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The pBINPLUS Binary Overexpression Construct and a Somatic Embryogenesis Protocol for *Agrobacterium*-Mediated Transformation of Cotton (*Gossypium hirsutum* L.) cv. Coker-312

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A B S T R A C T

Transferring sesquiterpenoid biosynthetic genes into a high-biomass crop has been proposed as one route to broadening the production base for plant-derived pharmaceutical compounds, which are frequently obtained from low-yielding source species. Cotton is an attractive candidate host because of its biomass, its non-food end use and its established tissue-culture systems, yet it has received little attention in this context and is known to be recalcitrant in culture. This study reports the design of a binary overexpression construct carrying a sesquiterpene synthase coding sequence and its stable introduction into cotton. The 1,641 bp coding sequence was placed between a constitutive cauliflower mosaic virus 35S promoter and a Rubisco small-subunit terminator using XbaI and NcoI sites, and the cassette was assembled into the pBINPLUS backbone together with an nptII selectable marker, giving a construct of 15,709 bp. The construct was introduced into *Escherichia coli* DH5α and then into *Agrobacterium tumefaciens* LBA4404; colony PCR and restriction analysis confirmed its integrity at each step. Hypocotyl explants of cv. Coker-312 were co-cultivated with the recombinant bacterium, and transformants were recovered on kanamycin-containing media through callus induction, somatic embryogenesis and plantlet conversion. Control explants inoculated with sterile water did not survive selection. The complete cycle from explant to acclimatized plant occupied approximately eight to nine months, and two independent lines were confirmed by PCR and Sanger sequencing, with BLAST analysis indicating complete identity between the integrated and donor sequences. Artemisinin and its precursor arteannuin B were subsequently detected in leaf material of these lines by UHPLC-QTOF-MS, as we reported previously in two national-language publications; the present article provides the full English-language account of the construct design and of the transformation and regeneration protocol, with the expanded methodological detail required for the procedure to be reproduced.

Introduction

A number of plant-derived pharmaceutical compounds are sesquiterpene lactones obtained from source species in which they accumulate to low levels that vary with genotype, season and growing conditions. Supply and price are consequently unstable, and a considerable body of work has been directed at alternative routes of production (Rakhmanov *et al.*, 2025).

The branch is entered when farnesyl diphosphate, the common C_{15} precursor of plant sesquiterpenes, is cyclized by a dedicated synthase; the coding sequence used in this work corresponds to that step, and its 1,641 bp open reading frame matches the original characterization of the enzyme (Mercke *et al.*, 2000). Downstream oxidative and reductive steps complete the route to the final product, while precursor supply is governed by enzymes of general isoprenoid metabolism, overexpression of which has been shown to raise product content in the source species (Alam and Abdin, 2011).

Engineering efforts have followed two broad directions, reconstitution in microbial hosts and transfer of pathway genes into plants, the latter including the movement of a complete pathway into a high-biomass crop (Fuentes *et al.*, 2016). Neither has displaced field cultivation of the source species, and a recurring conclusion is that the host matters as much as the gene, since the precursor pool, compartmentation and biomass of the chassis set the ceiling on what any introduced enzyme can achieve.

Cotton has been largely overlooked here despite properties that recommend it. It produces substantial vegetative biomass that is currently of low value, it is a non-food crop, and it is among the better-established targets for *Agrobacterium*-mediated transformation following the first reports of transgenic plants in the species (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987) and the development of regeneration through somatic embryogenesis (Trolinder and Goodin, 1987). The limitation is that regeneration competence is strongly genotype-dependent and few genotypes respond in culture (Trolinder and Xhixian, 1989). Coker-312 has therefore become the reference genotype, with traits subsequently moved into commercial cultivars by conventional crossing, a route demonstrated for insect resistance (Perlak *et al.*, 1990) and for fibre-quality traits (Abdurakhmonov *et al.*, 2014). Protocol refinements have shortened the cycle and improved embryo conversion (Sunilkumar and Rathore, 2001; Leelavathi *et al.*, 2004; Khan *et al.*, 2010), but the process remains long.

