## Review

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# Advanced technologies for genetically manipulating the silkworm *Bombyx mori*, a model Lepidopteran insect

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Genetic technologies based on transposon-mediated transgenesis along with several recently developed genome-editing technologies have become the preferred methods of choice for genetically manipulating many organisms. The silkworm, *Bombyx mori*, is a Lepidopteran insect of great economic importance because of its use in silk production and because it is a valuable model insect that has greatly enhanced our understanding of the biology of insects, including many agricultural pests. In the past 10 years, great advances have been achieved in the development of genetic technologies in *B. mori*, including transposon-based technologies that rely on *piggyBac*-mediated transgenesis and genome-editing technologies that rely on protein- or RNA-guided modification of chromosomes. The successful development and application of these technologies has not only facilitated a better understanding of *B. mori* and its use as a silk production system, but also provided valuable experiences that have contributed to the development of similar technologies in non-model insects. This review summarizes the technologies currently available for use in *B. mori*, their application to the study of gene function and their use in genetically modifying *B. mori* for biotechnology applications. The challenges, solutions and future prospects associated with the development and application of genetic technologies in *B. mori* are also discussed.

## 1. Introduction

Genetic technologies applicable to plants, vertebrates and insects began to become available in the early 1980s, beginning with simple tools for inserting transgenes into genomes. Since then, there has been a steady growth in the number and sophistication of genetic technologies such that today we have the capabilities of precisely editing the DNA sequence of an organism's genome. While these tools were often developed initially for use in popular reference organisms such as yeast, tobacco, mouse and fruit flies, today their applications are found in a wide range of organisms. When existing genetic technologies are used in combination with the abundance of genomic DNA sequence data that is increasingly available, biologists of all types have unprecedented opportunities to understand the relationship between genotypes and phenotypes. Not only do these technologies enable fundamental and basic questions regarding the biology of organisms to be addressed but they also enable the genetic manipulation of organisms for practical purposes—improving crop yields, developing therapeutics for human diseases and controlling agricultural pests to name just a few. Here, the genetic technologies that have been applied to the Lepidopteran model insect, the silkworm *Bombyx mori*, will be briefly reviewed along with the current challenges and future trends.

*Bombyx mori* is a domesticated insect that has been intimately associated with humans for over 5000 years [1]. It has been and continues to be employed to produce silk, and has contributed greatly to the economic development of countries along the ancient Silk Road and cultural exchanges between the East and West [2]. Since the nineteenth century, *B. mori* has begun to serve as a reference or model

organism for studies in the life sciences and has contributed many classic paradigms associated with our understanding of genetics and molecular biology [3]. While *B. mori* remains critically important to sericulture throughout the world, the significance of *B. mori* has grown with the sequencing and annotation of its genome [4,5] along with the development of genetic technologies that enable the genome to be manipulated. *Bombyx mori* larvae have also exhibited enormous potential to be used as living 'bioreactors' which, after appropriate genetic modification, can produce valuable proteins, therapeutics and silk-based biomaterials.

# 2. Transposon-based technologies

## (a) Transgene integration technologies

### (i) Transposons

DNA-type transposons from insects including *P*, *Mos1*, *Minos*, *Hermes* and *piggyBac* [6] are used as gene vectors to carry exogenous DNA into the chromosomes of insects to create transgenic insects. To date, over 40 species of insects have been genetically modified using transposon-based gene vectors [7]. Among the current collection of insect transposons that can serve as gene vectors, only the *piggyBac* element originally isolated from the genome of the moth *Trichoplusia ni* [8] functions efficiently in *B. mori*. Initially, microinjecting *piggyBac*-derived vectors into *B. mori* embryos yielded a transformation frequency of only 1.8% [9], but shortly thereafter optimization resulted in rates of up to approximately 25% by several groups [10–13].

