yonemura *et al.* 2013). It is expected that combination of targeted DNA cuts induced by TALENs or by the CRISPR/Cas system with the use of the ϕ C31 integrase will greatly improve transgene expression and spur the deployment of silkworm biotechnologies.

3. Silk glands as ideal organ for the production of recombinant proteins

The pair of silk glands (SG) is the organ of choice for the production of recombinant proteins because it is tuned to the synthesis of copious amounts of a few proteins in specific gland regions and at specific times of the last larval instar (reviewed by Fedič *et al.* 2002). Large posterior silk glands section secretes heavy chain fibroin (H-Fibroin), light chain fibroin (L-fibroin) and fibrohexamerin (Fhx, also known as P25) that are assembled into the fibroin core of the silk filament (Inoue *et al.* 2000). The column of jelly-like fibroin is pushed into the middle silk glands section where it is enveloped by several sericin proteins derived from the genes known as *Ser1*, *Ser2* and *Ser3* that are expressed in the middle SG region. *Ser1* was almost fully sequenced by Garel *et al.* (1997) who identified 9 exons spliced alternatively to 4 mRNAs (2.8, 4.0, 9.0 and 10.5 kb, respectively). *Ser2* gene was mapped with restriction enzymes and showed to generate at least 2 mRNAs (3.1 kb and 5.0 kb) (Michaille

et al. 1990a, b). Gene analysis in different strains of *B. mori* revealed variability in exon number in the *Ser2* gene (Kludkiewicz *et al.* 2009). The sequence of *Ser3*, which encodes a single mRNA of 4.5 kb, was identified several years ago (Takasu *et al.* 2007).

During spinning the column of protein gel moves in the SG lumen to the narrow anterior silk gland section where fibroin core solidifies into a strong and flexible filament. The filaments (one from each gland) align in the spinneret and are sealed into a single fiber by the inner-most layer of sericins. Next sericin layer stiffens the fiber, glues it to a substrate suitable for the cocoon attachment, and eventually glues fibers to one another in the cocoon wall. The expression of sericin genes and the splicing of the *Ser1* and *Ser2* transcripts follow precise time patterns.

All structural silk proteins are specifically and strongly expressed in defined silk gland regions. Their promoters (*in sensu lato*) are used to prepare DNA constructs driving expression of introduced transgenes in the silk glands. Genetic engineering aims at silencing the innate gene(s) whose products contaminate and reduce the yield of the desired protein(s) encoded by the transgene. In addition to the fibroin and sericin proteins, the silk contains minor proteins of different kinds. They include Kazal-type and Kunitz-type proteinase inhibitors, seroins (Nirmala *et al.* 2001), serpins (Yonemura *et al.* 2012a), anti-bacterial hemolin (Shaik and Sehna 2009) and a few other proteins. The functions of these proteins are little known and possible impact of their expression on the transgene expression remains to be examined.

The selection of promoters and other regulatory sequences is a key step in the preparation of transgene constructs. Xu (2014) has recently reviewed experience with the constructs based on putative regulatory regions of the *H-fibroin*, *L-fibroin*, *Fhx*, *Ser1* and *Ser3* genes. All constructs included regulatory 5' flanking region, promoter and part of the internal sequence of the respective gene. The 3' flanking region was included in the transgene constructs of the *H-fibroin*, *L-fibroin* and *Ser1* expression system, while the *Fhx* construct contained polyadenylation sequence from the simian virus (SV40) and the *Ser3* construct ended with the 240 bp terminal sequence of the *hsp70* gene. Silkworm transgenesis was achieved with all five systems, the constructs based on the *H-fibroin*, *L-fibroin* and *Ser1* seemed to be the best ones.

