



A Stepwise Approach to Plant Transformation: Integrating STTM Gene Editing, PCR Validation and RNA Expression Analysis

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ABSTRACT

Background: The research presents an organized procedure to transform plants through the integration of short tandem target mimic (STTM) gene editing followed by the validation using polymerase chain reaction (PCR) and RNA expression measurements for genetic modification testing.

Methods: The research established a recombinant plasmid to control plant dwarfism in the eggplant species *Solanum melongena* that holds STTM selection gene fragments. Scientific protocols begin with plasmid development, followed by bacterial transformation using *Agrobacterium* and culminate in molecular testing via PCR analysis of nucleic acids. Useful research methods were developed, enabling biological gene validation and improving plant characteristics through directed genetic modification. The research demonstrates that STTM-based transformation provides plants with a reliable method for performing precise, repeatable genetic modifications.

Result: The findings from this research have evolved plant biotechnology because they created an authenticated framework to analyze gene functions as well as advanced purposeful genetic transformation approaches for improving stem height through targeted modifications. The findings also validate *Agrobacterium*-mediated transformation as an effective method for stable gene expression in plant transformation. Analysis of RNA expression confirmed that STTM-mediated miRNA suppression has functional consequences, establishing a suitable technique for controlling plant height through genetic modification.

Key words: *Agrobacterium*-mediated transformation, Genetic modification, PCR validation, Plant transformation, RNA expression analysis, *Solanum melongena*, STTM gene editing.

INTRODUCTION

Genetic engineering relies on plant transformation as its foundational process to achieve exact modifications in plant traits to fulfil requirements. The research by Tsakirpaloglou *et al.* (2023) serves as a foundation for scientists who utilize STTM technology with CRISPR/Cas9 tools to boost desirable plant characteristics (Othman *et al.*, 2023; Krishna *et al.*, 2010). According to Jones (2016), this technology controls plant modifications by regulating the expression patterns of microRNAs (miRNAs), thereby silencing their biological activities through sophisticated biological methods (Tang *et al.*, 2012; Joshi *et al.*, 2012; Hahne *et al.*, 2019).

The development of an advanced plant transformation protocol required scientists to unite STTM gene-editing technology with *Agrobacterium tumefaciens* transformation, which had already demonstrated success in foreign gene insertion. Scientific evidence demonstrates that this unified method successfully modifies *Solanum melongena* DNA, representing the plant species known as aubergine or eggplant (Wan *et al.*, 2023; Keshavareddy *et al.*, 2018). The first stage requires scientists to create recombinant plasmids based on STTM for genetic modification of aubergine, followed by separate procedures for implementation. The identification of transformed DNA sequences is an essential step toward complete genetic transformation (Agrawal *et al.*, 2005); Nandy *et al.* (2020) explain that plants undergoing transformation require thorough testing to properly validate genetic modifications (Husaini *et al.*, 2011).

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The introduced gene functions properly, as indicated by RNA expression analysis and its presence in the plant genome is verified by polymerase chain reaction (PCR) testing (Stoddard *et al.*, 2016). The general purpose of this project is to deploy STTM technology for specific gene transformations in *Solanum melongena* (Wójcik *et al.*, 2020; Rahman and Mishra, 2025). Plant trait enhancement is expected

to experience substantial progress through this method. A major breakthrough achieved through STTM occurs when plants are controlled to remain short through genetic suppression combined with bacterial rhizospheric infection, as explained by Sundin *et al.* (2016). Additional molecular verification methods operate alongside these procedures to establish a strong foundation for the enlargement of multiple plant traits (Peng *et al.*, 2018; Pennell *et al.*, 2021; Wada *et al.*, 2022). Using STTM-based gene editing technology in combination with conventional transformation methods provides plants with an effective genetic engineering platform. Utilizing this thorough plant transformation method, companies will now be able to enhance agricultural output while making precise plant modifications, which creates potential for technology growth in this field (Ali *et al.*, 2023; Akgul and Aydinoglu, 2025; Arya *et al.*, 2017).