In our earlier studies, published in Uzbek, we reported the construct and the recovery of transformed lines (Rakhmanov *et al.*, 2026a) and gave a structural and metabolomic treatment of the same material (Rakhmanov *et al.*, 2026b). Neither reported the culture system in a form that another laboratory could follow. The present work therefore sets out the protocol in full, with three objectives: to describe the design and assembly of the binary overexpression construct; to report the complete transformation and regeneration scheme, stage by stage, with the media, timings and conditions needed to reproduce it; and to document the recovery and molecular confirmation of transformed lines.

Materials and Methods

Plant material.

Seeds of *Gossypium hirsutum* L. cv. Coker-312, held in the germplasm collection of the Center of Genomics and Bioinformatics, were used throughout. The cultivar was selected for its capacity to regenerate whole plants from single somatic cells (Trolinder and Goodin, 1987). Seeds were delinted with concentrated sulphuric acid, surface-sterilized in 70 per cent ethanol, Tween-20 and 20 per cent sodium hypochlorite, and rinsed in sterile water. Ten seeds per vessel were germinated on solid Murashige and Skoog medium (Murashige and Skoog, 1962) for 10 days at 28 °C under an 8/16 h photoperiod, after which hypocotyls were excised as explants.

Design of the construct

Sequences were retrieved from the GenBank database of the National Center for Biotechnology Information and assembled in SnapGene 6.1 (GSL Biotech, USA). A 1,641 bp coding sequence of the early artemisinin branch (GenBank HQ315833.1) was used as the target; the length corresponds to the open reading frame described in the original characterization of the enzyme (Mercke *et al.*, 2000). Transcription was placed under the constitutive cauliflower mosaic virus 35S promoter (Odell *et al.*, 1985) and closed by the Rubisco small-subunit (T-RbcS) terminator. Universal XbaI (tctaga) and NcoI (ccatgg) sites were appended to the fragment ends for directional ligation, and the complete fragment was obtained by commercial gene synthesis. The pBINPLUS backbone was selected because it offers a higher copy

number in *E. coli* than pBIN19, places the selectable marker adjacent to the left T-DNA border, and carries rare restriction sites flanking the cloning region (van Engelen *et al.*, 1995). The assembled construct is 15,709 bp and carries, within the T-DNA borders, an nptII cassette under nopaline synthase regulatory sequences and the overexpression cassette described above ; the backbone contributes the oriV and pBR322 origins of replication and a bacterial nptIII marker for plasmid maintenance (Figure 1).