Practical exploitation of silkworm transgenesis is targeted to the production of modified silk for the textile industry and/or to the fabrication of biomaterials for medical purposes. Several laboratories prepared silkworms carrying genes encoding spider-like silk proteins in addition to the endogenous silk genes (Kojima *et al.* 2007; Kurihara *et al.* 2007 Wen *et al.* 2010; Zhu *et al.* 2010). The expression of spider-like silk in transgenic silkworms was achieved with different constructs but the yields were low. Teulé *et al.* (2012) prepared composite silk that combined protein

copying part of the H-fibroin with a protein encoded by a gene designed on the basis of 3 different kinds of silk proteins of the spider *Nephila clavipes*. Composite silk obtained from the cocoons was stronger and more extensible than the parental silkworm and spider silks. Increased fiber toughness was also reported by Kuwana *et al.* (2014) for a fusion protein composed of a H-fibroin region and part of the dragline protein of the spider *Araneus ventricosus*. Although the content of the spider protein was only 0.37–0.61 % of the

native H-fibroin (w/w), the tensile properties (toughness) of the raw silk was enhanced by 53%. The authors demonstrated that their silk can be processed from the cocoons to textile products.

The success of silkworm transgenesis is usually monitored with the aid of a marker (Horn *et al.* 2002) such as GFP. When the gene encoding a fluorescent protein is included in the transgene construct driven by a promoter of a silk protein gene, the fluorescent protein is deposited in the



Figure 2. Silk from transgenic silkworms containing heavy chain fibroin gene fused with a gene encoding a fluorescent protein. Cocoons that are white or pink in normal light appear green and orange, respectively, in the fluorescent light (top panel). Fluorescent silk can be processed by low temperature reeling and used in textile industry (bottom panels).

secreted silk. Japan researchers used fusion genes encoding the non-repetitive region of H-fibroin and a chosen fluorescent protein and obtained stable transgenic lines producing fluorescent silks of different colors (Iizuka *et al.* 2013). A newly developed modification of the silk reeling and further silk processing preserves fluorescent colors in the manufactured textiles (figure 2) that have acquired higher commercial value. Mass production system for the fluorescent color silks and fashion designs of the apparel are being developed.

The production of recombinant proteins for medical purposes also affords great potential (Tomita 2011; Tatematsu *et al.* 2012). More than 20 reports on the production of medically important recombinant proteins in the silk glands of transgenic silkworms were listed by Xu (2014). The proteins include collagens, serum albumin, cytokines, and monoclonal antibodies. Transgene products are in some cases used as admixtures improving the properties of native silk. For example, Yanagisawa *et al.* (2007) showed that expression of partial sequences of collagen and fibronectin in the silk glands of transgenic silkworm enhanced cell adhesion to the spun-out silk.

