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REVIEW ARTICLE

Advancement in genetic transformation of mulberry: Current trends and future directions

P Mangammal[1], S Bhuvana[2]*, M Kiruba[3], N Vinothini[4], K Govindan[5], K Sasikumar[5], P Kumar[6], P S Devanad[7], P Senthil kumar[8] & B Sivakumar[9]

[1]Horticultural College and Research Institute, Tamil Nadu Agricultural University, Paiyur 635 112, Tamil Nadu, India
[2]Forest College and Research Institute, Mettupalayam 641 301, Tamil Nadu, India
[3]Krishi Vigyan Kendra, Sandhiyur 636 203, Tamil Nadu, India
[4]SRM College of Agricultural Sciences, Chengalpattu 603 201, Tamil Nadu, India
[5]Regional Research Station, Paiyur 635 112, Tamil Nadu, India
[6]Horticultural College and Research Institute, Paiyur 635 112, Tamil Nadu, India
[7]Forest College and Research Institute, Mettupalayam 641 301, Tamil Nadu, India
[8]Regional Research Station, Paiyur 635 112, Tamil Nadu, India
[9]Forest College and Research Institute, Mettupalayam 641 301, Tamil Nadu, India

*Correspondence email - bhuvana4997@gmail.com

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Abstract

A vital plant species that is important to the silk industry is the mulberry (*Morus* spp.). However, traditional breeding methods have limitations in enhancing key traits such as disease resistance, stress tolerance and leaf yield. Genetic transformation has emerged as a powerful tool for mulberry improvement, enabling precise modifications to enhance agronomic traits. This review explores recent advancements in genetic transformation techniques, including *Agrobacterium*-mediated transformation, biolistic methods and CRISPR/Cas genome editing, which have significantly improved the efficiency and precision of genetic modifications in mulberry. Genetic engineering of mulberry has led to the successful incorporation of specific genes like HVA1, osmotin, mAKR2A, MmSK, MaTCP, MuGABA-T that enhance pest and disease resistance, drought and salt tolerance, biomass yield and secondary metabolite production. The review also highlights the optimization of transformation protocols, including advancements in tissue culture, regeneration systems and selection marker strategies that have improved transformation efficiency. Despite these advancements, challenges such as low transformation efficiency, genotype dependency and limited genomic resources remain. We explore future directions, including the application of synthetic biology, genome-wide association studies and multi-omics approaches, to further accelerate genetic improvements in mulberry. Additionally, emerging gene-editing tools hold great promise for precise and efficient trait enhancement. Genetic modifications in mulberry have been explored to enhance leaf quality, biotic and abiotic stress resistance, aiming to improve the performance of the silkworm (*Bombyx mori*), cocoon weight and consistent silk production. By summarizing the latest developments and future prospects this review provides valuable insights into the potential of genetic transformation in mulberry. The advancements discussed here are expected to contribute to the genetic improvement of mulberry, ensuring its sustainability and productivity in the sericulture industry.

Keywords: abiotic stress; *Agrobacterium*-mediated transformation; biotic stress; CRISPR/Cas; genetic transformation; sericulture; transgenic mulberry

Introduction

The leaves of mulberry trees are the primary food source for silkworms (*Bombyx mori*), which are essential to sericulture and the production of silk (1, 2). Over the past decade, the mulberry cultivation area in India has gradually increased. According to the Central Silk Board, the mulberry plantation area expanded from 223926 hectares in 2017-18 to 263302 hectares in 2023-2024. India produced a total of 215642 metric tons of mulberry cocoons, yielding 29892 metric tons of mulberry silk (3). However, despite the numerous benefits and applications of mulberry, the plant faces significant challenges related to its cultivation, particularly in terms of environmental stress tolerance, pest and disease management and the improvement of leaf quality for sericulture (4-6). Traditional breeding methods have been instrumental in improving mulberry varieties; however, they often face limitations due to the plant's long generation time and complex genetics (7, 8). Genetic transformation offers a more precise and accelerated approach to introducing desirable traits (9, 10). Transgenic

technology has emerged as a promising tool for improving mulberry (*Morus* spp.) traits such as disease resistance, stress tolerance and enhanced leaf quality for sericulture (11, 12). Genetic transformation in mulberry has been successfully achieved using *Agrobacterium tumefaciens*-mediated transformation, biolistics and protoplast fusion techniques (2, 13). Several studies have reported the successful introduction of genes conferring resistance to fungal pathogens (*chitinase* and *β-1,3-glucanase* genes) and insect pests (*Bt* genes) (14, 15). Additionally, transgenic mulberry with stress-responsive genes, such as *DREB* and *NHX1*, has shown improved tolerance to drought and salinity stress (16, 17).