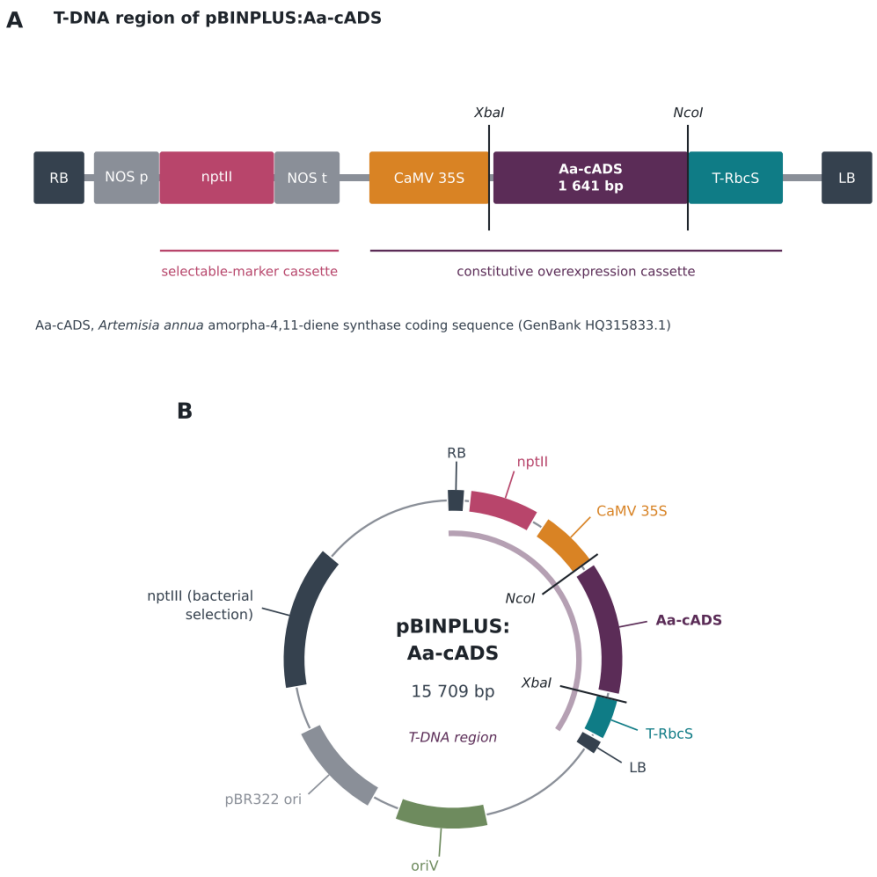


Figure 1. Organization of the overexpression construct. (A) T-DNA region, showing the order of elements between the right and left borders and the XbaI and NcoI sites flanking the target sequence; elements are shown in their designed order and are not drawn to scale. (B) Map of the complete construct (15,709 bp); the T-DNA region is indicated by the inner arc, and backbone elements comprise the origins of replication and the bacterial selection marker.

Bacterial strains

Competent cells of *Escherichia coli* DH5α and *Agrobacterium tumefaciens* LBA4404 were prepared by standard procedures. The construct was introduced first into *E. coli* DH5α, with transformants selected on solid Luria-Bertani medium containing kanamycin for 16 h at 37 °C. Plasmid DNA from verified colonies was then introduced into *A. tumefaciens* LBA4404. Colonies grown on yeast extract broth medium were screened by colony PCR with nptII-specific primers (nptII-F, 5′-GATAGCGGTCCGCCACAC-3′; nptII-R, 5′-CGAGGATCTCGTCGTGACACAT-3′), and plasmid DNA from positive colonies was subjected to restriction analysis. Plasmid DNA of the construct served as positive control; Coker-312 genomic DNA and a no-template reaction served as negative controls.

Transformation and regeneration

Recombinant *A. tumefaciens* was recovered from storage at −80 °C and grown in liquid YEP medium containing kanamycin (50 mg L^{-1}) and rifampicin (40 mg L^{-1}) for 3 days at 28 °C with shaking at 200 rpm. Cells were

pelleted, resuspended by vortexing in 10 mL of pre-induction medium (PIM), and held for a final 16 h at 4 °C in darkness with shaking. Hypocotyl explants were cut into 5–7 mm segments and placed on solid P1 medium (pH 5.8, 0.2 per cent phytagel). Cut surfaces were inoculated with 5–6 mL of the bacterial suspension; control explants received sterile water. Explants were then incubated in darkness at 22 °C for 3 days.

Explants were transferred to fresh P1 medium containing kanamycin (50 mg L^{-1}) and cefoperazone (500 mg L^{-1}) and cultured under a 16 h photoperiod for 21 days until callus formed. Calli of approximately 3 mm were moved to P7 medium (MS salts, myo-inositol, glucose, phytagel, pH 5.8) and subcultured monthly. After 13–16 weeks on selective P7 medium, calli were transferred to R5 induction medium, on which somatic embryos appeared after a further 12–16 weeks. Embryoids of 6–7 mm were transferred to SH-1 medium and incubated in darkness for 10 days; following desiccation and root initiation, embryos were moved to SH-2 medium under a 16 h photoperiod for 10 days, and then to SH-3 medium under increased light intensity (70 µmol m^{-2} s^{-1}) until four to five leaves and secondary roots had formed. Plantlets were transferred to soil and acclimatized in a non-sterile phytotron. The scheme is summarized in Figures 2 and 3.