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Escherichia coli DH5 α is a common laboratory strain that has been utilized as the primary host organism in this study for both standard cloning and the isolation of large quantities of recombinant plasmids. *Agrobacterium tumefaciens* is a soil-borne bacterium commonly used to introduce new genes into plants and was selected for these studies to transform plant tissues. The pCambia backbone provided a binary vector system for cloning the STTM (Short Tandem Target Mimic) gene segment. Bacterial cultures were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics to select for cells harbouring recombinant plasmids. *E. coli* DH5 α cultures were incubated at 37°C with agitation (shaking) at 180 rpm; whereas, cultures of *A. tumefaciens* were kept at 28°C and subjected to the same level of agitation as their *E. coli* counterparts. Antibiotics were added to each culture at concentrations that allowed selection of bacterial cells containing the recombinant plasmid, as required by the cloning vector.

Plasmid construction and cloning

Partnership between the pCambia plasmid backbone and the STTM (Short Tandem Target Mimic) gene fragment 163/164 enables cloning of plant dwarfism-related sequence for doping the pCambia vector. The ligation of the STTM fragment into the linearized pCambia vector was performed in a total volume of 20 μ L of a reaction consisting of; 2 μ L of the linearized pCambia vector, 4 μ L of the STTM fragment, 2 μ L of 10 $\times$ T4 DNA ligase buffer and 1 μ L of T4 DNA ligase with the remainder of the volume being made up with nuclease-free water, as listed in Table 1. The ligation mixture was incubated at 4°C overnight to allow efficient insertion of the STTM fragment into the pCambia vector.

Ligation

P-cambia Backbone	+	STTM insert
2 μ L		4 μ L

Bacterial transformation and plasmid isolation

Competent *E. coli* DH5 α cells were transformed using the heat-shock method with the ligation mixture. After transformation, the bacterial cells were plated on Luria-Bertani (LB) agar plates containing kanamycin (50 μ g/mL) and chloramphenicol (25 μ g/mL) and incubated at 37°C overnight. Putative transformant colonies were picked and re-streaked on new LB agar plates containing the same antibiotics to confirm plasmid stability.

Plasmid DNA was purified from three separate bacterial cultures via the alkaline lysis technique. The quality of the purified plasmids was evaluated and utilized for further molecular analysis. The recombinant plasmids were first confirmed by PCR-based screening, as previously described (Anvari *et al.*, 2021; Kumar *et al.*, 2019).

PCR confirmation

The STTM and nptII primers were used in PCR amplification to confirm the STTM gene and the nptII selection marker, which confer kanamycin resistance. Gene-specific primers designed for the STTM and nptII genes were used for amplification. The mixture for the PCR reaction required the following composition during the preparation of STTM primers, as listed in Table 2. The amplification cycle included an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Bacterial colony screening and PCR amplification

Colony PCR screening was performed to isolate recombinant *Escherichia coli* DH5 α colonies carrying the target construct. Colonies were randomly picked from LB agar plates

Table 1: DNA ligation reaction setup illustrating the preparation of a ligation reaction using the P-cambia backbone (2 μ L) and STTM Insert (4 μ L).

Components	Concentration
10 $\times$ Ligase buffer	2 μ L
T4 DNA ligase	1 μ L
NFW (Nuclease-free water)	11 μ L
Total	20 μ L

Table 2: PCR (Polymerase Chain Reaction) reaction setup, showing the components and their volumes.

Reagent	Control (Con.)	Test ($\times 3$)
Template DNA	1 μ L	3 μ L
10 mM dNTPs	0.4 μ L	1.2 μ L
10 μ M forward primer (FP)	0.4 μ L	1.2 μ L
10 μ M reverse primer (RP)	0.4 μ L	1.2 μ L
10 $\times$ PCR buffer	2 μ L	6 μ L
Taq polymerase	0.1 μ L	0.3 μ L
Nuclease-free water (NFW)	15.3 μ L	46 μ L
25 mM MgCl ₂	0.4 μ L	1.2 μ L
Total volume	20 μ L	60 μ L

Table 3: PCR reaction components and their concentrations for Npt2 primers.

