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AGROBACTERIUM-MEDIATED STABLE TRANSFORMATION IN COTTON (*GOSSYPIUM HIRSUTUM* L.) VIA SOMATIC EMBRYOGENESIS FOR THE *CAS9* GENE EDITING

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SUMMARY

Genetic transformation is essential for studying gene function and improving agronomic traits in cotton (*Gossypium hirsutum* L.). In this study, we developed a stable *Agrobacterium*-mediated transformation protocol using the pKSE401 vector carrying the *Cas9* gene. Seven-day-old hypocotyls of cotton cultivar Coker-312 sustained *Agrobacterium tumefaciens* strain inoculations, with transgenic plants regenerated via somatic embryogenesis. Out of 4,000 hypocotyl explants, seven embryos reached the embryogenesis stage, and five transgenic plants successfully matured. *Cas9* integration reached confirmation in T_0 transgenic plants through PCR analysis. Callus induction succeeded using a modified Murashige and Skoog (MS) medium with kinetin (0.1 mg/L), 2,4-D (0.1 mg/L), Kanamycin (50 mg/L) and cefotaxime (400 mg/L). Somatic embryogenesis enhancement using a hormone-free MS medium had supplements with $MgCl_2$, glutamine, asparagine, $CuSO_4$, ascorbic acid, and activated charcoal (2 g/L). Shoot and root induction emerged with MS medium containing 1 g/L activated charcoal, BAP (1 mg/L), and kinetin (0.1 mg/L) for shooting, and NAA (1 mg/L) for rooting. Among the *Agrobacterium* strains tested, EHA101 and LBA4404 exhibited the highest transformation efficiencies (40%). This study establishes a reliable and time-efficient transformation system for cotton with an overall nine months, less regeneration time of 80–90 days, and an optimized range of OD (0.5–0.8) for infection of explant. This protocol offers a valuable tool for molecular breeding and functional genomics research.

Keywords: Cotton, *Cas9* gene, gene transformation, cotton transformation

Key findings: An optimized *Agrobacterium*-mediated transformation in cotton was successful by identifying LBA4404 as the most efficient strain and enhancing transformation rates through hormone-free media and an optimized acetosyringone concentration (20 mg/L). Our improved somatic embryogenesis protocol reduced the regeneration timeline to 80–90 days, with activated charcoal (1–2 g/L) playing a key role in embryo development.

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INTRODUCTION

Cotton is an important crop, being the primary source of natural fiber and vegetable oil (Saeed *et al*., 2024). The economic significance of cotton is substantial, contributing approximately USD 500 billion annually from the worldwide production of 25 million tons (110 million bales). Additionally, its byproducts are widely useful for animal feed. Cottonseeds are rich in oil and protein, while cotton fiber remains a dominant material in the global textile industry (Saeed *et al*., 2024).

Plant transformation methods vary significantly based on species and could have broad categorizations as physical and biological techniques. Physical methods include particle bombardment, electroporation, microinjection, and sonication, while biological approaches involve *Agrobacterium tumefaciens*- and *Agrobacterium rhizogenes*-mediated transformations. Among these, the biolistic gene gun method and *Agrobacterium*-mediated transformation are the most commonly used techniques for genetic modification in plants (Yang *et al*., 2011; Ribeiro *et al*., 2021; Lin *et al*., 2022).

The introduction of several functional genes into cotton has been successful through *Agrobacterium*-mediated transformation. For instance, the *glucose oxidase* and *Cry1Ia5* genes, successfully introduced into cotton cultivars Coker-315 and Coker-310, conferred fungal resistance and resistance to lepidopteran insects, respectively (Leelavathi *et al*., 2004). Similarly, in Coker-312, introducing transgenes, such as *2,4-D mono-oxygenase*, *protease inhibitor*, *EPSP synthase*, *Cry1Ac*, and *Mn superoxide dismutase,* has been progressive to enhance resistance to herbicides, insect pests, glyphosate, lepidopteran insects, and oxidative stress (Perlak *et al*., 2001; Jakubovics *et al*., 2002). Despite these advancements, current transformation methods remain limited by slow processes, complex selection procedures, low efficiency, and poor regeneration rates. Successful transformation often relies on time-consuming and labor-intensive methods, further hindering the widespread application of these techniques (Leelavathi *et al*., 2004).

Transformation efficiency varies depending on the *Agrobacterium* strain used. The number of *Agrobacterium* strains has increased, such as AGL1, C58, C58C3, EHA101, EHA105, GV3101, GV3111, and LBA4404 (Jin *et al*., 2005; Tohidfar *et al*., 2005). The success of *Agrobacterium*-mediated transformation primarily depends on two key factors: (1) the ability of *Agrobacterium* to successfully transfer the gene into the target genome and (2) the regeneration capacity of the transformed cells (Wilkins *et al*., 2000).

Researchers working on cereal transformation have emphasized the importance of establishing embryogenic cultures before transformation rather than directly bombarding immature embryos. This strategy facilitates subsequent callus proliferation and has been successful in its application to wheat, barley, maize, rice, and other species (Sarker *et al.,* 2007; Zakaria *et al.,* 2012; Guruprasad *et al*., 2016; Noor *et al.,* 2022). This study presented an efficient and stable *Agrobacterium tumefaciens*-mediated transformation method and, for the first time, provided a comprehensive record of all stages of somatic embryogenesis with distinct, independent identification. Such a method entailed specific treatment for cotton regeneration via somatic embryogenesis using the Coker-312 cultivar.