4. Silkworm parthenocloning

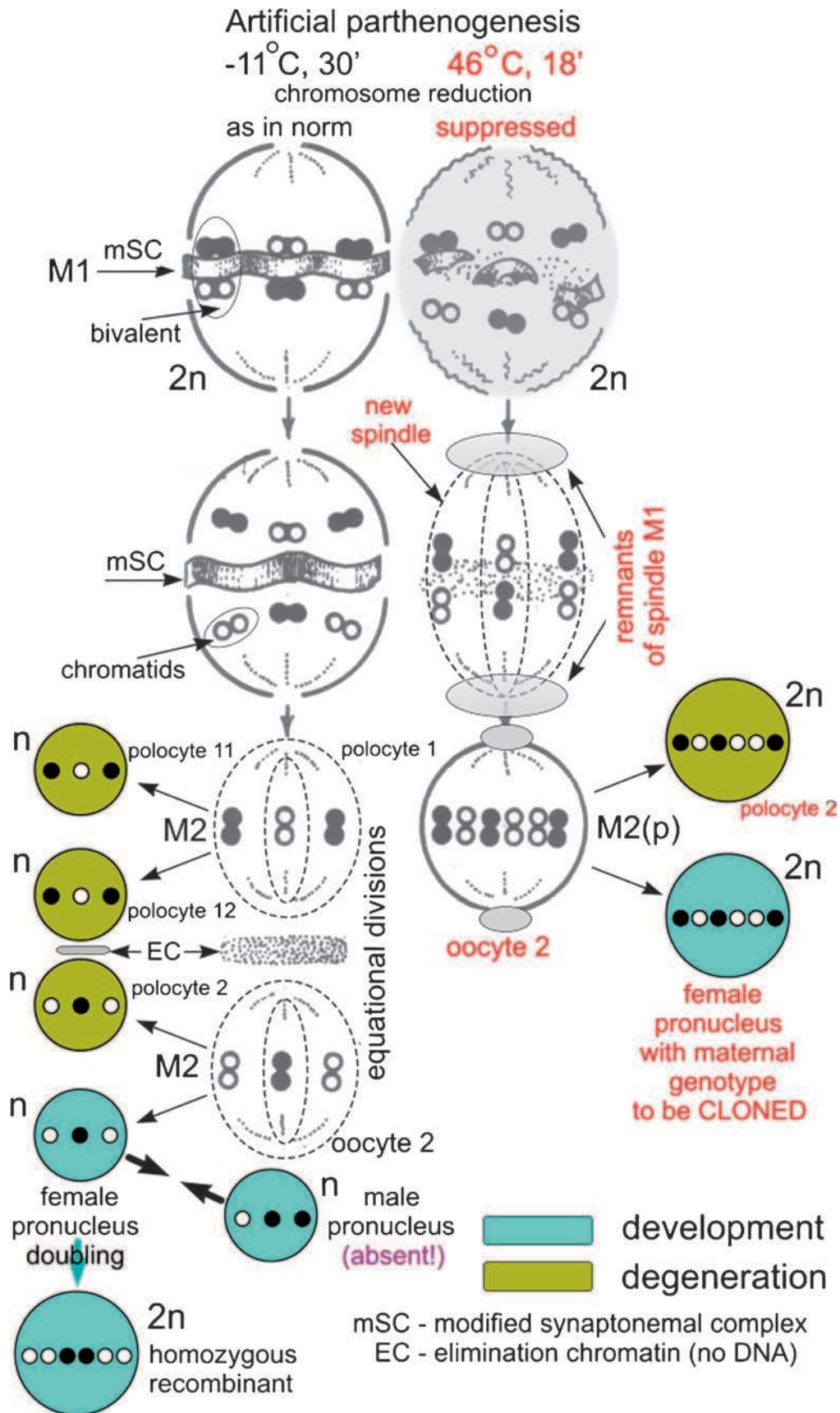
When a successful transgenic event is accomplished, it is desirable to obtain silkworms homozygous for the transgene inserted in a suitable genomic context. Novel methods of targeted transgene insertion, e.g. with the aid of TALENs, may mediate simultaneous transgene insertions into a certain locus in both chromosomes and thereby generate homozygous insects already in the F1 generation (Takasu *et al.* 2014). However, repeated sibling crosses of the F1 and subsequent generations must often be used and establishment of a suitable genetic strain may take several years. Grenier *et al.* (2004) proposed acceleration of the selection process by artificial parthenogenesis, i.e. induced production of viable progeny from unfertilized eggs. It is normally a very rare event in *B. mori* but it may be elicited by temperature shocks (Tichomiroff 1886). Efficient induction of parthenogenetic development was discovered by Astaurov (1936, 1940), who tested different temperature treatments. He found that exposure of unfertilized eggs (dissected from the ovaries of adult females) to 46°C for 18 min followed by 3 days at 15°C was most efficient for the induction of complete parthenogenetic development in several silkworm strains. The treatment generated exclusively female progeny that was further propagated as a clone using the heat treatment of unfertilized eggs in every generation.

On the basis of genetic analysis, Astaurov (1936) predicted the mechanism of the heat-induced parthenogenesis (figure 3). Like in other Lepidoptera, silkworm

oogenesis is blocked in the metaphase of the first (reducing) meiotic division when homologous chromatid doublets attach to the spindle and align in two plates adhering to the band-like synaptonemal complex (Rasmussen 1976). The continuation of meiosis is normally induced by egg interaction with the sperm. Chromatid doublets then detach from the synaptonemal band and are pulled to the daughter nuclei that continue meiosis with the equational division. Complete meiosis yields 1+2 haploid polocytes that are discarded, and a mature haploid oocyte. Half of the oocytes contain the sex chromosome W, the other half harbors chromosome Z. Embryogenesis is initiated by the entrance of haploid sperm with the Z chromosome. Zygotes with resulting ZZ configuration develop into males, and those with the ZW configuration become females. The pronuclei interact and initiate the first cleavage mitosis.