Component	Single reaction (20 µL)	Bulk mix (×5) (160 µL)	Control (20 µL)
Template DNA	1 µL	8 µL	-
10 mM dNTPs	0.4 µL	3.2 µL	0.4 µL
10 µM forward primer (FP)	0.4 µL	3.2 µL	0.4 µL
10 µM reverse primer (RP)	0.4 µL	3.2 µL	0.4 µL
10× Buffer (B)	2 µL	16 µL	2 µL
Taq polymerase	0.1 µL	0.8 µL	0.1 µL
Nuclease-free water (NFW)	15.7 µL	125.6 µL	16 µL
25 mM MgCl ₂	0.4 µL	3.2 µL	0.2 µL
Total volume	20 µL	160 µL	20 µL

Table 4: PCR reaction components for STTM confirmation in *Agrobacterium* colonies.

Component	Control (Con.)	Test reaction (×4)
Template DNA	1 µL	5 µL
10 mM dNTPs	0.4 µL	1.6 µL
10 µM forward primer (STTM-F)	0.4 µL	1.6 µL
10 µM reverse primer (STTM-R)	0.4 µL	1.6 µL
10× PCR buffer	2 µL	8 µL
Taq polymerase	0.1 µL	0.4 µL
Nuclease-free water (NFW)	15.7 µL	50 µL
25 mM MgCl ₂	0.4 µL	1.6 µL
Total volume	20 µL	80 µL

supplemented with kanamycin and resuspended in 20 µL of nuclease-free water. Bacterial cells were lysed by heat treatment at 95°C for 10 min, followed by centrifugation at 12,000 rpm for 5 min. The supernatant containing genomic and plasmid DNA was used as the PCR template.

PCR was performed using Npt2 primers to confirm the presence of the target sequence in the selected colonies. The reaction was set up in a total volume of 20 µL, with the components listed in Table 3. A negative control (without template DNA) was included to ensure specificity.

PCR confirmation of *agrobacterium* colonies

PCR analysis was performed to verify the existence of STTM sequences in *Agrobacterium* colonies after transformation. PCR was performed in a total volume of 20 µL, containing 1 µL of template DNA, 10 mM dNTPs, 10× PCR buffer, 25 mM MgCl, Taq polymerase and 10 µM forward and reverse primers specific to the STTM. To guarantee specificity, a control reaction was also incorporated, as listed in Table 4. The PCR cycling parameters included an initial denaturing step, followed by a series of denaturing, annealing and extension steps and a final extension step.

To confirm that the anticipated bands were present, the amplified products were examined on a 1.5% (w/v) agarose gel to confirm the presence of bands of the expected size. The solution involved dissolving 1.5 g agarose in 100 mL 1× TAE buffer before adding ethidium bromide (0.5 µg/mL). Each well received 5 µL of PCR product blended with 1 µL 6× loading dye. The analysis used a 100 bp DNA ladder to

provide molecular weight markers. The electrophoresis occurred at 100 V for a 45-minute duration, while the UV transilluminator revealed the bands.

Plasmid isolation from *agrobacterium*

Recombinant *Agrobacterium tumefaciens* cultures were grown overnight and 10 mL of bacterial culture was harvested by centrifugation at 6,000 rpm for 5 min at 4°C. Plasmid DNA was isolated using a modified alkaline lysis method. DNA precipitation was performed by adding 0.7 volumes of isopropanol, followed by centrifugation. The DNA pellet obtained was washed with molecular biology-grade ethanol, air-dried and resuspended in 20 µL of nuclease-free water for further molecular analysis.

Plant tissue culture (PTC) and *agrobacterium* transformation

Seed sterilization and germination

Seeds were sterilized in aseptic conditions using autoclaved Falcon tubes (Fig 4). Seeds were first washed three to four times with sterile distilled water. Then, they are washed with 0.1% HgCl₂ for 2-3 minutes. After that, again wash the seeds with autoclaved distilled water 2-3 times to remove any traces of the sterilizing agent. A final wash with 70% (v/v) ethanol was done and the seeds were then air-dried in aseptic conditions. At last, they are dried and then placed in the MS media. Preparation of MS media involves adding 2.2 g of MS media to 500 mL of autoclaved water containing 15 g of sucrose. pH is adjusted to 5.6.