Furthermore, transgenic approaches have been employed to enhance mulberry leaf nutritional quality by modifying secondary metabolite pathways (18). Despite these advancements, concerns related to biosafety, regulatory approvals and the stability of transgenes remain key challenges in the widespread adoption of transgenic mulberry (19, 20). Future research integrating CRISPR/Cas9-based genome editing may provide more precise and stable genetic modifications in mulberry (21, 22). In this review, we explore the recent advancements in genetic transformation techniques for mulberry, focusing on their role in improving agronomic traits and biomass production. We begin by discussing the significance of genetic transformation in mulberry and its applications in the sericulture industry. Next, we provide a detailed examination of transformation methods and highlight key advancements in regeneration protocols, selectable marker systems and transgene expression optimization. Following this, we discuss major challenges such as low transformation efficiency and genotype dependency, along with emerging strategies to overcome these limitations. Finally, we conclude with future perspectives and potential research opportunities to enhance genetic transformation in mulberry.

2. Methods used in Genetic Transformation of Mulberry

2.1 Agrobacterium-mediated transformation

Agrobacterium tumefaciens-mediated transformation is the most commonly used method for genetic engineering in mulberry due to its efficiency and stable gene integration (2, 13, 23-25). This method involves co-cultivation of mulberry explants (such as leaves, callus, or nodal segments) with *A. tumefaciens* carrying the gene of interest within the *T-DNA* region of a binary vector. Several studies have successfully transformed mulberry with stress tolerance genes, such as *DREB* and *NHX1*, using this approach (16, 17, 26). Optimization of co-cultivation conditions, explant selection and bacterial strains significantly improves transformation efficiency (27, 28). The overall process of *Agrobacterium* mediated transformation is illustrated in Fig. 1. There are two ways of gene transfer by *Agrobacterium*-mediated gene transfer.

i) **Co-culture with tissue explants:** Co-culture in *Agrobacterium*-mediated gene transfer involves incubating plant explants with *Agrobacterium* to facilitate T-DNA transfer into the plant genome. The explants are placed on co-culture medium for 24-72 hr, allowing gene integration. After co-culture, *Agrobacterium* is eliminated using antibiotics and transformed cells are selected. The explants then regenerate into transgenic plants for further analysis. The regeneration capacity of mulberry plants depends on the explant used and the culture conditions (29-31). Regeneration through direct induction of adventitious shoot buds was examined and the results show that the highest transformation frequency (18.6 %) acquired by adventitious shoot buds followed by transformation frequency obtained by somatic embryogenesis (14.2 %) and least from the callus cultures (8.5 %) (32). Several regeneration techniques and effective transformation could pave the way for the transfer of desired genes (against stress and disease) into economically significant *Morus* clones (33, 34).

ii) **In planta transformation:** In planta transformation is a simple and efficient method for transforming plants. An avirulent mutant of *Agrobacterium tumefaciens* is used to infect the plantlets. The steps of in vitro regeneration of plants are avoided. The transformants exhibit an altered phenotype due to a hormone imbalance. *In planta* transformation method was used for mulberry plants (35). Following a needle prick, young mulberry trees in containers were