Insertions of a single copy of a transgene per genome is often preferred for the analysis of gene function and, in most cases, only a single copy of *piggyBac* was shown to integrate into the *B. mori* genome, and all integrations occurred in the sequence TTAA, the known target site for *piggyBac* [14]. *Bombyx mori* has about 100 endogenous *piggyBac*-like elements in its genome, some of which have the potential to encode an intact transposase, whereas the most conserved element shares only 34% amino acid identity with the transposase of *T. ni piggyBac* [15,16]. It was thought by some that the existence of these elements might influence the genetic stability of integrated *piggyBac* gene vectors but it was shown recently that after 11 successive generations integrated *piggyBac* gene vectors remained stable [17]. *piggyBac* gene vectors are able to carry large fragments of exogenous DNA into genomes. In *B. mori*, the largest fragment integrated using a *piggyBac* vector was 12.4 kb [18]. Ma *et al.* [13] reported that fragments 2–8 kb in length had no significant effect on the transformation efficiency of *B. mori* using *piggyBac* vectors. Collectively, these characteristics make the *piggyBac* transposon an indispensable platform for the development of advanced tools for gene manipulation.

### (ii) Site-specific recombination

Transposon-mediated transgenesis can result in unexpected transgene expression patterns as a result of local chromatin structure as well as promoters and enhancers found at or near the integration site. These insertion site-dependent influences on transgene expression, or position effects, make transgene expression from randomly integrated transposons unpredictable. To overcome this problem, several site-specific recombination systems originating from yeast (Flp/FRT) or bacteriophage (Cre/loxP and ΦC31/att) were

developed into transgene integration systems. These systems work through the action of specific recombinases that mediate recombination between two precisely defined sequences specific for each system. Transposon-based technology is used to introduce a system-specific recombination site into the host genome. After confirming that the recombination site is not subject to unwanted position effects, it can be used as a landing site to recombine transgenes of interest via microinjection of plasmid DNA. Recombinase-mediated recombination between the chromosomal and plasmid-borne recombination-sequences results in the plasmid being integrated into the chromosome. This allows for repeated transgene integration into a site in the genome that will result in predictable transgene expression. These systems have been successfully used in *B. mori* [19–21] although the recombination efficiency has been very low compared with efficiencies seen in other organisms such as *Drosophila*. Although potentially useful in *B. mori*, improvements are needed in the performance of these site-specific recombination systems to make them practical options for those working with this species.

## (b) Transgene expression technologies

### (i) Heat-inducible expression system

For some *B. mori* genes, functional analysis can be easily achieved via transgene expression regulated directly by its native promoter, but sometimes this can cause unexpected effects such as developmental defects and lethality, making it difficult to maintain the transgenic lines. To avoid these effects, Uhlírová *et al.* [22] developed a heat-inducible expression system by using the promoter of *Drosophila heat-shock protein 70* gene (*Dmhsp70*). Uhlírová *et al.* [22] created transgenic *B. mori* in which the *Bombyx* nuclear receptor *Ftz-F1* (*BmFtz-F1*) gene was controlled by the *Dmhsp70* promoter. *BmFtz-F1* is normally highly expressed for short periods during each moult [23], but under *Dmhsp70* promoter control when transgenic larvae were treated at 42°C for 1 h, ectopic expression of the *BmFtz-F1* was easily induced at different developmental stages and in diverse tissues [22]. This heat-inducible system was the first tool developed for the conditional control of transgene expression in *B. mori* and showed that some promoters from species of insects other than *B. mori* could be used to control transgene expression in various tissues or developmental stages of this species.

### (ii) Gal4/upstream activating sequences expression system

This system is a binary expression system that is extensively used in gene analysis studies in model organisms and consists of a transcription factor (*Gal4*) and a promoter whose activity requires *Gal4* binding and which contains multiple *Gal4*-binding sites referred to as upstream activating sequences (UAS) [24,25]. Using this system, genes of interest under the regulatory control of UAS-containing promoters can be expressed in well-defined temporal and spatial patterns determined by the expression of *Gal4*. The modularity of this system in which one creates transgenic lines with various *Gal4* expression patterns and lines harbouring UAS-controlled gene expression-cassettes enables investigators to easily make different combinations of *Gal4*-expressing and UAS-regulated transgenes to yield a variety of spatio-temporal patterns of gene expression by simply performing genetic crossing.