Cotyledon excision and Pre-treatment

Cotyledons excised from in vitro Agro-germinated seedlings at 7 days were used as explants for transformation. The excised cotyledons were pre-cultured on full-strength MS medium for 2 days to improve their ability for *Agrobacterium*-mediated transformation.

Agrobacterium transformation and co-cultivation

The pre-cultured cotyledon explants were infected with a secondary culture of *Agrobacterium tumefaciens* carrying the recombinant plasmid, adjusted to an optical density (OD₆₀₀) of 0.5. After infection, the explants were blotted dry and placed on co-cultivation MS plates, incubated for an additional 2 days under controlled conditions.

Selection and callus induction

After co-cultivation, the explants were transferred to selection media (MS with kanamycin and cefotaxime) to screen for successful transformation. Callus formation started after 3 weeks.

Rooting and acclimatization

Actively growing calli were transferred to half-strength MS medium to induce root development. Well-developed plantlets were gradually acclimatized and transferred to soil-filled pots under controlled conditions for acclimatization.

PCR confirmation in *Agrobacterium*

PCR was performed to verify the presence of the STTM gene construct in the transformed *Agrobacterium tumefaciens* colonies, as described by Ebrahimzadegan *et al.* (2022). The STTM (Short Tandem Target Mimic) primers with nptII (neomycin phosphotransferase II) primers were used to screen colonies 4, 6, 12 and 15. The primers used amplified specific target DNA regions of the transformed bacterial genome to confirm correct gene insertion. The PCR reactions were set up as described in Table 5 and the reactions underwent the same thermal cycling parameters, including

optimized annealing temperatures and extension times. The results were then confirmed by agarose gel electrophoresis, which showed the presence of DNA fragments of the expected sizes, indicating the successful transfer of the gene constructs into *Agrobacterium* strains.

RESULTS AND DISCUSSION

Molecular verification of recombinant *agrobacterium tumefaciens*

Recombinant plasmids that were positively verified in *Escherichia coli* DH5α colonies were successfully transferred into *Agrobacterium tumefaciens* strain LBA4404. The growth of transformed *Agrobacterium* colonies on selective YEP medium containing rifampicin, kanamycin and chloramphenicol confirmed the successful uptake and retention of the recombinant plasmid (Fig 1). Plasmid DNA extracted from selected *Agrobacterium* colonies met the required quality and quantity for subsequent molecular analyses. The presence of the recombinant plasmid in *Agrobacterium* was also verified by PCR, confirming the successful transfer of the STTM-containing plasmid before the commencement of plant tissue culture experiments.