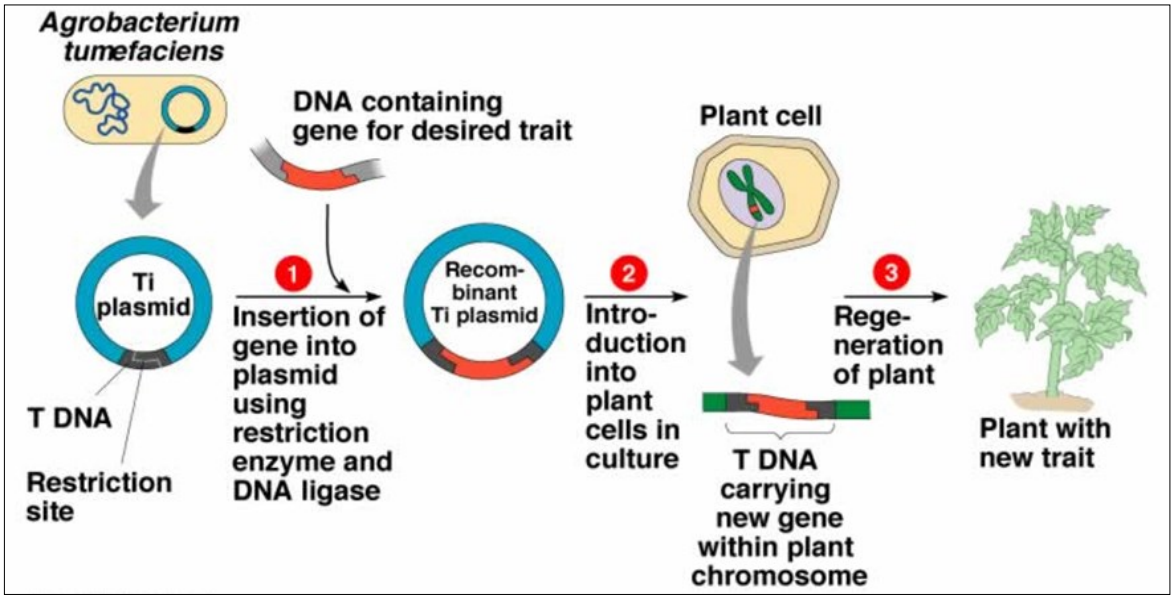

Fig. 1. Overview of *Agrobacterium*-mediated gene transfer.

decapitated and axillary bud meristems were infected with a water suspension of *A. tumefaciens*. Pots were used to cultivate the four inoculated plants. Compared to non-transformed plants, all four of the transformants in the T0 generation displayed distinctive phenotypes, which appeared to be caused by an excess of cytokinin. Nestled PCR revealed that the transgene (Tn5-inserted T-DNA) was present in all four transformants. Stem cutage-produced transformants' (T0) progeny also displayed notable characteristics that were distinct from those of non-transformed plants' progeny. All of the transformants' progeny carried the transgene.

Advantages

- High efficiency and stable gene integration.
- Cost effective method.
- Reduce the risk of gene silencing and rearrangement by transfers genes in low copy numbers.
- Targeted gene insertion.
- Minimal DNA damage.

Disadvantages

- Successful transformation requires efficient regeneration systems, which can be challenging for recalcitrant genotypes.
- Longer transformation process compared to direct gene transfer methods.
- Limited by host specificity.
- Risk of vector contamination.

2.2 Biolistic (gene gun) transformation

Biolistic or particle bombardment is another widely used method for transgenic transformation in mulberry, particularly for species with low susceptibility to *Agrobacterium* (19, 36). For the bombardment of mulberry leaf tissue, plants were aseptically grown from surface-sterilized seeds on a seedling medium. For preconditioning experiments, various leaf treatments were carried out prior to bombardment: (1) osmoticum treatment; (2) heat treatment; (3) dimethyl sulfoxide treatment. Chimeric plasmid DNA, pBI 221, which has a β-glucoronidase (GUS) gene under the control of the cauliflower mosaic virus 35S promoter, was used. The DNA of the plasmid was purified in its closed circular form. Gold particles with an average diameter of 1.0 µm were coated with the DNA. From Table 1 it can be concluded that smaller microprojectile size results in a higher number of blue spots, indicating greater bombardment efficiency. The particle inflow gun (PIG) device and general methods for bombardment have been followed as per method (37). This technique has been successfully used to introduce disease resistance genes, such as *chitinase* and *β-1,3-glucanase*, into mulberry to improve resistance against fungal pathogens (15, 20). The percentage of GUS positive ex plants was very low with tungsten (20 %) as compared to gold particles (36 %) indicating tungsten toxicity to the tissue (38). When explants were double-blasted with 10 µg of DNA loaded on macrocarriers, the results were obviously better (up to 56 %) than when they were only bombarded once (30 %). pBI221 produced the highest (100 %) GUS positive explants in the leaf callus out of all the plasmids that were examined. The results of bombardment efficiency for single and double shots are presented in Table 2.

Table 1. Effect of microprojectile size on bombardment efficiency

Microprojectile size (µm)	No. of blue spots/cm²
1	116.7 ± 27.2
2	24.9 ± 9.5

Table 2. Effect of single and multiple shot on bombardment efficiency

	No. of shot	No. of blue spots/cm²
Exp. 1*1	Single	8.4 ± 6.5
	Double	43.2 ± 17.7
Exp. 2*1	Single	33.4 ± 19.5
	Double	64.2 ± 22.8
	Triple	49.3 ± 16.2
Exp. 3*2	Single	89.6 ± 49.1
	Double	163.0 ± 78.3

*1: nine leaves which were precultured for 9 days were used for each bombardment.