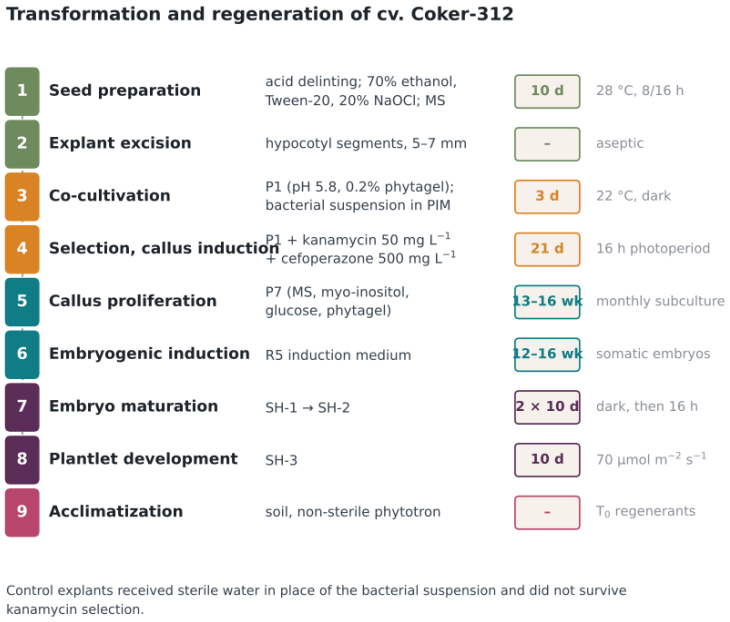


Figure.2 Sequence of culture stages used for Agrobacterium-mediated transformation and regeneration of cv. Coker-312, with the duration and principal conditions of each stage.

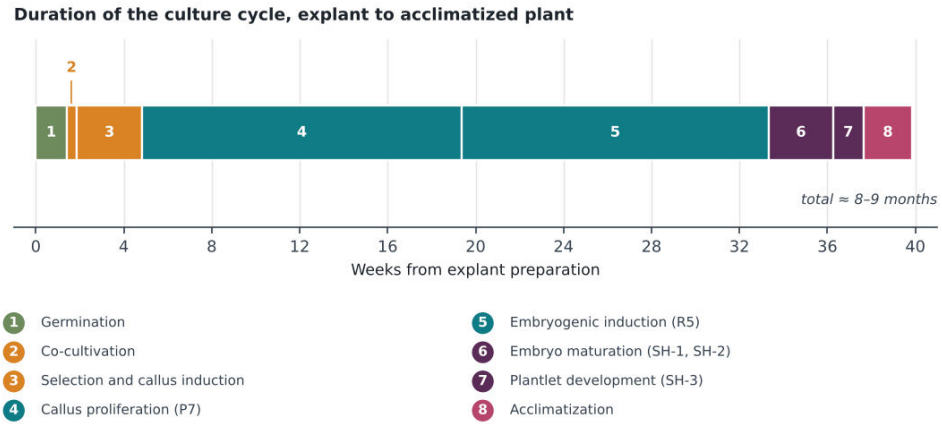


Figure 3. Duration of the culture cycle from explant preparation to acclimatized plant. Bars are drawn to scale on the week axis; numbers correspond to the stages listed in the key.

Photographic documentation.

Cultures were photographed at each stage. The images are the authors' own and document the material described in our earlier Uzbek-language reports (Rakhmanov *et al.*, 2026a, 2026b).

Confirmation of transformants.

Genomic DNA was extracted from leaves of putative transformants and controls. Integration was assessed by PCR with insert-specific primers (forward, 5′-GGGGTGGAACAGATAGTGAATG-3′; reverse, 5′-GCATGATCAATCTCCCGTTC-3′), followed by digestion with HindIII and separation on 0.9 per cent agarose gels. PCR products from T_0 regenerants were purified and sequenced with the ABI PRISM BigDye Terminator kit, and reads were compared with the donor sequence using BLAST.

Results and Discussion

Architecture of the construct

The assembled construct is 15,709 bp (Figure 1). Within the T-DNA region the elements are arranged in the order right border, nptII cassette under nopaline synthase regulatory sequences, CaMV 35S promoter, the 1,641 bp coding sequence, T-RbcS terminator and left border. The XbaI and NcoI sites flanking the coding sequence were retained in the final construct, which was a deliberate choice: they allow the coding region to be exchanged in subsequent work without rebuilding the vector.