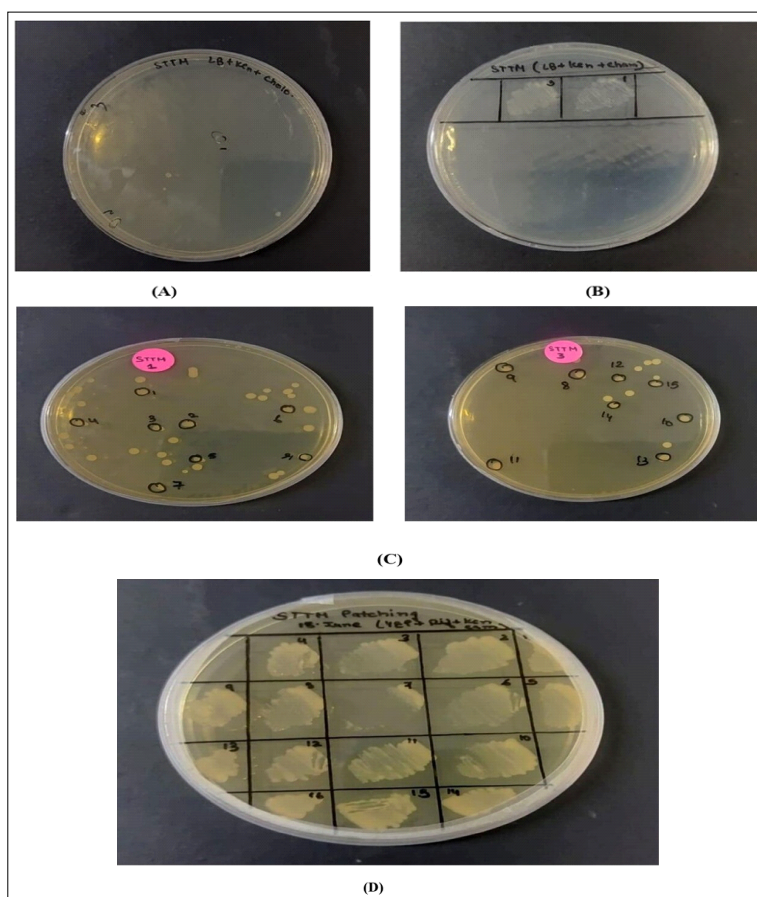


Fig 1: Transformation in Dh5 alpha (A) Ligation product is used for transformation in dh5 alpha (B) LB+Ken+cham selection is used. Colonies are patched on LB+Ken+cham plates. (C) *Agrobacterium* transformation. (D) Growth of *Agrobacterium tumefaciens* transformants on YEP plates supplemented with rifampicin (50 µg/mL), kanamycin (50 µg/mL) and chloramphenicol (25 µg/mL).

Establishment of *agrobacterium*-mediated plant transformation and callus induction

Surface-sterilized seeds exhibited successful *in vitro* germination on MS medium, yielding healthy seedlings (Fig 2-6). Seven-day-old seedlings offered ideal cotyledon explants for subsequent transformation experiments.

Excised cotyledons remained alive throughout the pre-culture phase and were successfully transformed using *Agrobacterium*-mediated transformation. After co-cultivation, the explants were placed on selection medium, where callus development was observed after about 3 weeks (Fig 3). The developed calli exhibited vigorous growth, confirming successful tissue transformation.

Transformed calli were further grown for regeneration and were placed on rooting medium, where root development occurred. Regenerated plantlets displayed normal growth and were successfully acclimatized and transferred to soil-filled pots (Fig 4).

These findings confirm the successful development of an *Agrobacterium*-mediated transformation and plant regeneration system.

PCR Confirmation in *Agrobacterium*

PCR screening of *Agrobacterium* colonies was carried out using STTM and nptII primers (Table 5). Colonies 4, 6, 12 and 15 gave amplification of the target fragments. All the reactions were carried out under the same conditions. Agarose gel electrophoresis confirmed the presence of bands of the expected sizes, indicating successful incorporation of the gene constructs into the transformed *Agrobacterium* strains.

Molecular analysis

High-quality genomic DNA was isolated from the leaves of transformed and control plants and its quality was verified by agarose gel electrophoresis. The isolated DNA was of good quality and suitable for further molecular studies.

PCR validation with gene-specific primers confirmed the integration of the STTM gene and the nptII selectable marker in transformed plants (Table 6). Amplification of the expected STTM (163/164 bp) and nptII (700 bp) fragments was detected in transgenic plants, but not in non-transformed control plants. Amplification of the expected fragments was detected in the positive control plasmid DNA.

RNA profiling indicated the quality of total RNA isolated from transgenic and control plants. Quantitative RT-PCR analysis showed higher STTM gene expression in transgenic plants than in control plants. The qRT-PCR detection sequence used SYBR Green with target-specific primers, as shown in Table 7. The STTM construct was associated with lower expression of miRNA target genes. Expression levels were normalized with the actin reference gene using the $\Delta\Delta C_t$ method and there was little variation among biological replicates, as shown by the error bars.

Phenotypic analysis of transgenic plants

Phenotypic analysis of transgenic plants was conducted over 4 weeks to determine the impact of STTM gene expression on plant development. Transgenic plants demonstrated a relative reduction of about 35% in plant height compared to wild-type plants. In addition to reduced plant height, transgenic plants also exhibited compact growth with wider leaves.