In this study, we demonstrated cotton transformation, in which transformed callus progressing through embryogenic stages marked by *Cas9* expression, which can give rise to stably transformed plantlets within a period of nine months using this protocol. This proposed method is better in transformation using hypocotyls as explants, efficient, and reliable for gene functional studies, offering higher transformation efficiency and success rates compared with other transformation methods via somatic embryogenesis in literature. A strong *Agrobacterium* transformation ability was evident using optimized strains and systematically evaluated key factors influencing transformation efficiency. Furthermore, optimized culture media compositions served to enhance the regeneration of transformed cells. Additionally, comparing multiple *Agrobacterium* strains and

their transformation efficiencies provided valuable insights for researchers aiming to achieve high transformation rates in targeted tissues used as explants, thereby facilitating precise gene targeting in functional studies.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of cotton (*Gossypium hirsutum* L. cv. Coker-312) were soaked in deionized water for 2 h to break dormancy. After soaking, seed sowing commenced in plastic cell trays filled with autoclaved peat moss before incubating in a growth room at a maintained temperature of 28 °C ± 2 °C with a 16/8-h (light/dark) photoperiod and 70% relative humidity. Seven-day-old cotton seedlings with fully expanded cotyledonary leaves succeeded in selecting for transformation.

Vector and bacterial strain transformation

For introducing the plant binary expression vector pKSE401 into *Agrobacterium tumefaciens* strains used the freeze-thaw method (Höfgen and Willmitzer, 1988), a widely used technique for transferring DNA constructs into *Agrobacterium*. The *A. tumefaciens* strains GV3101, LBA4404, and EHA101 reached transformation with the pKSE 401, a vector containing the *Cas9* expression cassette (Table 1), for the introduction of the transgene into *Gossypium hirsutum*.

Transformed *Agrobacterium* colonies harboring the pKSE401 vector (Figure 1) sustained culturing on solid Luria-Bertani (LB) medium supplemented with 50 mg/L

Table 1. zCas9 start and end sequence on the sequence retrieval of Addgene NGS result of pKSE401.

No.	zCas9 Sequence	Nucleotides (nts)
1	Starting 10nts of zCas9 sequence	GATGGATTAC
2	Ending 10nts of zCas9 sequence	GAAGAAGTGA

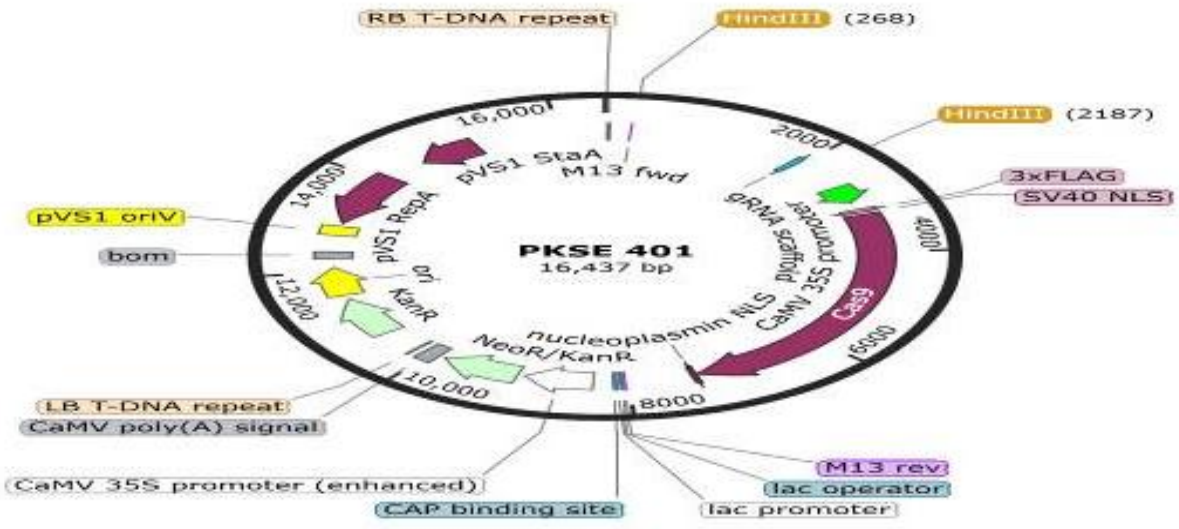

Figure 1. A construct of vector/plasmid (pKSE401) and T-DNA details=LF, left border; kanamycin, selection marker; AtU6-26, *Arabidopsis U6-26* gene as Promoter; zCas9, endonuclease; restriction site of HindIII; polyA, site; 2x CaMV 35S promoter, promoter, AtU6-26 terminator, terminator=RB, right border. The gene used the vector, PKSE 401, which contained RB and LB (right and left borders).

Table 2. Restriction enzyme HindIII sites, their starting nucleotides, and the position on the sequence retrieval of Addgene NGS result of PKSE 401.

No.	Restriction Digestion	Nucleotide (nt)	Position on nucleotide Number
1	First Restriction site of the enzyme HindIII location	A	268
2	Second Restriction site of enzyme HindIII	A	2187

kanamycin before their incubation overnight at 28 °C. A single colony from each strain, as independently used to inoculate 10 mL of liquid LB medium containing 50 mg/L kanamycin, continued to overnight incubation at 28 °C in a shaker incubator until the cultures reached an optimized optical density (OD600) of 0.5–0.8.

The final expression vector confirming post-transformation through restriction enzyme digestion analysis used HindIII with restriction sites (Table 2). The validated construct proceeded in preparing for transformation into cotton plants.

Agrobacterium transformation of cotton hypocotyls

Explant selection and preparation

Cotton hypocotyls became options as the preferred explant for transformation due to their sharp-cut ends, which provided a larger surface area for *Agrobacterium* infection and facilitated successful transformation.