In 2003, the Gal4/UAS system was established in *B. mori* [26], and further optimized by increasing the transcriptional activation activity of Gal4 [27]. So far, there have been

dozens of ubiquitously active and tissue-specifically active promoters identified in *B. mori* and some have been used to produce *Gal4*-expressing lines [26,28–34]. Using these lines, many *B. mori* genes have been analysed (electronic supplementary material, table S1) and the broad utility and versatility of the Gal4/UAS system has made it a crucial tool for analysing gene function in *B. mori*. In combination with an active transposon, the *Gal4*-coding region can be randomly inserted throughout the genome and used to screen for and identify regulatory regions with specific patterns of expression (see Gene- and enhancer-trap technologies, below).

### (iii) Tetracycline-On/tetracycline-Off expression system

The Tet-On/Tet-Off systems are also binary gene expression systems consisting of a tetracycline (Tet)-controlled trans-activator (tTA) and tTA-responsive promoter containing tTA-binding sites (Tet operators; TetO). However, binding of the tTA to the TetO, which is strong in the absence of Tet or doxycycline (Dox), is inhibited in the presence of either of these molecules (Tet-Off). On the other hand, binding of Dox to a reverse form of tTA (rtTA) results in binding of the rtTA to TetO and initiation of transcription (Tet-On) [35–37].

Karasaki *et al.* [38] showed that the Tet-On system did not function in *B. mori* but when rtTA was modified by addition of the activation domains of herpes virus protein 16 (VP16) and the immediate-early gene 1 of the *Autographa californica* nuclear polyhedrosis virus (*ie1*), the transactivator showed typical responsiveness to Dox (Tet-On). Further optimization of the rtTA resulted in transcription activation of a reporter gene up to 300-fold in the presence of Dox [39]. Recently, the Tet-Off system was also shown to function in *B. mori*. Tan *et al.* [40] inserted the coding region of tTA into a transgene containing the coding region of the *doublesex* gene from *Pectinophora gossypiella* in such a way the functional tTA-encoding messages will only arise in females as a result of sex-specific splicing. This tTA transgene was regulated by a promoter containing TetO resulting in an auto-activating transgene that was subsequently integrated into the genome of *B. mori*. In the absence of Tet, the tTA was highly expressed as a result of auto-activation but only in transgenic females. Expression of the auto-activating transgene leads to levels of tTA transcription and protein production that result in cell-autonomous lethality by mechanisms that remain unclear. In the presence of Tet, the tTA transgene was not expressed, demonstrating the functionality of the Tet-Off system in *B. mori*. Overall, these results indicate that the Tet-On/Tet-Off systems are functional tools available for manipulating gene expression in *B. mori*.

### (c) Transgenic RNA interference-based gene silencing technology

Transient RNA interference (RNAi)-based gene silencing using double-stranded RNA (dsRNA) [41] is widely used in insects for downregulating gene expression for the purposes of analysing gene function, but in *B. mori* this method of gene silencing has rarely been successful. However, co-expressing the gene *systemic RNA interference-deficient-1* (*sid-1*) from *Caenorhabditis elegans* [42,43], can enhance RNAi potency in *B. mori* ovary-derived cells but has no effect on gene silencing in transgenic individuals expressing *sid-1* [44]. It has been shown that insects with *sid-1*-like genes (e.g. *Tribolium castaneum*) exhibit a robust systemic RNAi response, while insects without *sid-1*-like genes

(e.g. *Drosophila*) show only cell-autonomous RNAi, leading to a hypothesis that the presence of *sid-1*-like genes correlate tightly with the presence of systemic RNAi in an organism [45,46]. *Bombyx mori* carries three *sid-1*-like genes, yet it does not show a robust systemic RNAi [46], implying that this species might lack another component of the RNAi pathway, or possess a suppressor. On the other hand, RNAi is effective in *B. mori* when dsRNA is produced through the expression of short-hairpin RNAs from chromosomally integrated transgenes using *piggyBac*-derived gene vectors. The success of this strategy depends on the construction of promoter-controlled dsRNA expression-cassettes in which identical target-gene sequences are arranged in an inverted repeat orientation and separated by a short 'stuffer' sequence. Expression of such transgenes produces dsRNA in the shape of a hairpin and will trigger RNAi against transcripts containing the sequences found in the inverted repeat.