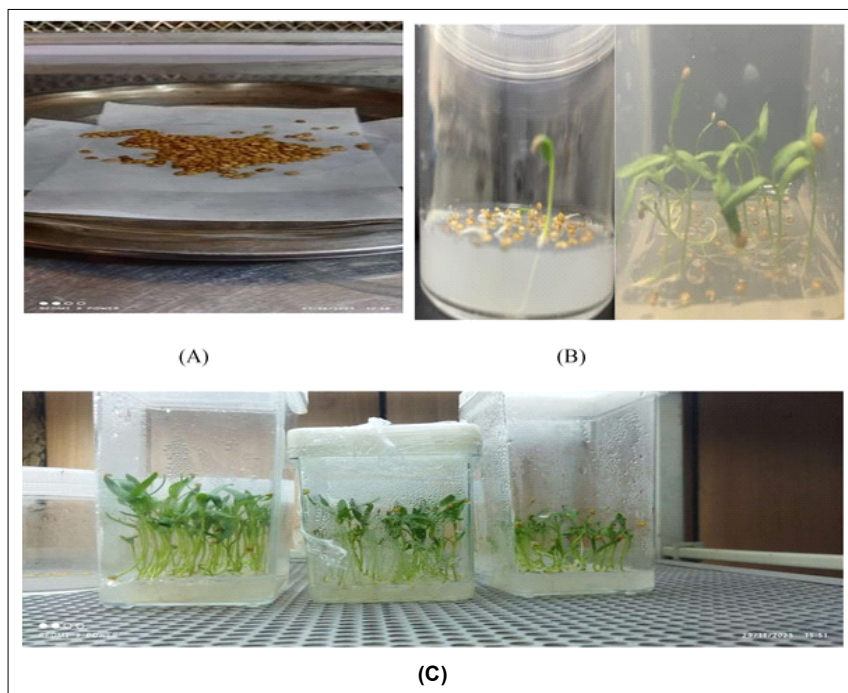


Fig 2: (A) Seed germination, (B) Early-stage seed germination *in vitro*, along with initial sprouting. Multiple seedlings at an advanced stage of germination in a sterile medium. (C) Large-scale seedling production under plant tissue culture (PTC) conditions.

Transgenic cotyledon explants initiated callus after 10-14 days of selection, confirming successful transformation (Fig 5). Root induction in transgenic plants occurred later than in wild-type plants.

Survival rate on selection media

Stable transformation occurred when the regenerated shoots proved their resistance to kanamycin. The shoots carrying the *nptII* gene survived on the selection media, while the non-transformed plants did not survive on kanamycin media.

PCR verification of recombinant plasmid in *E. coli* DH5α

PCR analysis of *E. coli* DH5α colonies was performed using primers specific to STTM to verify the presence of the recombinant plasmid. Of the colonies analyzed, Colonies 1 and 3 were able to amplify the expected STTM fragment, while Colony 2 did not amplify, indicating a failed transformation (Fig 6A, 6B).

Subsequent verification using *nptII* (*Npt2*) primers amplified the expected fragment in Colonies 1, 2 and 3

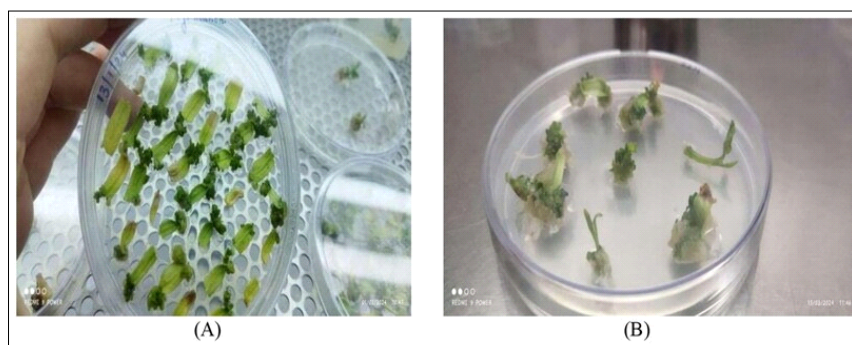
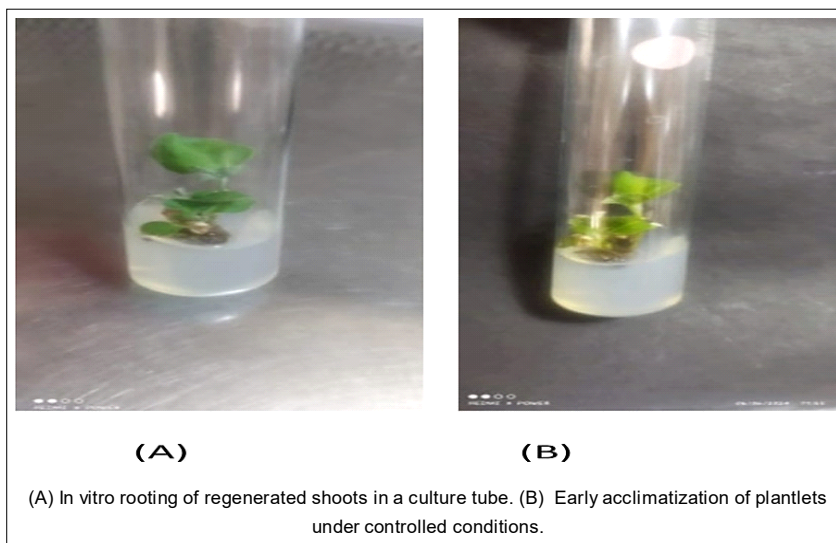