The hypocotyl explants underwent surface sterilization using 0.1% $HgCl_2$ for 10 min, followed by three to four washes with autoclaved distilled water to remove any residual sterilizing agent. The explants then incurred precise cutting into ~1 cm segments before placing them on autoclaved filter paper to remove excess moisture from the surface before further processing.

Inoculum preparation

Applying a 2-day-old *Agrobacterium* culture with an OD600 of 0.5–0.8 served to infect the hypocotyl explants. The bacterial culture in pellet form by centrifugation underwent resuspension in a magnesium glycine liquid (MGL) medium to prepare the final transformation suspension for cotton hypocotyls. The MGL medium consisted of 5 g/L tryptone, 5 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L glycine, and 0.25 g/L KH_2PO_4 (Garfinkel and Nester, 1980).

Explant inoculation

Approximately half an inch from both ends of the hypocotyls bore trimming, with the explants submerged in 30 mL of *Agrobacterium* culture for 40 min to facilitate infection. After incubation, the hypocotyls' placement on autoclaved sterile filter paper helped remove any excess bacterial culture from the surface before further processing.

Co-cultivation

The inoculated hypocotyls proceeded in transferring to a solid co-cultivation medium (CCM) with slight modification. The CCM composition included Murashige and Skoog (MS) salts (4.43 g/L) with vitamins, 30 g/L (3%) sucrose, 0.1 mg/L kinetin, 0.1 mg/L 2,4-D, 1 g/L $MgCl_2$, 20 mg/L acetosyringone, and 7.5 g/L plant agar as a solidifying agent, with no selection agents added (Murashige and Skoog, 1962; Sunilkumar and Rathore, 2001; Harjeet, 2005).

The co-cultivation of hypocotyls continued in darkness at 19 °C–21 °C for 36–48 h to enhance *Agrobacterium* attachment and virulence, as darkness has been shown to promote bacterial interaction with plant cells, improving transformation efficiency. Moreover, researchers used a low concentration of acetosyringone (20 mg/L), unlike other protocols in literature (Jin *et al*., 2012).

Callus induction and maturation

After two days of co-cultivation, the transfer of hypocotyls to a callus induction medium (CIM) ensued, supplemented with selection agents for callus formation with slight modification.

Approximately 10–15 hypocotyls reached culturing on each Petri plate containing MS salts (4.43 g/L) with vitamins, 30 g/L (3%) sucrose, 0.1 mg/L kinetin, 0.1 mg/L 2,4-D, 400 mg/L cefotaxime, 7.5 g/L plant agar, and 50 mg/L kanamycin for selection (Murashige and Skoog, 1962; Harjeet, 2005; Juturu *et al*., 2021).

For sub-culturing, the transfer of 8–10 hypocotyls to fresh CIM plates used sterilized forceps. The cultures acquired incubation in a growth chamber (HPP-750 Ecol, Memmert, Germany) at 28 °C under a 16-h light/8-h dark photoperiod to facilitate callus induction, maturation, and embryogenic development. The plates, monitored daily, had explants transferred to fresh media as needed to prevent bacterial and fungal contamination. The study used low concentrations of hormones, both with the same concentration of kinetin and 2,4-D (0.1 mg/L), unlike other protocols. Callus induction was noticeable within two weeks, while callus maturation and embryogenic development required up to three months.

Somatic embryogenesis

Somatic embryogenesis occurred in two distinct stages. In the first step, the transfer of transgenic calli to the regeneration medium I (RM-I) remained for 40 days before progressing to the second stage.

The RM-I composition: Murashige and Skoog (MS) salts (4.43 g/L) with vitamins, supplemented with 30 g/L (3%) glucose, 0.22% $MgCl_2$, 50 mg/L KNO_3, 50 mg/L kanamycin, and 7.5 g/L plant agar as the solidifying agent (Murashige and Skoog, 1962; Sunilkumar and Rathore, 2001; Harjeet, 2005; Juturu *et al*., 2021).

After 40 days, transferring the calli to the regeneration medium II (RM-II) received incubation for 40–50 days. The RM-II composition: MS salts (4.43 g/L) with vitamins, supplemented with 30 g/L (3%) glucose, 1 g/L $MgCl_2$, 1 g/L glutamine, 0.5 g/L asparagine, 1.25 g/L $CuSO_4$, 100 mg/L ascorbic acid, 2 g/L activated charcoal, 50 mg/L kanamycin, and 7.5 g/L plant agar as a solidifying agent (Murashige and Skoog, 1962; Harjeet, 2005; Juturu *et al*., 2021). RM-II was hormone-free, as minimal concentrations of plant growth regulators (PGRs) were essential at this stage for somatic embryogenesis and regeneration of kanamycin-resistant calli.

During callus induction, the use of a high concentration of hormones, such as kinetin and 2,4-D (0.1 mg/L), transpired. However, to evaluate the role of hormones in somatic embryogenesis, we employed hormone-free media to compare the experiment results with previously reported methods. In this study, applying different media formulations took place with a varied set of combinations of $MgCl_2$, KNO_3, and kanamycin, as given above.

Shooting, rooting, and hardening

Following somatic embryogenesis, shoot induction progressed on a shooting medium containing MS salts (4.43 g/L), 30 g/L sucrose, 1 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L kinetin, 1 g/L activated charcoal, 50 mg/L kanamycin, and 7.5 g/L plant agar (Murashige and Skoog, 1962; Sunilkumar and Rathore, 2001; Harjeet, 2005; Juturu *et al*., 2021).

For root development, the explants involved transferring to rooting medium, which consisted of MS salts (4.43 g/L), 30 g/L sucrose, 1 mg/L naphthalene acetic acid (NAA), 1 g/L activated charcoal, 50 mg/L kanamycin, and 7.5 g/L plant agar. Throughout the study, all media remained at a pH range of 5.6–5.8 to optimize plant growth (Murashige and Skoog, 1962; Sunilkumar and Rathore, 2001; Harjeet, 2005; Juturu *et al*., 2021). Activated charcoal treatment in both rooting and shooting media occurred, unlike other protocols in literature.