*1: twelve leaves which were precultured for 5 days were used for each bombardment.

Advantages

- Suitable for many different genotypes.
- Direct DNA delivery into various target tissues.
- Fast transformation process.
- Multiple gene delivery for complex trait modifications in mulberry.

Disadvantages

- High copy number and random integration increasing the risk of gene silencing.
- Reducing regeneration efficiency in mulberry in tissue damage.
- Lower transformation efficiency.
- Expensive method.

2.3 Electroporation

Electroporation involves applying a high-voltage electric pulse to create temporary pores in plant cell membranes, allowing foreign DNA to enter (21). This method has been explored in mulberry protoplasts and embryogenic callus for introducing genes related to secondary metabolite biosynthesis (18). Although electroporation can be efficient, it requires protoplast isolation, which is a complex and time-consuming process in mulberry. Electroporation can achieve transient expression of the GUS gene in approximately 20-30 % of mulberry protoplasts at pulse voltages between 500 and 750 V/cm with a capacitance of 330 µF (8).

Advantages

- Efficient DNA delivery.
- Fast and simple method.
- No risk of vector contamination.
- Allows for transient and stable transformation.

Disadvantages

- Cell damage and low viability.
- Limited to protoplasts.

- Random DNA integration.
- Requires specialized equipment.

2.4 Protoplast fusion and PEG-mediated transformation

Protoplast fusion is another approach used in mulberry transformation, particularly for somatic hybridization and genome modification (2). This technique involves enzymatically digesting the plant cell wall to generate protoplasts, which are then fused using polyethylene glycol (PEG) treatment or electrofusion. PEG-mediated transformation has been used for direct gene transfer into mulberry cells, allowing the expression of transgenes related to flavonoid biosynthesis and leaf quality improvement (18, 28). Transformation efficiency of approximately 47 % using PEG-mediated methods has been achieved through the establishment of an efficient mesophyll-derived protoplast manipulation system in mulberry (39). This efficiency is higher than previously reported electroporation-mediated protocols.

Advantages

- Allows hybridization between genetically distant species or incompatible genotypes.
- No foreign DNA required.
- Potential for Novel variants.

Disadvantages

- Random genome recombination due to non-specific genetic mixing.
- Low regeneration efficiency.
- Somoclonal variation.
- Time consuming process.

2.5 Plastid transformation in mulberry

The benefits of genetically transforming chloroplasts include the accumulation of proteins at high levels, the possibility of many genes being expressed as polycistronic units and the containment of transgenes in most crops due to pollen transmission (40). The process of chloroplast transformation involves several steps. The transformation vector used contains selective plastid markers, spectinomycin-streptomycin and kanamycin resistance, conferred by the expression of chimeric *aad*A11 and *aph*A6, genes, respectively. In the case of plastid transformation, this includes having a transcription terminator and a suitable plastid promotor. The next step is to add flanking sequences (600-1000 bp each) to the construct at the 5' and 3' ends, respectively. The chloroplast DNA from the intended location on the chromosome for the selection cassette insertion is the source of these flanking nucleotides. The flanking sequences in chloroplasts enable homologous recombination, which disrupts or replaces genes. On plant regeneration media, the medications used in the selection process prevent the growth of shoots and the accumulation of chlorophyll. The transformed lines can be identified by their ability to form green shoots on bleached wild-type leaf sections (41, 42).

Advantages

- High level gene expression.
- Maternal inheritance reduces gene flow.
- Stables gene integration.
- Consistent gene expression.

Disadvantages

- Limited protocols for woody plants.
- Difficult regeneration.
- Requires gene gun transformation.
- Time consuming process.

3. Scope of Genetic Transformation in Mulberry

3.1 Abiotic stress tolerance

One of the most pressing issues affecting mulberry cultivation is its sensitivity to various environmental stresses such as drought, extreme temperatures, soil salinity and heavy metal contamination adversely affect mulberry growth and leaf yield (43-45). Salinity stress in mulberry plants disrupts growth, metabolism and productivity by causing ion toxicity and osmotic imbalance. Understanding calcium signaling and salt stress in mulberry can aid in enhancing salt tolerance and developing stress-resistant transgenic plants for better soil management (46). Water stress leads to reduced leaf moisture content, affecting silkworm nutrition and cocoon quality (47, 48). Mulberry, like many other plants, requires specific environmental conditions for optimal growth and deviations from these conditions can result in decreased productivity, poor leaf quality and compromised silk production (49, 50). Finding superior mulberry cultivars with more biomass under stress is therefore crucial. Drought tolerant mulberry genotypes exhibit minimal plasticity in foliar gas exchange characteristics and sustain longer periods of transpiration and stomatal conductance under soil water deficit (51, 52). Genetic engineering has the potential to enhance mulberry's tolerance to environmental stresses (53). For instance, by introducing genes that encode stress-protective proteins or enzymes, transgenic mulberry plants can be made more resistant to drought, salinity and cold (54).