The transgenic RNAi system has not only been used to silence the expression of individual genes within the genome of *B. mori*, but also multiple genes simultaneously [18]. Moreover, conditional gene silencing is also feasible in *B. mori* using transgenic RNAi technology in combination with available transcription regulatory systems. For example, heat-inducible promoters such as *Dmhsp70* have been used to control transcription of dsRNA expression-cassettes in *B. mori* [47]. Likewise, *Gal4*-mediated expression of UAS-regulated dsRNA expression-cassettes has been used to efficiently regulate gene expression in specific tissue and temporal patterns [48]. Collectively, these results demonstrate that the transgenic RNAi system can serve as a powerful tool when used alone or jointly with other technologies for gene expression analyses that benefit from the creation of hypomorphic or loss-of-function genotypes and phenotypes.

### (d) Gene- and enhancer-trap technologies

Enhancer-, gene- and protein-trap technologies all depend on active transposons to carry and move tunable transgenes within a genome that are used as sensors to detect the presence or activity of genes or regulatory elements. Tunable transgenes are constructed such that their expression is highly context-dependent and will be expressed and readily detected only when the transgene is within the domain of an active enhancer (enhancer-trap) or inserted into a transcribed gene (gene-trap) or a protein-coding region of a gene (protein-trap) [49–51].

In *B. mori*, an effective enhancer-trap system has been established and requires the use of three transgenic lines: (i) a line that expresses the *piggyBac* transposase ubiquitously or at least within the germ cells (this line is sometimes referred to as the 'jumpstarter'); (ii) a line containing a functional non-autonomous transposon (e.g. *piggyBac*) carrying the *Gal4*-coding region under the regulatory control of a minimal promoter and sensitive to the influences of enhancers (this line is sometimes referred to as a 'mutator'); and (iii) a line with a reporter gene such as a fluorescent protein under the regulatory control of a UAS-containing promoter that is responsive to Gal4 and capable of reporting on the 'enhanced' expression of Gal4 from the mutator (this line is sometimes referred to as a 'reporter'). Genetically introducing the jumpstarter and mutator into the same genome will result in remobilization of the mutator in the germline. Progeny arising from germlines in which the mutator was remobilized to a new genomic position are mated to individuals of the

reporter line. Progeny from this mating that have new patterns of reporter-gene expression compared with the parents contain the mutator element in a new genomic location in which the expression of *Gal4* is under the influence of an enhancer element. Lines with spatially and temporally distinct patterns of Gal4 expression can be used and re-used to regulate the expression of any transgene under the regulatory control of a UAS-containing promoter. This system has been very successfully used in *B. mori* [52,53] and hundreds of enhancer-trap lines showing ubiquitous patterns of *Gal4* expression and highly specific patterns of expression in various tissues and at different developmental stages have been created. Descriptions of these lines can be found in the *Bombyx* Trap DataBase [54]. Not only have these lines been useful for controlling transgene expression but they have also been used to isolate genes such as *non-susceptibility to densovirus type 2* (*Bmnsd-2*) and *biogenesis of lysosome-related organelles complex1, subunit 2* (*BmBLOS2*) using positional cloning methods [55,56].

Shimomura *et al.* [54] reported the existence of a gene-trap system for *B. mori*, in which a transgene present in the mutator element would be expressed only when the mutator was inserted into an actively transcribed gene, but details have not been published.