The careful transfer of regenerated cotton plants from culture plates to jars containing peat moss followed to facilitate hardening. The jars received a covering of polyethylene bags to maintain humidity and support acclimatization. Once hardened, the plants succeeded in transferring to the field for further analysis.

T-DNA integration analysis

Genomic DNA extraction continued from fresh leaves of stably transformed cotton plants carrying the pKSE401 plasmid. DNA extraction, as performed, used the CTAB method with slight modifications, utilizing 0.2 g of leaf tissue as the starting material. The extracted DNA then attained quantification using a NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, USA).

PCR reactions, when carried out in a 10 µL volume, utilized a Bio-Rad thermocycler (±0.2 °C accuracy and ±0.4 °C uniformity). The reaction mixture contained 1 µL Taq buffer, 1 µL $MgCl_2$, 1 µL dNTPs, 0.25 µL Taq polymerase, 0.5 µL each of forward and reverse primers, 2 µL DNA template, and 3.75 µL dH_2O (Thermo Scientific, USA).

The PCR conditions comprised an initial denaturation at 96 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 45 s, with a final extension at 72 °C for 5 min. The PCR products separation via 2% agarose gel electrophoresis had the band visualization performed using a Gel Documentation System (Bio-Rad, USA).

Transformation efficiency of *Agrobacterium* strains

A total number of 4000 hypocotyls used for transformation with the pKSE401 plasmid. The hypocotyls underwent division into 25 equal transformation batches, with each batch infected by one of three *Agrobacterium tumefaciens* strains: GV3101, LBA4404, and EHA101. The mean transformation frequency entailed calculation by dividing the number of positively transformed plants by the total number of hypocotyls infected by *Agrobacterium*.

RESULTS

Molecular characterization of plasmid pKSE401 in bacterial strains

The confirmed construct succeeded in introduction into three different *Agrobacterium tumefaciens* strains (GV3101, LBA4404, and EHA101). *Agrobacterium* culturing on solid LB media contained kanamycin as a selection marker. This resulted in the growth of numerous colonies, while the negative control showed no colonies. The entire plasmid containing the *Cas9* gene gained confirmation via restriction digestion with the HindIII enzyme, a dual-cutter enzyme. Selected positive colonies proceeded to prepare a bacterial suspension for the subsequent hypocotyl infection.

***Agrobacterium*-mediated cotton stable transformation**

Plant growth and explant preparation

Healthy Coker-312 cotton seeds were samples used for germination in peat moss under controlled conditions in a growth room maintained at 28 °C. Germination began within 2–3 days, with some viable seeds showing emergence above the peat moss. The hypocotyls, which began to grow, reached a length of 6–8 inches, representing the optimal stage for explant preparation (Figure 2A).

Callus induction

The cut hypocotyls (Figure 2B), used as explants, commenced transferring to CCM media for co-cultivation (Figure 2C). Callus initiation was evident at both sharp-cut ends, where they developed into swollen portions (Figure 2D), with 2–3 weeks required for callus induction.

After three weeks, the transformation involving the pKSE401 plasmid resulted in greenish callus growth (Figure 2E). This green callus gradually turned light yellow with slightly dark brown hues as the calli became more embryogenic, and 3–4 months were necessary for full maturation (Figure 2F).

The transformed hypocotyls then incurred placement on a selection plate containing the antibiotic kanamycin. Callus multiplication was successful by carefully dividing the callus, resulting in increased callus mass that covered the entire plate. Only the transformed calli survived on the kanamycin selection plate, while the untransformed

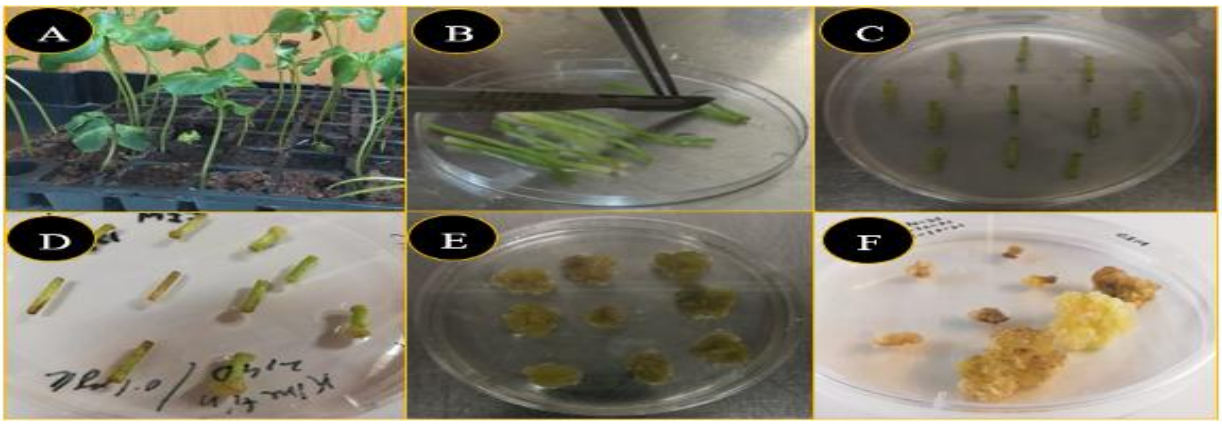

Figure 2. *Agrobacterium*-mediated transformation of cotton *(G. hirsutum L.)* variety Coker-312 using hypocotyls. From the growth of nursery to callus maturation: A) Grown cotton nursery; B) Sharp cutting of hypocotyls; C) Hypocotyls on solid co-cultivation (CCM) without selection of kanamycin; D) Hypocotyls further shifted on Callus induction media (CIM) with selection of kanamycin; E) Callus multiplication; and F) Mature callus obtained in approximately 14 weeks.