Fig 3: Selection and callusing (A) Callus induction in explants under selection pressure. (B) Early-stage callusing and shoot initiation in selected explants.



(A) In vitro rooting of regenerated shoots in a culture tube. (B) Early acclimatization of plantlets under controlled conditions.

Fig 4: Rooting and acclimatization in plant tissue culture.

Table 5: PCR Reaction Mix for STTM and *nptII* primer screening in *Agrobacterium*.

Components	Concentration (single reaction)	Concentration (×4 reactions)
Template	1 µL	5 µL
10 mM dNTPs	0.4 µL	1.6 µL
10 µM forward primer (STTM-F)	0.4 µL	1.6 µL
10 µM reverse primer (STTM-R)	0.4 µL	1.6 µL
10× Buffer (B)	2 µL	8 µL
Taq polymerase	0.1 µL	0.4 µL
NFW (Nuclease-free water)	15.7 µL	50 µL
25 mM MgCl ₂	0.4 µL	1.6 µL
Total volume	20 µL	80 µL



Fig 5: Callus induction from explants visible green callus formation is observed, along with some browning in certain regions.

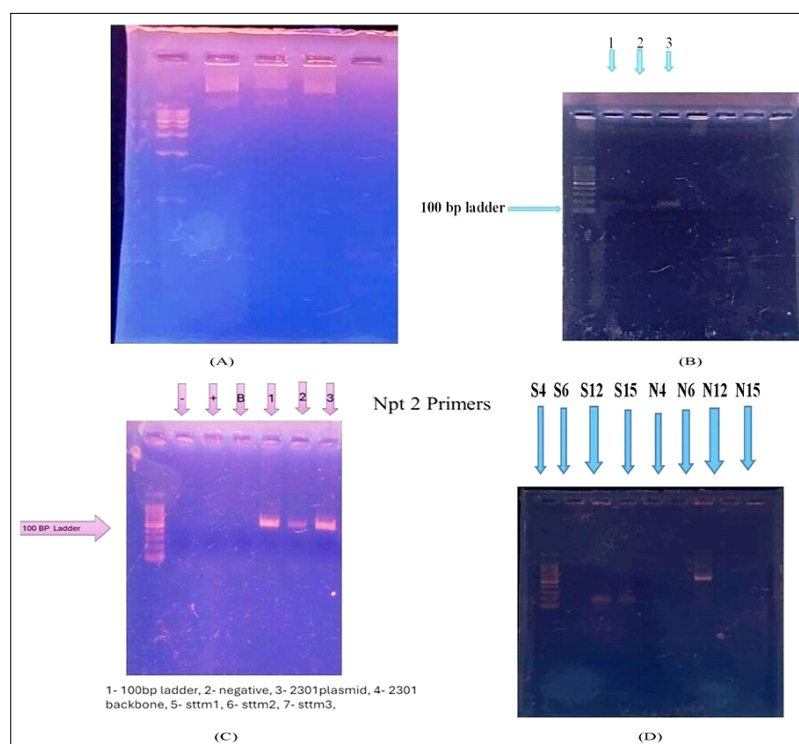


Fig 6: (A) PCR confirmation of DH5α colonies, (B) Dh5 alpha colonies by STTM primers, (C) Confirmation of DH5α colony by PCR with Npt2 Primers, (D) PCR confirmation *Agrobacterium* colony using.