# 3. Genome-editing technologies

Genome editing allows for precise modification of genomic DNA sequences *in vivo* and there are at least three systems currently available. All rely on the creation of DNA double-strand breaks (DSBs) at a target locus under the guidance of either tunable modular proteins such as zinc fingers (ZFs) and transcription activator-like effectors (TALEs), or short guide RNAs (gRNAs) [57–60]. DSBs in DNA are repaired either by non-homologous end-joining that can result in disruption of the target sequence through insertion or deletion of nucleotides (creating a knockout mutation) or by homology-directed repair that can be used to insert exogenous DNA sequences at the target locus via homologous recombination (HR) (creating a knock-in mutation) [61–63]. Those studying *B. mori* were some of the first to use these technologies successfully in insects.

## (a) Zinc finger nucleases

The zinc finger nuclease (ZFN) system was the first genome-editing technology to be devised [64] and consists of pairs of user-designed proteins each based on three tunable ZF motifs that are capable of interacting with known trinucleotide sequences and that are linked to a monomer of the non-specific DNA endonuclease domain from the *Fok* I enzyme. The tuned ZF-containing domains determine where the proteins will interact within a genome, while dimerized *Fok* I domains act as 'DNA scissors' to create DSBs at the target locus [57,65]. In 2010, ZFNs were successfully used in *B. mori* to mutagenize the genes *BmBLOS2* and *white egg 3* (*Bmwh3*), for which null mutations are recessive [66]. Both genes were selected as mutations in either results in readily recognizable visible phenotypes in which the larval cuticle appears translucent and 'oily' [56,67]. Microinjection of embryos with ZFNs targeting either *BmBLOS2* or *Bmwh3* yielded 72% (*BmBLOS2*) and 22% (*Bmwh3*) of larvae with mosaic patterns of translucent and normal skin. Rearing those individuals to adults and backcrossing them to wild-type individuals resulted in 0.28%

of the larval progeny in which *BmBLOS2* was targeted having the oily phenotype [66]. More recently, *fibroin heavy chain* gene (*BmfibH*), encoding the largest and most abundant silk protein, was successfully disrupted using ZFNs [68]. These examples, while demonstrating the functionality of ZFNs in *B. mori*, also showed that obtaining heritable germline mutations using this technology was inefficient and complex—it is generally a difficult and time-consuming task to genetically modify genes using ZFNs owing to the challenge of designing and building ZFs with sufficient specificity, which are mainly restricted by the lack of known ZFs for some trinucleotide sequences and context effects of individual ZFs in an array [69,70].

## (b) Transcription activator-like effector nucleases

TALE nucleases (TALENs) are an effective and versatile genome-editing technology [71] which, like ZFNs, consists of a pair of user-designed proteins each based on 17 nearly identical approximately 34 amino acid motifs, each capable of interacting with a single nucleotide and linked to a monomer of the non-specific DNA endonuclease domain from the *Fok* I enzyme. Because a single approximately 34 amino acid motif binds a single nucleotide, users can easily design proteins capable of targeting almost any sequence in a genome [58,72]. The utility of TALENs in insects was quickly demonstrated by Ma *et al.* [73] and Sajwan *et al.* [74] who reported the creation of somatic and germline mutations in the *B. mori* gene *BmBLOS2*. The somatic mutations in $G_0$ larvae was 22–46% [73] and 6–15% [74], respectively. Notably, the heritable germline mutations (0.4–61% [73] and 0.05–0.69% [74]) was higher than that of ZFNs [66]. Differences in efficiency reported by Ma *et al.* [73] and Sajwan *et al.* [74] were probably owing to differences in the target sites chosen and the biochemical performance of the different TALENs. Optimization of TALENs through the careful selection of target sites can greatly increase both the efficiency of somatic and germline mutagenesis [75] as can changes in the quantity of TALENs delivered to cells and the accuracy and efficiency of delivery, which in *B. mori* relies on injecting developing embryos directly.