3.1.1 Drought and salinity resistance

***Expression of hva1 gene*:** Transgenic mulberry (*Morus indica*) produces resistance to water stress and salinity through overexpression of the barley hva1 gene. ABA and water deficiency circumstances trigger the expression of hva1 gene, which codes for a group 3 LEA protein. Through *Agrobacterium* mediated transformation, we describe the overexpression of hva1 in mulberries under a constitutive promotor. The steady integration and expression of the transgene in the transformants were demonstrated by molecular analysis of the transgenic plants. To investigate how hva1 contributes to tolerance, transgenic plants were exposed to drought and salinity stressors (55).

Most of the plant stressors first target membranes and preserving their integrity is crucial to a plant's ability to withstand drought and salinity, therefore, in the present study, cellular membrane stability (CMS) index was used to assess salt (NaCl, 400 mM) and drought (PEG, 2 %) tolerance amongst different lines. At the end of 48 h of PEG mediated water stress cellular membrane stability was reduced to 70 % in the transgenic lines ST31, ST11 and ST8 overexpressing *hva1* and 50 % in the non-transgenic plants. Plants treated

with 400 mM NaCl for 6 hr showed cellular membrane rupture, in contrast to a considerably delayed reaction to simulated drought stress. However, non-transgenic plants were more susceptible to membrane injury and CMS was reduced to 35 % and transgenic lines ST6, ST11, ST30 and ST34 showed better membrane stability ranging from 47-60 %. As far as dehydration stress is concerned, all lines differed significantly from the non-transgenic plants with respect to different durations of stress and are numerically superior to them. Similarly, after 6 hr of salinity stress, transgenic plants showed superiority (ST6 and ST11) over non-transgenic plants and differed considerably from non-transgenic plants. Carbon isotope ratio $\delta^{13}C$ has been proposed as an indirect selection criterion for water use efficiency. The ratio of dry matter generated to total transpiration over a given growth period is known as water use efficiency (56).

Plants discriminate against 13C ($\delta^{13}C$) during photosynthesis and the extent of this discrimination shows a strong inverse relationship with WUE. Foliar $\delta^{13}C$ values have been used as an integral measure of the response of photosynthetic gas exchange to environmental variables such as water availability, humidity and salinity. *hva1* confers various degrees of tolerance against both drought and salinity conditions in *Morus indica* has been examined (55). The overexpression of barley Hva1 confers drought, salt and cold tolerance in transgenic mulberry were found (57).

Expression of osmotin: To assess stress treatment, non-transgenic controls, transgenic plants with osmotin driven by the CaMV 35S (MT1) constitutive promoter and transgenic plants with the stress inducible promoter rd29A (RO2) were used. To test their tolerance, mature transgenic and non-transgenic plants in pots (5-6 months) were exposed to water deficit stress (by ceasing watering) and salinity stress (200 mM NaCl). In osmotin transgenic plants with the constitutive promoter, approximately 50 % of the mature leaves showed signs of senescence, whereas non-transgenic plants had lost all their leaves by day 15 days of salt stress and water stress treatments. Following 15 days of salt stress, osmotin transgenic plants with the constitutive promoter and non-transgenic plants showed signs of NaCl toxicity, notably fewer lateral shoots, smaller leaves and fewer young leaves. Even after 20 days, the transgenic plants containing the stress-inducible promoter continued to grow normally in both stress treatments. In both salinity and drought, the rd29A transgenic plants performed better than the 35S transgenic plants.