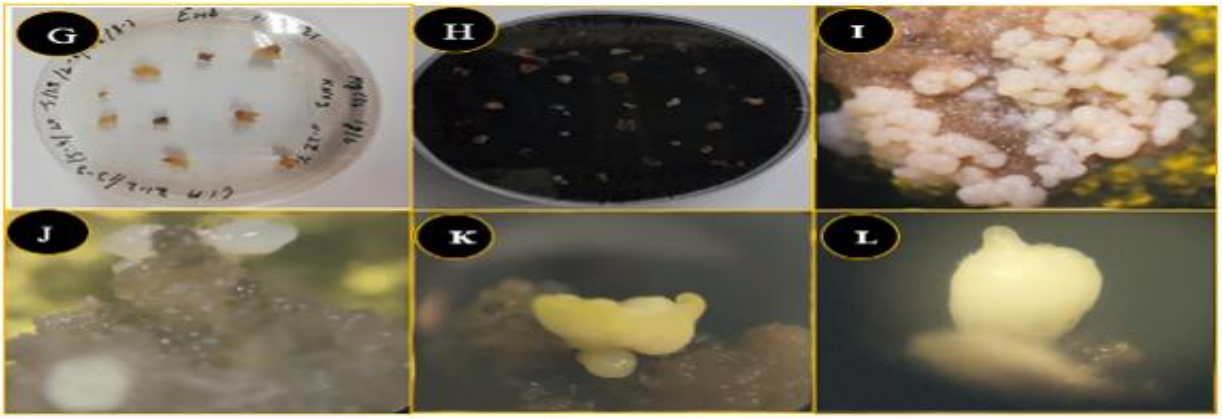

Figure 3. Somatic embryogenesis: G) Calli placed at 28 °C with 16/8 hours photoperiod and maintained on medium for same formulation for 40 days on RM I medium; H) Callus hardening on RM II medium with embryogenic callus; I) Microscopic view of developmental stages of embryo regenerated from explant, Globular stage; J) Heart shape; K) Torpedo stage; and L) Cotyledonary stage.

hypocotyls were killed and destroyed. Overall, callus induction, multiplication, and maturation took approximately three months.

Embryogenesis

After 40 days of culture, somatic embryos were noticeable in Coker-312 cotton. The cotton calli initially appeared dark brown before progressing through various embryonic stages. Somatic embryos were notable at different developmental stages on hormone-free media.

In this study, the careful documentation of each stage of somatic embryogenesis took place, independently identifying each stage. Figures 3G-L illustrated the different developmental stages of embryos regenerating from the callus over 50 days. For Figure 3I, it showed a microscopic view of embryos at the globular stage. Figure 3J

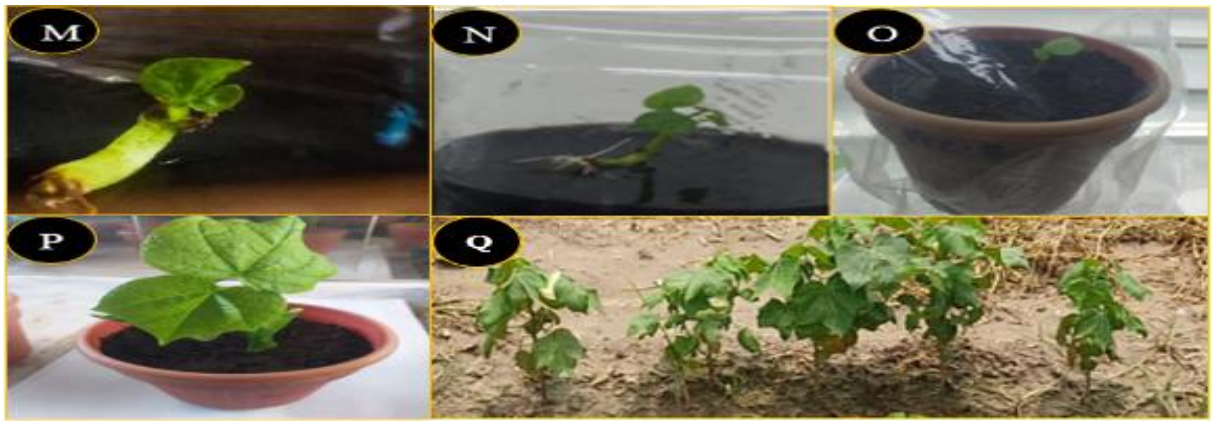

Figure 4. Shooting, rooting, and hardening: M) Shoot generation from embryo; N) Plant growing roots from embryo transferred in glass jar with more media and space for better growth at 3–4 leaf stage; O) Acclimatized putative Cas9 transgenic cotton plant; P) Plant properly shifted in jar with peat moss without any cover and ready to transfer in field conditions; and Q) Wild-type and putative Cas9 transgenic cotton plant shifted in field conditions.

depicted embryos at the heart-shaped stage, ranging in color from whitish to light green. Figure 3K represented embryos at the torpedo stage, showing a whitish to pale yellow shade, while Figure 3L illustrated embryos at the cotyledonary stage with a pale yellow hue.

It typically took 30–40 days for the embryos to reach a stage suitable for rooting and shooting, indicating further growth and development. Overall, somatic embryogenesis took between 80 and 90 days. Scientists observed the addition of 1 g/L of activated charcoal improved the growth of somatic embryos (Figure 3G-L).