Three features of the design merit comment. First, a constitutive promoter was used. The 35S promoter directs high-level expression in most organs of transgenic plants and shows little tissue specificity (Odell *et al.*, 1985), which suits a first-generation construct intended to establish that the sequence is transcribed in the host at all. It is also, for that reason, unlikely to be optimal: in the source species the pathway is confined to glandular trichomes, and a tissue-preferential promoter is the obvious refinement for a second-generation construct.

Second, placement of the selectable marker adjacent to the left border, a feature of the pBINPLUS backbone (van Engelen *et al.*, 1995), has a practical consequence. Because T-DNA transfer proceeds from the right border, a marker at the left border is transferred last, so plants surviving selection are more likely to carry the complete insert rather than a truncated fragment. Third, the rare restriction sites of the backbone were retained, which will facilitate analysis of insertion junctions in later work.

Transfer to Agrobacterium

Introduction of the construct into *E. coli* DH5α and then into *A. tumefaciens* LBA4404 proceeded without difficulty. Colony PCR with nptII-specific primers gave the expected product in the screened *Agrobacterium* colonies, matching the amplicon from the plasmid positive control, while no product was obtained from Coker-312 genomic DNA or from the no-template reaction. Restriction analysis of plasmid DNA from positive colonies was consistent with the designed map. These checks establish that the construct was maintained intact in the bacterial host, which is worth confirming rather than assuming, since large binary constructs can rearrange during transfer between strains.

Regeneration through somatic embryogenesis

Explants co-cultivated with the recombinant *Agrobacterium* and transferred to kanamycin-containing P1 medium produced callus within 21 days. Control explants inoculated with sterile water did not survive selection, confirming that kanamycin was an effective primary screen at the concentration used. Calli transferred to P7 medium and subcultured monthly proliferated over 13–16 weeks before transfer to R5 induction medium, on which somatic embryos appeared after a further 12–16 weeks. Embryoids converted to plantlets through the SH-1 to SH-3 sequence and were acclimatized in soil (Figures 2 and 3). The morphology of the cultures at each stage is shown in Figure 4.

Three transitions in the sequence proved the most demanding in practice and are worth describing in detail, since they are where material is most often lost. The first is the shift from proliferating callus to embryogenic callus. Callus that remains friable, translucent and pale does not go on to form embryos; the tissue that does is firmer, more compact and yellow-green, with a granular surface (Figure 4B, C). Selecting this fraction at each monthly subculture, and discarding the rest, was more effective than attempting to induce the whole mass. The second is embryo maturation, where the dehydration step preceding transfer to SH-1

determines how many embryoids convert rather than arrest; embryoids moved before 6 mm converted poorly in our hands. The third is acclimatization, where plantlets with four to five expanded leaves and visible secondary roots (Figure 4E) transferred to soil far more reliably than smaller material, however healthy the latter appeared in vitro. The morphology at each stage is shown in Figure 4: explants bearing proliferating tissue at the cut surfaces on P1 (Figure 4A), nodular callus on selective P7 (Figure 4B), somatic embryoids at the stage of transfer (Figure 4C), germinating embryos on SH-1 (Figure 4D), a rooted plantlet in the culture vessel (Figure 4E) and an acclimatized plant in soil (Figure 4F).