The use of TALEN-mediated genome editing in *B. mori* is now routine, including the design and assembly of TALENs, *in vitro* synthesis and evaluation of TALEN mRNA, delivery by embryo microinjections, mutation detection and maintenance of mutant lines [76–78]. To date, at least six *B. mori* genes have been edited using TALENs (electronic supplementary material, table S1), including the creation of large deletions by the coordinated use of multiple pairs of TALENs [77]. These results indicate that TALENs should be a more attractive genome-editing technology in *B. mori* than ZFNs, because they can be easily designed and built according to a simple cipher to target desired DNA sequences, and can be used not only for routine knockouts of single gene, but also for simultaneously knocking out two or more genes.

## (c) Clustered regularly interspersed short palindromic repeats/CRISPR-associated 9

The clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system [79] is the most recent genome-editing technology to emerge. Unlike ZFNs and TALENs, CRISPR/Cas9-mediated genome editing does not

depend on the design and synthesis of sequence-specific DNA-binding proteins but instead relies on a DNA endonuclease whose target-site specificity is determined by a user-designed gRNA. Creating the reagents necessary for altering a gene using CRISPR/Cas9 requires only the synthesis of a short gRNA rather than large, target-specific proteins such as with ZFNs and TALENs, which are often difficult to design and more time-consuming and costly to produce due to their inherent repetitiveness. Besides, CRISPR/Cas9 uses RNA–DNA base-paring to target DNA sequences, which is more efficient than ZFNs and TALENs as RNA–DNA base-paring is usually more stable than protein–DNA interaction [80]. Since the first application of CRISPR/Cas9 in human and mouse cells [59,60], it has been rapidly adopted by scientists working on insects, mammals, plants and bacterias.

CRISPR/Cas9 genome editing in insects was first reported in *B. mori* and, again, the gene *BmBLOS2* was selected as a target because of the easily scored phenotypes observable during larval development (transparent 'oily'-appearing cuticle) following mutagenesis [81]. Wang *et al.* [81] designed two 23 bp gRNAs that directed the Cas9 endonuclease specifically to two sites separated by 3.48 kb in exons 2 (site S1) and 4 (site S2) of *BmBLOS2*. Microinjection of the targeted CRISPR/Cas9 plasmids into young, preblastoderm-stage *B. mori* embryos resulted in an extremely high proportion of the larvae showing evidence of somatic mutations. S1-specific editing resulted in 95.6% of the surviving larvae showing evidence of somatic mutations, while 94% of those arising from S2 targeting had evidence of somatic mutations. When both S1 and S2 sites were targeted simultaneously, Wang *et al.* [81] observed that 95.5 – 100% of the $G_0$ larvae had evidence of somatic mutations and 35.6 – 100% of those individuals carried germline mutations recoverable in the next generation. CRISPR/Cas9 was used not only to induce minor insertion/deletion mutations, and large chromosomal deletions (approx. 3.5 kb) or inversions at a single gene locus, but also mediate the mutagenesis of up to six genes simultaneously in *B. mori* (electronic supplementary material, table S1). Collectively, these results demonstrate that the CRISPR/Cas9 is currently the most efficient technology for genetically modifying insect genomes and paves the way for large-scale genome editing of *B. mori* in the near future.

## 4. Challenges

Current genetic technologies depend heavily on effective methods by which they can be delivered to appropriate cells, usually presumptive germ cells. Currently, for *B. mori* and all other insects, delivering various genetic technologies to presumptive germ cells requires microinjecting all essential reagents into preblastoderm embryos. Microinjecting *B. mori* embryos is a well-established method and this has facilitated the testing and adoption of contemporary genetic manipulation technologies as they have become available. Nevertheless, some technical challenges remain, such as improving the efficiency of transposon- and site-specific recombination-based technologies. Optimizing codon usage in transgenes encoding transposases and recombinases may be the most straightforward means by which efficiencies could be improved as most of the current genetic technologies were designed initially to function in the cells of mice, *Drosophila* or some other model organism.