(Fig 6C). The positive control amplified well, while no amplification was observed in the blank control, ensuring the specificity of the PCR reaction. The size of the fragments was estimated using a 100 bp DNA ladder.

PCR confirmation of recombinant plasmid in *agrobacterium tumefaciens*

PCR analysis of *Agrobacterium tumefaciens* colonies was performed using primers specific for STTM and nptII genes. Colonies 4, 6, 12 and 15 were analyzed for the presence of the recombinant plasmid. Colony 6 was positive for the amplification of both STTM and nptII genes, while colonies 4, 12 and 15 were partially or not amplified (Fig 6D). The positive control was positive and no amplification was observed in the negative control.

STTM and Npt2 primers

A complete three-step protocol was established, using the STTM gene-editing method, followed by genetic testing and phenotype scanning across eggplant (*Solanum melongena*) plants. The pCambia backbone containing the STTM 163/164 gene fragment was confirmed by PCR before introducing it into bacteria during transformation. The isolated plasmid DNA from transformed DH5α strains showed the presence of both STTM and nptII-targeted sequences as determined by PCR. Plasmid uptake was verified when *Agrobacterium tumefaciens* cultures yielded positive colonies that tested positive by PCR. The eggplant cotyledons transformed with *Agrobacterium* produced efficient callus and shoot regeneration when cultured on medium containing kanamycin and cefotaxime, thereby ensuring stable integration of transgenes.

Table 6: PCR reaction mixture for transgene integration validation.

Component	Volume (μL)
Genomic DNA	1.0
10 mM dNTPs	0.4
10 μM Forward primer (STTM/nptII)	0.4
10 μM Reverse primer (STTM/nptII)	0.4
10× Taq buffer	2.0
Taq polymerase	0.1
25mM MgCl ₂	0.4
Nuclease-free water	15.3
Total volume	20

Table 7: qRT-PCR reaction mixture for gene expression analysis.

Component	Volume (μL)
Cdna	1.0
10 μM Forward primer	0.4
10 μM Reverse primer	0.4
SYBR green mix	10.0
Nuclease-free water	8.2
Total volume	20

PCR tests on transgenic plants confirmed that they contained both STTM and nptII genes and qRT-PCR results indicated high STTM expression together with decreased expression of height-related miRNA targets. The genetically modified eggplants displayed 35% reduced plant height due to expression of the STTM construct. The findings validate *Agrobacterium*-mediated transformation as an effective method for stable gene expression in plant transformation. Analysis of RNA expression confirmed that STTM-mediated miRNA suppression has functional consequences, establishing a suitable technique for controlling plant height through genetic modification.

FUTURE PROSPECTIVE

Multiplexed STTM

Improving and developing the polygenic traits by simultaneously silencing several miRNAs.

CRISPR/STTM hybrid systems

These systems combine CRISPR/Cas tools with STTM to regulate and control the genes in a synergistic manner.

Tissue-specific or inducible expression

To precisely regulate miRNA inhibition, use tissue-specific or inducible promoters.

Delivery based on nanotechnology

Creation of non-transgenic STTM delivery systems through the use of exosome-like vesicles or nanoparticles.

Integration with omics

For systems-biology knowledge of STTM impacts, combine transcriptomics, proteomics and metabolomics.

Crop improvement programs

Usage STTM to increase productivity, fertiliser usage, efficiency and stress resilience in commercial crops.

CONCLUSION

PCR and qRT-PCR analysis confirmed the stable integration and expression of the STTM gene in transgenic eggplant plants. Gene expression mediated by STTM led to the development of a dwarf plant, which confirmed the efficient suppression of miRNA as a method for plant height control. Stable transformation was also confirmed by the survival of transgenic plants on kanamycin-containing selective media. The results collectively confirm the successful use of STTM genetic transformation. This study presents a simplified, efficient method combining STTM gene editing with PCR and RNA expression analyses, confirming the potential of STTM technology for future crop genetic transformation.

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Conflict of interest

All authors have no conflict of interest.

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