Shooting, rooting, and hardening

The callus required specific conditions for tissue differentiation to regenerate a complete plant. Figure 4M depicted shoot generation from the embryo, while Figure 4N showed rooting from an embryo at the 3–4 leaf stage in a glass jar. Rooting and shooting took about two months, from placement on the appropriate media to transfer into a jar with peat moss. The jar's covering with a porous polyethylene bag (Figure 4O) promoted acclimatization. The jar further received placement in a growth chamber with appropriate conditions.

The plant required approximately two weeks for proper acclimatization before being transferred to the open field. During this period, the polyethylene bag bore initial removal for two hours daily to facilitate acclimatization. This duration gradually increased by two hours each subsequent day. After 12 days, the polyethylene bag attained complete removal (Figure 4P), and the plant was ready for field transfer (Figure 4Q). Researchers observed that 2 g/L activated charcoal was effective in proper shoot and root development (Figures 4M and 2N).

Molecular characterization of T_0 generation in cotton

The confirmation of the presence of the *Cas9* gene in putative transgenic kanamycin-resistant calli necessitated performing PCR amplification using *Cas9*-specific primers (Table 3). The vector construct containing the *Cas9* gene reached an expression in putative transgenic cotton plants. The integration of the transgene succeeded in confirming through PCR amplification. The transformation of the pKSE401 vector associated with *Cas9* attained validation in the transformed cotton plants. Cas9 screening emerged as the most effective method for this confirmation. Designing a pair

Table 3. zCas9 sequence and primer location on the sequence retrieval of Addgene NGS result of pKSE401.

No.	Primer ID	Primer sequence (5'-3')	Product size	Description
1	Cas9-F	ATGAATTTCTTCAAGACAGAGATC	988bp	For Cas9 detection in putative transgenic cotton plants
2	Cas9-R	CTGGCCGCTTGTCGCCCCCG		

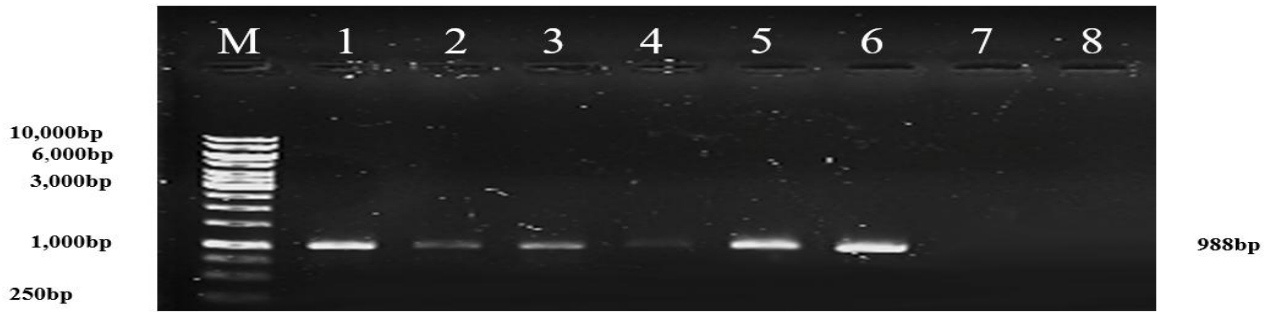

Figure 5. Detection of the existence of Cas9 in T0 generation of strains transformant colonies. Cas9 was used with Cas9-specific primers. A PCR gel image: Lanes 1-5 = transgenic plants; Lane 6 = positive control of construct; Lanes 7 and 8 = negative control (d_3H_2O) and the wild type; and M = 1kb Mass ruler DNA ladder. A pair of primers (Cas9-F and Cas-R) used for Cas9 integration in putative plants is available in Table 3.

Table 4. Comparison of transformation efficiency among all *agrobacterium* strains used during the conduct of the experiment.

No.	Description	Total no. of explants used for transformation event	Total no. of putatively transformed ex-plants	Transformation efficiency	Comparison of transformation efficiency among all strains used
1	Total No of hypocotyls used	4000	5	0.125%	-
2	With strain GV3-101	1333	1	0.075%	20%
3	With strain LBA4404	1334	2	0.150%	40%
4	With strain EHA101	1334	2	0.150%	40%

of primers specific to the *zCas9* gene resulted in a product size of 988 bp (Table 3; Figure 5). The results confirmed the successful transformation of the *Cas9* gene into the target kanamycin-resistant cotton plants via *Agrobacterium*-mediated transformation.

Transformation efficiency of strains used

In this study, the hypocotyls transformed with the vector pKSE401 across 25 transformation batches totaled 4000. Cotton calli selection relied on two key checkpoints. The first checkpoint assessed kanamycin resistance during the early callus induction and plant regeneration. The second checkpoint focused on confirming the integration of the *Cas9* gene into the targeted cotton plant.

Out of the 4,000 hypocotyls infected across the different batches, only seven embryos occurred successfully, and five survived through field transfer, resulting in a transformation efficiency of 0.125% (Table 4). All five independent transgenic plants (Coker-312) reached maturity. PCR amplification confirmed the presence of bands of the expected size in all five lines, validating the transformation of these PCR-positive transgenic lines (Figure 5).

DISCUSSION

Cotton is a major contributor to the global economy, primarily through fiber production for the textile industry and other byproducts (Ahmar *et al*., 2020). In this study, we successfully improved a stable *Agrobacterium tumefaciens*-mediated transformation method for cotton cv. Coker-312 with more time efficiency. Moreover, the study documented all stages of the transformation process, from hypocotyl inoculation to regeneration via somatic embryogenesis.