Genome-editing technologies also present some immediate challenges, most notably, difficulty in screening for mutants that do not result in visible phenotypes. Currently the most common method is to determine genotypes using PCR and DNA sequencing technologies but this tends to limit the number of individuals that can be assessed in a given screen and maintaining lines with cryptic mutations can also be challenging. Because identifying genome-editing mutations can be challenging in the absence of a visible mutation, recombining in a dominant visible marker gene via HR, while less efficient, results in a mutant line that can be more easily identified and maintained. Although the rates of HR are low, overexpressing the genes crucial for homology-directed recombination or silencing the genes necessary for non-homologous end-joining can improve the efficiency with which dominant marker genes can be 'knocked into' target genes. Optimizing the HR vector, such as selecting longer homology arms with lengths of approximately 2 kb or using single-stranded DNAs [82], may also help to increase the knock-in efficiency. Most recently, Nakade *et al.* [83] developed a knock-in strategy based on TALEN and CRISPR/Cas9, termed TALE-PITCh (precise integration into target chromosome) and CRIS-PITCh, respectively. The method was shown to be efficient for introducing donor DNA fragments into the genomes of human, *B. mori* and other cells *in vitro*, suggesting its potential for targeted knock-in in a wide range of cells and organisms, even those with low HR activity.

In *B. mori*, maintaining lethal mutations arising from genome editing is very challenging. However, by combining transposon-based technologies with genome-editing technologies, one can circumvent many of these problems. For example, each of the two *Fok* I-based TALENs can be expressed independently in transgenic lines regulated by the same promoter and resulting in gene mutagenesis that will occur only in progeny expressing both TALENs. Likewise, for CRISPR/Cas9 mutagenesis, it can be combined with the Gal4/UAS system such that Cas9 and a gRNA can be expressed in any cell or tissue within the organism. By using different patterns of *Gal4* expression, tissue- or stage-specific gene editing can be realized. These strategies have proved feasible in *B. mori* and *Drosophila* [84–86].

## 5. Conclusion and future trends

As the challenges associated with using contemporary genetic technologies in *B. mori* are met, the use of these technologies will increase and significantly impact a number of important areas of *B. mori* research.

Functional analysis of genes will be greatly enhanced leading to increased understanding of silk protein synthesis, immunity and disease resistance, development and metamorphosis, and sex determination. Progress in the study and understanding of silkworm biology will certainly enhance the study and understanding of other insects.

Enhanced capabilities for genetically manipulating *B. mori* will enable the creation of insects with new or enhanced phenotypes leading to improved commercial success of sericulture. Silk fibre used to produce fabrics and materials comes mainly from commercial *B. mori* varieties, and while traditional breeding practices have increased silk quality and quantity from individual silkworms, further increases using these methods have not been forthcoming. Contemporary genome

modification and manipulation technologies hold great potential for creating novel commercial varieties by modifying endogenous genes or introducing exogenous genes. Genome modification technologies will allow the creation of novel *B. mori* varieties with increased yields and quality of silk, high resistance to virus and other special properties.

The silk gland of *B. mori* is an ideal tissue for producing large quantities of valuable proteins and while several expression systems have been developed using the promoters of silk protein-encoding genes, improvements are needed. First, silk protein production needs to be reduced or eliminated in strains of *B. mori* used for recombinant protein production. Recently, some progress towards this end was made with the creation of *B. mori* lines in which the *BmfibH* gene was disrupted using ZFNs and the resulting mutants were successfully used to produce recombinant proteins in the silk gland, with a significant increase in protein yield [68]. This result demonstrated the practicality of using silk protein knockout mutants to produce recombinant proteins including enhancing protein purification from cocoons. Second, modular gene expression technologies are need for the production of proteins

toxic to *B. mori*. Some valuable proteins are difficult to produce using existing silk gland expression systems because of toxicity. Development of modular expression systems by combining silk gland-specific promoters with modular tools such as the Gal4/UAS system would make the production, isolation and purification of toxic proteins in silk glands more feasible.

After years of effort, the silkworm *B. mori* has been developed into one of just a few insect systems for which the most advanced genetic technologies are available. The application of these technologies has not only facilitated the functional analysis of *B. mori* genes, and enabled the sophisticated genetic modification of silkworms to improve their commercial value, but also contributed to the development and use of similar technologies in non-model insects.

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