Real-time PCR was also used to examine the expression of osmotin using the constitutive and stress-inducible promoters, respectively. In contrast to the non-stressed transgenic plants using the CaMV 35S promoter, it was discovered that the constitutive promoter-driven transgenic plants had higher levels of osmotin transcripts following 20 days of drought stress. Interestingly, real-time reverse transcriptase–PCR showed that osmotin transgenic plants with the inducible rd29A promoter accumulated more osmotin transcripts than the non-stressed inducible transgenic plants with the rd29A promoter. The impact of osmotin protein on silkworm larvae, eating and rearing was examined using a biotic assay. We observed that larvae feeding on transgenic lines were healthier than those feeding on non-transgenic controls at every instar and before mounting and that the weight of larvae and cocoons of transgenic lines was somewhat higher that of those on the control non-transgenic plants (58). Transgenic plants with antifungal properties and improved resistance to salt, drought and cold stress have been created using tobacco osmotin. The biotic experiment using transgenic plants further shown that these plants have no negative effects on the feeding and rearing of silkworm larvae. Based on these results, these improved mulberry plants can be used for rearing silkworms in the future (58).

Expression of MmSK gene: Shaggy-like protein kinase (SK) is involved in signal transduction, abiotic and biotic stress, plant growth and development and the regulation of drug metabolism. Several abiotic stress treatments increased the expression of MmSK in mulberries. In comparison to the negative control infected with empty vector pTRV2-00 (CK), the expression of MmSK in the pTRV2-MmSK-VIGS plant (transgenic mulberry) decreased to 34.02 % after MmSK gene silencing. By using virus-induced gene silencing (VIGS), the mulberry MmSK gene was effectively silenced. In comparison to the CK, the transgenic mulberry's soluble protein content, proline content, superoxide dismutase (SOD) and peroxidase (POD) activity all declined to varying degrees under drought stress.

On the other hand, transgenic mulberry showed a substantial increase in malondialdehyde (MDA) accumulation. The soluble protein, proline and MDA contents progressively increased as the duration of the drought stress treatment was prolonged. In line with the trend of changing MmSK gene expression, the SOD and POD activities under drought stress progressively increased to their peak on the fifth day before declining. The MmSK gene might act as a positive regulator of drought stress in mulberries (28).

Expression of MaTCP gene: An examination of the 15 MaTCP genes' expression patterns in mulberry roots revealed a correlation between root development and the transcriptional levels of MaTCP2, MaTCP4-1, MaTCP8, MaTCP9-1 and MaTCP20 -2. These five MaTCP genes are involved in root growth and may improve mulberry drought resistance, as evidenced by changes in their expressions following drought treatment (59).

Expression of AtDREB2A and AtSHN1 gene: One significant study involved the co-expression of AtDREB2A and AtSHN1 genes from *Arabidopsis thaliana* in transgenic mulberry plants (61). These modifications aimed to improve drought and salinity tolerance, key factors affecting leaf quality in mulberry cultivation. The transformed plants demonstrated reduced post-harvest water loss due to an increase in cuticular resistance, which helped maintain leaf moisture and prevent early wilting. Additionally, they exhibited enhanced photosynthetic efficiency, with a 26-46 % increase in photosynthetic rate and a 9-15 % improvement in water-use efficiency under stress conditions. The relative water content of transgenic plants also increased by 15-18 %, which directly contributes to improved leaf quality and nutritional value for silkworms (60).

Expression of MuGABA-transaminase gene: Plants under salt

stress have been shown to accumulate gamma-aminobutyric acid (GABA) (61) and the primary enzyme in the GABA shunt pathway that breaks down GABA is GABA-transaminase (GABA-T). The GABA-T gene's cDNA, known as MuGABA-T, was cloned from mulberries and the MuGABA-T protein has several conserved traits in common with its homologs from other plant species. In mulberry tissues, the MuGABA-T gene was constitutively expressed at varying levels and was significantly stimulated by SA, ABA and NaCl. The mulberry hairy roots showed overexpression of the MuGABA-T gene and the transgenic mulberry plants showed comparable responses in terms of susceptibility to salt stress. According to the findings, the MuGABA-T gene is essential for GABA catabolism, causes a reduction in salt tolerance and may be connected to the ROS pathway during the reaction to salt stress (59).

3.2.2 Chilling tolerance

Expression of mAKR2A: The chilling-tolerant mulberry variety had higher levels of expression of mAKR2A, mSOD, mFAD and mKCS1 than the chilling-sensitive variety. The chilling-tolerant species has higher levels of unsaturated fatty acids and superoxide dismutase (SOD) activity than the chilling-sensitive variety. mFADII expression rose in the chilling-tolerant variety compared to the chilling-sensitive variety after chilling treatment, while mSOD1, mKCS1 and mAKR2A expression decreased to lower levels than in the chilling-tolerant variety. This suggests that the increased expression of the molecular chaperon mAKR2A helped to maintain or elicit the chilling-related proteins in the chilling-tolerant variety (62).