Plant regeneration from protoplasts is often slow and inefficient. In contrast to conventional physical transformation techniques, *Agrobacterium tumefaciens*-mediated transformation offers several advantages, including ease of use, cost-effectiveness, and the insertion of low-copy-number transgenes into the host genome (Leelavathi *et al*., 2004; Shang *et al*., 2009). In this study, we selected cotton hypocotyls as the explant material for *Agrobacterium tumefaciens*-mediated transformation. Kumar *et al*. (2015) reported hypocotyl explants exhibited the highest callus induction rate (94.90%), followed by cotyledon explants (85.20%). Similarly, we observed efficient callus induction using hypocotyl explants, enabling stable cotton transformation via somatic embryogenesis with the same *Agrobacterium* strains. Callus formation, achieved successfully in bulk, was within a shorter period of two weeks.

In plant transformation, antibiotics, such as kanamycin, and herbicides, like glufosinate and hygromycin, are commonly applicable as selectable markers (Sundar and Sakthivel, 2008). However, these agents can negatively impact plant cell differentiation and proliferation. In mitigating these effects, researchers used kanamycin at a concentration of 50 mg/L, similar to previous studies (Jin *et al*., 2012), ensuring effective selection without compromising plant regeneration.

The complete transformation process, from infection to mature plant development, required approximately nine months. The timeline included callus induction (two weeks), callus maturation (three months), somatic embryogenesis (three months), rooting and shooting (two months), and acclimatization (two weeks). Compared with previous reports on Coker-312 regeneration via hypocotyl explants, which required approximately 10 months (Kumar *et al*., 2013), our approach reduced the total duration. Zhang *et al*. (1999) reported regeneration plants from the Chinese elite cultivar CRI 12 emerged via somatic embryogenesis in 120–150 days. This study's protocol took only 80–90 days.

Previous studies indicated loose, light-yellow callus is more competent in inducing embryogenesis (Wu *et al*., 2004). We observed cotton calli remained dark brown until they progressed through all stages of embryogenesis. Kumria *et al*. (2003) reported and illustrated a mixture of milky white and slightly greenish globular-stage embryos in normal cotton plants. Comparatively, our microscopic analysis of developing embryos at the globular stage in transformed *Gossypium hirsutum* plants showed over 50% similarity, but with a uniformly milky white appearance (Figure 3I). At the heart-shaped stage, we observed whitish embryos in *Cas9* gene-transformed cotton (Figure 3J), whereas previous studies on normal cotton plants recorded a very light-yellow shade (Rao *et al*., 2006).

The presented study incorporated activated charcoal into both shooting and rooting media, unlike previous studies that did not include charcoal (Jin *et al*., 2005). This approach yielded well-developed shoots and roots in their respective media. The addition of acetosyringone (50–100 µM) to the co-cultivation medium has been reported to enhance transformation efficiency by two-to-threefold in Coker-312 and other cotton cultivars (Jin *et al*., 2005; Tohidfar *et al*., 2005). However, our study achieved the best transformation efficiency in Coker-312 using acetosyringone at 20 mg/L, demonstrating its potential to significantly improve stable cotton transformation.

The method employed in this study proved to be highly efficient and precise, achieving a transformation efficiency of 0.125%. This high transformation rate had the facilitation of using more virulent

Agrobacterium tumefaciens strains, specifically EHA101 and LBA4404, which have associations with higher transformation frequencies compared with GV3101. Among the 4,000 hypocotyls inoculated with all three strains, LBA4404 and EHA101 each achieved a 40% successful transformation rate, while GV3101 resulted in only a 20% transformation rate (Table 4).

Agrobacterium culture density plays a crucial role in transformation efficiency. Jin *et al*. (2005) reported a higher frequency of resistant calli at an *Agrobacterium* culture density (OD600) of 0.5, compared with densities of 1.0 and 1.5. In this study, we found an optimal culture density range of 0.5–0.8 yielded the highest transformation rates. Overall, this research provided a valuable tool for functional gene analysis in cotton and has potential applications in molecular breeding and genome editing, including both single and multiple genome editing.

CONCLUSIONS

This study presented a strategy for the effective and comprehensive gene transfer of the *Cas9* gene into cotton cv. Coker-312 using *Agrobacterium*-mediated transformation. The *Cas9* gene had a successful introduction into the Coker-312 cotton variety through the stable transformation of freshly cut hypocotyl explants using *Agrobacterium* sp. strains (GV3101, EHA101, and LBA4404). This research presented a streamlined, genotype-dependent strategy for producing genetically modified cotton cultivars using the CRISPR/Cas9 system, offering a valuable tool for advancing genetic studies and crop improvement in cotton.

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REFERENCES

Ahmar S, Gill RA, Jung KH, Faheem A, Qasim MU, Mubeen M, Zhou W (2020). Conventional and molecular techniques from simple breeding to speed breeding in crop plants: Recent advances and future outlook. *Int. J. Mol. Sci.* 21(7): 2590.

Garfinkel DJ, Nester EW (1980). *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. *J. Bacteriol*. 144(2):732–743.

Guruprasad M, Sridevi V, Vijayakumar G, Kumar MS (2016). Plant regeneration through callus initiation from mature and immature embryos of maize (*Zea mays* L.). *Indian J. Agric. Res*. 50 (2):135–138.

Harjeet K (2005). Studies on plant regeneration and *Agrobacterium*-mediated genetic transformation in cotton. Ph.D. Thesis, CCSHAU.

Höfgen R, Willmitzer L (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res.* 16(20): 9877.

Jakubovics NS, Smith AW, Jenkinson HF (2002). Oxidative stress tolerance is manganese (Mn^{2+}) regulated in *Streptococcus gordonii*. *Microbiol.* 148(10): 3255–3263.

Jin S, Zhang X, Liang S, Nie Y, Guo X, Huang C (2005). Factors affecting transformation efficiency of embryogenic callus of upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens*. *Plant Cell Tiss. Organ Cult.* 81: 229–237.