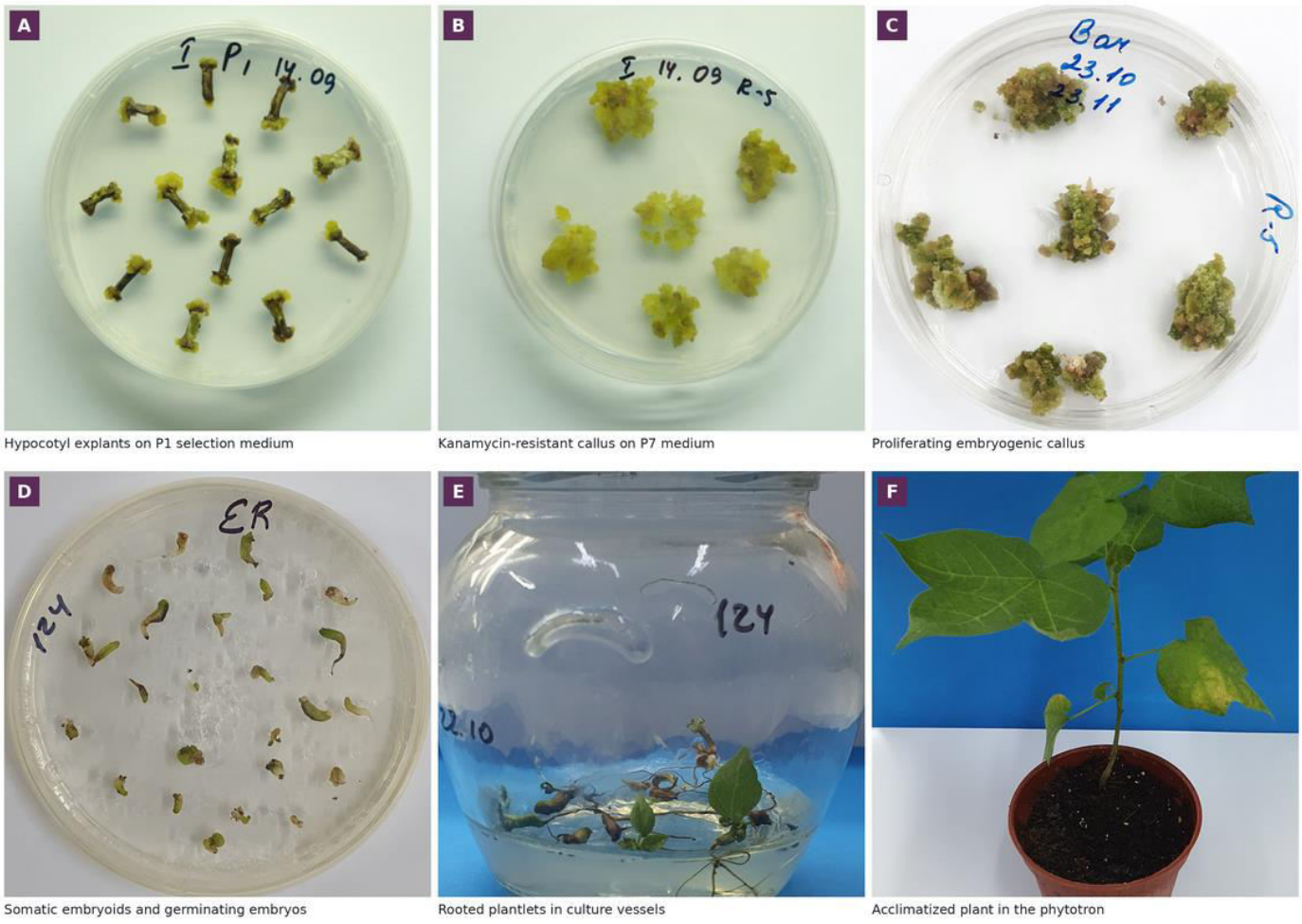


Figure 4. Stages of transformation and regeneration of cv. Coker-312. (A) Hypocotyl explants on P1 selection medium. (B) Kanamycin-resistant callus on P7 medium. (C) Proliferating embryogenic callus; note the firmer, granular, yellow-green tissue that goes on to form embryos. (D) Somatic embryoids and germinating embryos. (E) Rooted plantlets in culture vessels. (F) Acclimatized plant in the phytotron. Photographs are the authors' own and record the cultures from which the lines described in our earlier studies (Rakhmanov et al., 2026a, 2026b) were derived; panels have been cropped and assembled for presentation, with no adjustment of tone or colour.

The two long stages, callus proliferation and embryogenic induction, together account for more than two-thirds of the cycle (Figure 3). The total of roughly eight to nine months from explant to acclimatized plant falls within the range reported for Coker-312 but towards its slower end; protocols starting from embryogenic callus or imposing controlled metabolic stress have reported shorter cycles (Leelavathi *et al.*, 2004; Khan *et*

al., 2010). This is a real constraint rather than a presentational one, and it shapes what is feasible in a programme of this kind. Where each transformation cycle occupies the better part of a year, the number of independent events that can realistically be screened is small, and the value of recovering well-characterized events from each cycle rises accordingly. Media composition and the dehydration regime during embryo maturation are the variables most often identified as limiting (Sunilkumar and Rathore, 2001), and they are the obvious targets for shortening the cycle.