3.2 Biotic stress tolerance

Mulberry is susceptible to several pests and diseases, including fungal pathogens like *Cercospora moricola* and *Phyllactinia corylea*, bacterial infections such as *Pseudomonas syringae* and nematode infestations (63, 64). Insect pests, including leafhoppers (*Empoasca flavescens*) and mealybugs (*Maconellicoccus hirsutus*), also pose significant threats (65). These pest and diseases can significantly damage mulberry leaves, reducing their nutritional value for silkworms and leading to yield losses. Conventional pest control measures often rely on the use of chemical pesticides, which can harm the environment and lead to pesticide resistance in pests. Transgenic transformation allows for the incorporation of genes that confer resistance to pests and diseases (63). For example, genes from the *Bacillus thuringiensis* (Bt) bacterium can be inserted into mulberry to produce proteins that are toxic to specific insect pests but harmless to humans and animals. The introduction of Bt genes has already been successful in other crops like cotton and maize, offering a model for similar applications in mulberry. Additionally, genes that confer resistance to fungal diseases can be incorporated into mulberry trees, reducing the need for chemical fungicides and promoting more sustainable farming practices.

3.2.1 Disease resistance

Osmotin gene expression: The plant PR-5 group of proteins includes the stress proteins osmotin and osmotin-like proteins, which are produced in a number of plant species in reaction to different kinds of biotic and abiotic stresses. The over-expression of tobacco osmotin in transgenic mulberry plants under the control of a constitutive promoter (CaMV 35S) as well as a stress-inducible *rd29A* promoter was also reported. Fungal challenge undertaken with three fungal species known to cause serious losses to mulberry cultivation, namely, *Fusarium pallidoroseum*, *Colletotrichum gloeosporioides* and *Colletotrichum dematium* (58).

MnGolS2 gene expression: The study identified the MnGolS2 gene in mulberry, which encodes galactinol synthase, an enzyme involved in the synthesis of raffinose family oligosaccharides (RFOs) (66). The expression of MnGolS2 was found to increase in response to *B. cinerea* infection. To assess its role in disease resistance, the MnGolS2 gene was cloned and ectopically expressed in *Arabidopsis thaliana*. Transgenic Arabidopsis plants exhibited decreased malondialdehyde (MDA) content and increased catalase (CAT) activity after inoculation with *B. cinerea*, indicating enhanced resistance to the pathogen. This study demonstrated the involvement of MnGolS2 in biotic stress responses and suggested its potential utility in developing disease-resistant mulberry varieties.

MnANR and MnLAR genes expression: The study focused on the MnANR and MnLAR genes, which are involved in the flavonoid biosynthesis pathway (67). These genes were cloned from mulberry and expressed in *Nicotiana tabacum* (tobacco) to evaluate their function. Transgenic tobacco plants expressing MnANR and MnLAR exhibited increased resistance to *B. cinerea*, as evidenced by less severe disease symptoms compared to wild-type plants. In vitro assays further demonstrated that crude leaf extracts from these transgenic plants inhibited the growth of *B. cinerea*. These findings suggest that MnANR and MnLAR play a role in enhancing disease resistance, potentially through the modulation of flavonoid compounds that may have antifungal properties.

MnASI1 gene expression: The MnASI1 gene, encoding an α-amylase/subtilisin inhibitor, was investigated for its role in mulberry's defense against *B. cinerea*. Expression analysis revealed that MnASI1 was significantly upregulated following *B. cinerea* infection. To further explore its function, MnASI1 was overexpressed in both *Arabidopsis thaliana* and mulberry. Transgenic plants exhibited smaller necrotic lesions upon *B. cinerea* inoculation compared to control plants, indicating enhanced resistance. Additionally, these transgenic plants showed increased catalase activity and reduced accumulation of reactive oxygen species (ROS), suggesting that MnASI1 contributes to disease resistance by modulating oxidative stress responses (68).