Astaurov (1936) suggested that heat shock suppresses the reducing division of meiosis (hence the name ameiotic parthenogenesis), while the equational division takes place. Both daughter nuclei (one will become female pronucleus; the other is discarded as polocyte) receive one diploid set of chromatids. Since no crossing over occurs in silkworm oogenesis (Sturtevant 1915), the genomes are identical with that of the mother, including presence of both Z and W chromosomes that determine female sex. By means of many outcrosses and repeated selection of females yielding high proportion of parthenogenetic progeny, Astaurov created several parthenoclones in which heat treatment induced development in 30-60% of unfertilized eggs. He eventually

Figure 3. Two types of silkworm parthenogenesis induced by temperature treatments of unfertilized eggs. In the meiotic parthenogenesis (left), and ameiotic (right) parthenogenesis. In case of meiotic parthenogenesis, oocyte development is blocked in metaphase I (M1), when homologous chromatid doublets are attached to the band-like synaptonemal complex, mSC). The block is normally removed by fertilization: two subsequent divisions then yield 3 discarded polocytes and one mature oocyte (note random distribution of chromatids represented by black and white circles). Haploid female pronucleus fuses with the male pronucleus from the sperm and the cleavage begins (not shown). The sex chromosome (W or Z) in female pronucleus determines the sex of future embryo (male pronucleus always carries a Z chromosome). Haploid oocyte is produced in similar way in meiotic parthenogenesis but diploidy is restored in the 2n homozygous recombinant by the fusion of nuclei of the first cleavage division. The ameiotic parthenogenesis (right) is induced by the heat treatment that destroys chromosome anchoring to the mSC and thereby prevents completion of the first meiotic division (hence the name 'ameiotic'). Oocyte enters the second (equational) meiotic division (M2) with full number (4n) of chromosomes (including the WZ sex chromosomes determining the female development) and generates development-promoting diploid (2n) female pronucleus and abortive diploid polar body (Klimenko and Spiridonova 1979).



constructed a genotype with almost 100% hatchability of unfertilized eggs exposed to the heat shock (Astaurov 1973). This clone named P29 has been maintained for more than 40 years without any visible loss of its parthenogenetic potential and other phenotypic characteristics. Its genome has been reproduced faithfully at least for 100 serial generations. The ability to reproduce parthenogenetically was recognized as a dominant or semi-dominant polygenic trait that can be passed to P29 hybrids with the non-parthenogenetic silkworm strains. Numerous F₁ and F₂ crosses were made between the P29 clone and various silkworm lineages. The results demonstrated that the progeny of such outbred crosses retains high capacity of parthenocloning. Female parthenocloned silkworms with five morphological markers in homozygous state were obtained and can be used in the development of silkworm biotechnologies (Klimenko and Spiridonova 1977; Klymenko 2001).

The technique of silkworm transgenesis has been worked out for the strains without egg diapause, while the parthenocloned females produce diapausing eggs. Fortunately, non-diapausing eggs can be obtained from the parthenogenetic females when their ovaries are transplanted into male larvae in which they complete oogenesis to the chorionated eggs (Zabelina and Klymenko 2008). The availability of parthenocloned silkworms also offers the opportunity to establish parthenogenetic lineages from the standard (non-parthenogenetic) transgenic silkworms. Ovaries transplanted from a parthenocloned larva into a standard larva yield eggs responding to proper heat treatment by parthenogenetic development nearly as readily as the eggs from the ovaries left *in situ* (Zabelina and Klymenko 2008; Doroshenko and Klymenko 2010). The sex of the recipient is irrelevant. This observation is consistent with the genetic determination of the capacity for parthenogenesis. However, the potential of parthenogenetic development is also procured in the eggs that develop in ovaries transplanted from a standard larva into a parthenocloned larva, implants of ovaries from standard larvae acquire the capacity of ameiotic parthenogenesis by unknown factors in the internal milieu of the parthenocloned larva (Doroshenko and Klymenko 2010).