Jin SX, Liu GZ, Zhu HG, Yang XY, Zhang XL (2012). Transformation of upland cotton (*Gossypium hirsutum* L.) with *gfp* gene as a visual marker. *J. Integr. Agric.* 11(6): 910–919.

Juturu V, Gurramkonda C, Wu JC (2021). Biotechnological advances in cotton transformation: Current trends and future prospects. *Plant Cell Rep*. 40(12):2257–2271.

Kumar GP, Subiramani, S, Govindarajan S, Sadasivam V, Manickam V, Mogilicherla K, Narayanasamy J (2015). Evaluation of different carbon sources for high frequency callus culture with reduced phenolic secretion in cotton (*Gossypium hirsutum* L.) cv. SVPR-2. *Biotechnol. Rep.* (*Amst*). 7: 72–80.

Kumar M, Singh H, Shukla AK, Verma PC, Singh PK (2013). Induction and establishment of somatic embryogenesis in elite Indian cotton cultivar (*Gossypium hirsutum* L. cv Khandwa-2). *Plant Signal. Behav.* 8(10): e26762.

Kumria R, Sunnichan VG, Das DK, Gupta SK, Reddy VS, Bhatnagar RK, Leelavathi S (2003). High-frequency somatic embryo production and maturation into normal plants in cotton (*Gossypium hirsutum*) through metabolic stress. *Plant Cell Rep.* 21: 635–639.

Leelavathi S, Sunnichan VG, Kumria R, Vijaykanth GP, Bhatnagar RK, Reddy VS (2004). A simple and rapid *Agrobacterium*-mediated transformation protocol for cotton (*Gossypium hirsutum* L.): Embryogenic calli as a source to generate large numbers of transgenic plants. *Plant Cell Rep.* 22: 465–470.

Lin H, Han X, Feng X, Chen X, Lu X, Yuan Z, Yin Z (2022). Molecular traits and functional analysis of rapid alkalinization factors (RALFs) in four *Gossypium* species. *Int. J. Biol. Macromol.* 194: 84–99.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15(3): 473–397.

Noor W, Lone R, Kamili AN, Husaini AM (2022). Callus induction and regeneration in high-altitude Himalayan rice genotype SR4 via seed explant. *Biotechnol. Rep. 36:*e00762.

Perlak FJ, Oppenhuizen M, Gustafson K, Voth R, Sivasupramaniam S, Heering D, Roberts JK (2001). Development and commercial use of Bollgard® cotton in the USA–early promises versus today's reality. *Plant J.* 27(6): 489–501.

Rao AQ, Hussain SS, Shahzad MS, Bokhari SYA, Raza MH, Rakha A, Riazuddin S (2006). Somatic embryogenesis in wild relatives of cotton (*Gossypium* spp.). *.J. Zhejiang Univ. Sci. B* 7: 291–298.

Ribeiro TP, Lourenço-Tessutti IT, de Melo BP, Morgante CV, Filho AS, Lins CBJ, Grossi-de-Sa MF (2021). Improved cotton transformation protocol mediated by *Agrobacterium* and biolistic combined-methods. *Planta* 254(2): 20.

Saeed M, Naeem M, Alia J, Perveen S, Iqra S, Yousaf MZ, Chohan MSM, Riaz M, Sana U, Song X (2024). Characterization of water-deficit tolerance in upland cotton (*Gossypium hirsutum* L.) assessing morphological, biochemical, molecular and yield attributes. *Acta Physiol. Plant.* 46(1): 13.

Sarker KK, Kabir AH, Sharmin SA, Nasir Z, Alam MF (2007). Improved somatic embryogenesis using L-asparagine in wheat (*Triticum aestivum* L.). SJVOE6, 24(3-4): 187–196.

Shang HH, Liu CL, Zhang CJ, Li FL, Hong WD, Li FG (2009). Histological and ultrastructural observation reveals significant cellular differences between *Agrobacterium* transformed embryogenic and non-embryogenic calli of cotton. *J. Integr. Plant Biol.* 51(5): 456–465.

Sundar IK, Sakthivel N (2008). Advances in selectable marker genes for plant transformation. *J. Plant Physiol.* 165: 1698–1716.

Sunilkumar G, Rathore KS (2001). Transgenic cotton: Factors influencing *Agrobacterium*-mediated transformation and regeneration. *Mol. Breed. 8*(1):37–52.

Tohidfar M, Mohammadi M, Ghareyazie B (2005). *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a heterologous bean chitinase gene. *Plant Cell Tiss. Organ Cult.* 83: 83–96.

Wilkins TA, Rajasekaran K, Anderson DM (2000). Cotton biotechnology. *CRC Crit. Rev. Plant Sci.* 19(6): 511–550.

Wu J, Zhang X, Nie Y, Jin S, Liang S (2004). Factors affecting somatic embryogenesis and plant regeneration from a range of recalcitrant genotypes of Chinese cottons (*Gossypium hirsutum* L.). *In Vitro Cell Dev. Biol. Plant* 40: 371–375.

Yang S, Li G, Li M, Wang J (2011). Transgenic soybean with low phytate content constructed by *Agrobacterium* transformation and pollen-tube pathway. *Euphytica* 177: 375–382.

Zakaria AL, Ajlouni M, Shatnawi M, Shibli R, Makhdmeh I, Saeid ABU, Al-Ghazawi AL (2012). Callus induction, plant regeneration, and growth on barley (*Hordeum vulgare* L.). *South West J. Hortic. Biol. Environ.* 3: 25–39.

Zhang BH, Feng R, Liu F, Yao CB (1999). Direct induction of cotton somatic embryogenesis. *Chin. Sci. Bull.* 44: 766–767.