3.2.2 Pest resistance

Expression of oryzacystatin gene: Insect pests of mulberry are one of the main factors which cause the damage of mulberry leaves and loss of cocoon. It is a future in agricultural practice that applying plant anti-insect genetic engineering controls insect pests of mulberry. Currently, the gene used for plant anti -insect genetic engineering mainly is *Bacillus thuringiensis* (Bt), insecticidal crystal protein and proteinase inhibitor (PI) genes in *Oryza sativa* (called as oryzacystatin) (69, 70). Bt insecticidal crystal protein and PI are toxic to lepidopterous insects, while silkworm belong to their species. Therefore, Bt insecticidal crystal protein gene and the majority of PI gene are not used for plant anti-insect genetic engineering. Oryzacystatin gene

itself is a natural anti-insect gene, which strongly inhibit coleoptera insects in a lot of reports but not in lepidopterous insects. Biolistic method used to introduce the OC (oryzacystatin) into callus tissues of mulberry (70). Tissue culture and resistance selection were used to obtain the regenerative mulberry plants. Finally, it was demonstrated that the OC (oryzacystatin) had already introduced into mulberry by PCR Southern blotting and RNA dot blotting, which set the foundation for selecting the fight worm mulberry species.

3.3 Leaf quality improvement

In sericulture, the quality of mulberry leaves is directly related to the growth and productivity of silkworms (71, 72). Silkworms consume large quantities of mulberry leaves and any variation in leaf quality can affect the quantity and quality of silk produced. Factors such as leaf size, nutrient content and leaf texture are critical for optimal silkworm growth (72). However, the seasonal nature of mulberry leaf production presents another limitation to traditional breeding. Transgenic transformation can potentially overcome these challenges by introducing genes that enhance leaf quality, prolong leaf production and improve the nutritional content of the leaves (72-74).

3.3.1 Expression of glycinin gene: Soybean seed is rich in protein, 70 % of which is glycinin. It is an important protein source. Soybean protein usually is one of very important nutrition components in silkworm artificial diet and a highly valuable nutrient for silkworm. Based on constructing plant expression vector with glycinin AlaBlb subunit gene and the engineered *A. tumifaciens*, the glycinin gene was introduced into mulberry plants successfully with the use of an *A. tumifaciens* vector system (75).

3.3.2 Expression of AtSHN1 gene: The AtSHN1 gene, when overexpressed in mulberry, significantly improved leaf quality by increasing the epicuticular wax load, which reduced cuticular water loss. This modification resulted in improved post-harvest leaf quality, as the leaves retained moisture for a longer duration after detachment. Such an improvement is particularly beneficial in sericulture, as it ensures that silkworms receive fresh, high-quality leaves for an extended period, thereby promoting their healthy growth and maximizing silk yield (76).

3.3.3 Expression of PEPC gene: This study focused on overexpressing the PEPC gene from *Flaveria trinervia* in mulberry to enhance its photosynthetic potential. Phosphoenolpyruvate carboxylase (PEPC) plays a vital role in carbon fixation and improves drought tolerance. The transgenic mulberry plants exhibited a 1.91 to 2.66-fold increase in PEPC activity, which resulted in higher photosynthetic rates, better stomatal conductance and improved transpiration regulation under water-deficient conditions. These enhancements not only supported better growth but also maintained the nutritional integrity of the leaves, thereby benefiting silkworm rearing (77).

Conclusion

Genetic transformation is one of the attractive means of introducing desired traits to pre-existing genotypes within a short period. The availability of efficient transformation and different regeneration systems opens the way for transferring desirable genes against diseases and stresses into commercially important mulberry clones. The biotic assay with transgenic plants proved that these transgenic plants have no deleterious effect on silkworm rearing and feeding. Therefore, these improved mulberry plants can be used for rearing silkworms in future. To fully harness the potential of genetic transformation in mulberry, future research must focus on optimizing transformation protocols, enhancing regeneration efficiency and overcoming species-specific limitations. Plastid transformation, despite its advantages, remains largely unexplored in mulberry, necessitating the development of species-specific techniques and improved selection strategies. The adoption of transgenic mulberry is subject to strict regulatory frameworks that vary by country, focusing on biosafety, environmental impact and food/feed safety assessments. Regulatory bodies like the GEAC (India), USDA-APHIS (USA) and EFSA (EU) evaluate genetically modified organisms (GMOs) before commercialization. Compliance with risk assessment protocols, gene flow containment strategies and public acceptance measures is essential for approval. Future policies should aim to balance innovation with ecological sustainability and economic viability for successful adoption. Collaborative efforts between biotechnologists, molecular geneticists and breeders will be crucial in translating these advancements into practical applications for mulberry improvement. Investing in cutting-edge research and innovative approaches will pave the way for more efficient, stable and high-yielding transgenic mulberry varieties.

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Authors' contributions

SB helped in literature collection, manuscript writing and research contribution. PM contributes to research contribution, conceptualization preparation and correction of final manuscript. All other authors read and approved the manuscript.

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