Strunnikov (1987) and Terskaya and Strunnikov (1975) investigated the induction of parthenogenesis by exposing unfertilized eggs to the cold shocks. Transfer of eggs to –11°C for 30 min proved most efficient and became standard procedure for the induction of the ‘meiotic’ parthenogenesis leading to males (figure 3). By mimicking termination of the meiotic block by sperm, the cold treatment triggers completion of the first (reducing) meiotic division that is associated with random distribution of chromatid doublets into the daughter nuclei. The division generates nuclei of oocyte II and polocyte I, both with haploid number of the randomly distributed chromatid doublets. The second meiotic division (equational) takes place, whereby oocyte II produces female pronucleus and polocyte II, and the polocyte I gives rise to two daughter polocytes (figure 3). All four nuclei contain

haploid number of separated chromatids. All polocytes perish, while the oocyte initiates haploid cleavage (sperm nucleus is absent). The diploidy is restored by the fusion of the first two daughter nuclei (they have identical genomes). Homozygous diploid ‘parthenozygotes’ with ZZ chromosomes can develop into fertile males able to transmit the capacity of parthenogenesis and any transgene to the desired inbred lines. The rate of hatching in the male-generating parthenogenesis much depends on the genotype – in case of P29 it did not exceed 2–3%, in the new parthenocloned P14 it may reach 5–6% (Klymenko *et al.* 2013; Lysenko *et al.* 2013).

The transgenesis of females of the P14 parthenocloned silkworm has been accomplished (Zabelina *et al.* 2015) using procedures outlined in this paper. The numbers and sites of transgene insertions have been stable for four generations completed so far. It was also shown that complete male homozygotes can be obtained from these parthenocloned silkworms by the cold shock. The cross of such a meiotic homozygous parthenogenetic son with its maternal clone is likely to yield homozygous transgenic progeny. The bisexual homozygous lines can be used for further breeding, studies of transgene functions, harvesting the transgene product, etc. Their hybrids with non-parthenogenetic females acquire the potential of parthenocloning.

The potential of silkworm parthenogenesis has not been yet fully exploited. For example, females and males homozygous in the transgene locus can also be obtained through meiotic parthenogenesis in tetraploid eggs (Klimenko and Spiridonova 1977; Klymenko and Liang 2012). The multiple uses of parthenogenetic silkworms will accelerate the production and cloning of transgenic silkworms and facilitate practical exploitation of this technique.

5. Conclusions: Trends in silkworm biotechnologies

Currently developed silkworm biotechnologies focus on silk modifications and on the production of proteins for medical applications. Baculoviruses have been engineered to produce desired proteins under the control of viral promoters in the infected caterpillars or in insect cell lines propagated *in vitro*. However, more attention is paid to the use of transgenic silkworms that are mostly produced with the aid of the *piggyBac* transposon. Transgene constructs include a marker gene that is usually expressed in eyes and encodes a fluorescent protein. GAL4-UAS system is available for investigations on the tissue and time specific gene expression. Most of the transgenes used today contain promoters derived from the genes encoding the major silk proteins. Relatively low transgene expression with these promoters is probably due to inferior sites of the promoter/transgene insertion and to interactions with endogenous genes. New methods of targeted mutagenesis employing ZFNs, TALENs and CRISPR/Cas

system have been adopted with promising results. Targeted transgene insertion is facilitated with the enzyme integrase from the bacteriophage ϕ C31. So far hypothetical transgene insertion to identical site in both homologous chromosomes would yield silkworms homozygous for the trait already in the G1 progeny, whereas in case of random transgene insertion the selection of homozygous strain requires sibling crosses and is tedious. The selection can be accelerated with the use of parthenogenetic females that establish clones with stable and inheritable genotypes.

All females of a transgenic parthenoclone produce oocytes of identical genotype (including the number and insertion sites of the transgene) that are used for clonal female reproduction by thermal ameiotic parthenogenesis. In response to the cold shock, the oocytes may undergo meiotic parthenogenesis with random distribution of chromatid doublets into the daughter nuclei. Haploid pronucleus of the oocyte begins cleavage in the absence of the sperm pronucleus. The haploid set of chromosomes is then doubled, rendering the embryos homozygous. However, due to meiotic chromosome recombination, the genotypes of the embryos are diversified. Viable progeny is produced infrequently. When sons are crossed with their mothers of the female parthenoclone, half of their progeny will be homozygous for the transgene insertion. Hence, transgenesis is performed in G1, transgenic clones selected in G2, adults of both sexes are obtained in G3, and homozygous lineages and clones established in G4 – all this without expensive molecular biology checks.

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