It should also be said that genotype remains the dominant factor. Coker-312 was chosen precisely because it responds in culture, and the regeneration scheme reported here cannot be assumed to transfer to commercial Upland cultivars, most of which are recalcitrant (Trolinder and Xhixian, 1989). The established route is to transform the responsive genotype and then move the trait by conventional crossing, as has been done for insect resistance (Perlak *et al.*, 1990) and for fibre quality in locally adapted cultivars (Abdurakhmonov *et al.*, 2014). That is the route anticipated for the lines described here.

Molecular confirmation

Two independent lines were taken forward. PCR with construct-specific primers gave the expected products in both lines and not in the regenerated non-transformed control. Sanger sequencing of the amplicons, followed by BLAST comparison, indicated complete identity with the donor coding sequence. The absence of sequence change is the expected outcome for a synthesized cassette delivered through *Agrobacterium*, but it is worth verifying, since the construct passed through two bacterial hosts and an extended culture period before recovery.

Taken together,

These results establish that the construct was assembled as designed, remained intact through two bacterial hosts, and integrated into the cotton genome in two independent lines recovered through somatic embryogenesis.

The metabolic consequences of the introduction have been examined separately and are not repeated here. In our earlier studies, untargeted UHPLC-QTOF-MS profiling of leaf extracts, prepared by the extraction and identification procedures we described previously (Rakhmanov *et al.*, 2024), detected the target sesquiterpenoid and its precursor arteannuin B in the transformed lines (Rakhmanov *et al.*, 2026a, 2026b), which indicates that the introduced sequence is not only transcribed but gives rise to detectable product in the heterologous host. Those analyses, together with transgene copy number and transcript quantification, are the subject of a separate and more detailed account, and readers are referred to our earlier reports for the primary data. The present manuscript is confined to the construct and the culture system.

Two caveats are worth stating. Detection of a compound establishes its presence but not the yield at which it accumulates, and quantitative evaluation across generations and growing conditions remains to be completed. Further, in a host that does not normally support high sesquiterpene flux, precursor supply rather than enzyme abundance may prove limiting, which is the constraint that upstream engineering is intended to address (Alam and Abdin, 2011); experience with pathway transfer to other hosts indicates that multi-gene approaches are generally required before output becomes agronomically meaningful (Fuentes *et al.*, 2016).

A binary overexpression construct of 15,709 bp carrying a sesquiterpene synthase coding sequence was designed in silico, assembled in the pBINPLUS backbone and introduced into *Gossypium hirsutum* L. cv. Coker-312 by *Agrobacterium*-mediated transformation. Two independent lines were recovered through somatic embryogenesis and confirmed by PCR and Sanger sequencing. The length of the culture cycle, approximately eight to nine months, and the small number of independent events recovered remain the principal practical constraints of the system. In our earlier studies, metabolomic analysis of the same material detected the target sesquiterpenoid and arteannuin B in the transformed lines (Rakhmanov *et al.*, 2026a, 2026b). The construct and the regeneration scheme described here provide a working platform for sesquiterpenoid-pathway engineering in cotton, in which the addition of precursor-supply and downstream oxidative genes, the substitution of the constitutive promoter by a tissue-preferential one, and quantitative evaluation of product accumulation are the priorities for further work..

Author Contributions

Rakhmanov B.K.: transformation and tissue culture, molecular confirmation, writing of the original draft.

Buriev Z.T.: supervision of the project, review and editing. Ubaydullayeva Kh.A.: transformation and tissue culture. Usmanov D.E.: investigation, review and editing. Shermatov Sh.E.: methodology, review and editing. Abdurakhmonov I.Y.: conceptualization, project administration, supervision. All authors read and approved the final manuscript.

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Data Availability

Data generated during the current study may be made available by the corresponding author upon request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish All authors consent to the publication of this manuscript.

Conflict of Interest The authors declare no competing interests.

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