

A Histone Demethylase GbJMJ25 Regulates Somatic Embryogenesis Cotton by Modulating ROS

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Abstract

Somatic embryogenesis in *Gossypium barbadense* is highly genotype-dependent, which constrains its application in cotton molecular breeding. The formation of primary embryogenic cells from black-brown non-embryogenic callus represents a critical step in this process. Among the genes significantly differentially expressed during this transition, we identified a histone demethylase gene, *GbJMJ25*. In this study, we demonstrate that the emergence of primary embryogenic cells is accompanied by pronounced changes in cellular redox status, concurrent with the down-regulation of *GbJMJ25* expression. Quantitative PCR analysis indicated that *GbJMJ25* expression is modulated by abiotic stress factors such as ABA and PEG. Silencing *GbJMJ25* enhanced peroxidase accumulation and maintained lower intracellular ROS levels in cotton plants under abiotic stress. In non-embryogenic callus of *G. barbadense*, knockdown of *GbJMJ25* promoted the accumulation of anthocyanins, SOD, and CAT, thereby facilitating the conversion to embryogenic cells. Nevertheless, this transition remained dependent on auxin supplementation, suggesting that auxin induces substantial intracellular changes—including rendering cells into a “stressed” state—during the shift from non-embryogenic to embryogenic cells. By regulating cellular ROS homeostasis, *GbJMJ25* appears to influence cell survival and subsequent differentiation. In summary, our findings indicate that modulation of *GbJMJ25* expression can enhance plant regeneration through the somatic embryogenesis system, offering a candidate gene for improving genetic transformation techniques mediated by somatic embryogenesis in *G. barbadense*.

Key message

This study identifies the histone demethylase as an epigenetic switch that fine-tunes redox homeostasis to break genotype-dependent constraints on cotton somatic embryogenesis and plant regeneration.

Introduction

Gossypium barbadense (Sea Island or extra-long staple cotton) is renowned for producing the world's highest-quality natural fibers and represents a vital strategic resource for high-end textiles (Meng et al., 2025). A key objective in cotton breeding is to develop *G. barbadense* cultivars with improved stress tolerance, superior fiber quality, and adaptability to mechanical harvesting. The application of biotechnology to achieve these goals often depends on efficient genetic transformation, which in turn requires a robust plant regeneration system based on somatic embryogenesis.

However, the establishment of such regeneration systems in cotton remains constrained by strong genotype dependence. Somatic embryogenesis is influenced by multiple factors, including phytohormones, developmental gene regulation, epigenetic modifications, and reactive oxygen species (ROS) homeostasis. Notably, abiotic stress can promote the transition of somatic cells into embryos, and redox balance plays a pivotal regulatory role (Grafi and Barak, 2015; Fehér, 2015; Tognetti et al., 2017). Stress-induced ROS and nitric oxide (NO) affect plant development and interact with key hormone networks governing meristem formation (Su et al., 2017; Cui et al., 2015; Diaz-Vivancos et al., 2015;

Schaller et al., 2015). Key regulators such as RADICAL-INDUCED CELL DEATH1 (RCD1) modulate stress responses and development, and *OsBOC1* reduces ROS-mediated callus browning and improves transformation efficiency (Kun et al., 2020). Plants employ specific developmental regulators (e.g., PLT) to activate the evolutionarily conserved autophagy pathway, thereby regulating ROS levels and organelle turnover, which ultimately promotes stem cell fate transition and organ regeneration (Ganguly et al., 2026). In cotton, a transcriptional cascade involving *GhRCD1* temporally regulates ROS accumulation to influence cell fate during embryogenesis (Yuan et al., 2023).

Concurrently, the somatic-to-embryonic transition involves chromatin-level reprogramming (Grafi and Barak, 2015). The cotton cultivar Lumian 1, which has very low embryogenic capacity (<10%), exhibits high CHH-type DNA methylation, whereas the cultivar Yumian 1 with high embryogenic capacity (>80%), shows low CHH-type DNA methylation. Herein, it indicates the importance of genotype-dependent methylation patterns for somatic embryo differentiation capacity in cotton (Guo et al., 2020). And histone modifications, particularly H3K9 methylation, are dynamically regulated by Jumonji C (JMJ) domain-containing demethylases (Li et al., 2021; Lu et al., 2008). JMJ's enzyme activity depends on Fe²⁺ and α -ketoglutarate (α -KG), a tricarboxylic acid (TCA) cycle intermediate produced by α -ketoglutarate dehydrogenase (KGDH) (Klose and Zhang, 2007; Salminen et al., 2014). Intriguingly, α -KG promotes stem cell self-renewal by sustaining epigenetic modifiers, while its derivative succinate favors cell differentiation (Carey et al., 2015; Zhang et al., 2019). And KGDH is also a key node in cellular ROS homeostasis (Lain et al., 2011). Therefore, a potential link where α -KG and KGDH may coordinate ROS levels with JMJ-mediated epigenetic activity to jointly regulate cell differentiation processes.

In the study, we identified a histone demethylase gene, *GbJMJ25*, associated with primary embryogenic callus initiation in *G. barbadense* with Virus-Induced Gene Silencing (VIGS) and antisense RNA technology. Our work investigates the impact of *GbJMJ25* expression on the ROS status in cotton and elucidates its role in the formation of primary embryogenic callus.

Materials and methods

1.1. Plant Materials

Plant materials included primary embryogenic callus of the cotton (*G. barbadense*) cultivar Xinluzao 47 (XLZ 47) and hypocotyls of the cultivar Jin668. All materials were pre-cultured and subsequently maintained in our laboratory.

1.2. Experimental Methods

1.2.1. Primer Design and Bioinformatics Analysis

The nucleotide sequence of the *GbJMJ25* gene (Accession No.: GB_A12G1572) was retrieved from the CottonGen database. Preliminary sequence analysis was performed using DNAMAN software. Gene-specific primers were designed with Primer Premier 5 software following the study design. Bioinformatics analysis was conducted as follows: the coding sequence was translated using DNAMAN to predict the amino acid sequence. Conserved domains were identified using the online SMART tool to

determine functional domain composition. The deduced *GbJMJ25* amino acid sequence was aligned with other JMJ protein sequences from the NCBI database and members of the *Arabidopsis thaliana* JMJ family using MEGA5 software, which was also employed to construct a phylogenetic tree. Putative *cis*-acting elements in the *GbJMJ25* promoter region were predicted and analyzed using the online PlantCARE software.

1.2.2. Sample Treatment and RNA Extraction

Samples were collected from various tissues of *G. barbadense*, including root, stem, leaf, non-embryogenic callus, embryogenic cells, globular embryos, torpedo embryos, and cotyledonary embryos. For stress treatments, the plants were treated with 100 μ M abscisic acid (ABA), 100 μ M indole-3-acetic acid (IAA), or 10% PEG-6000. Samples were collected at 0, 3, 6, 9, 12, and 24 hours post-treatment. For the virus-induced gene silencing (VIGS) system, cotton plants or callus exhibiting the albino phenotype (pTRV2::CLA1) were sampled. For antisense RNA experiments, primary embryogenic callus from both the experimental group (induced with 30 mg/L dexamethasone, DEX) and the non-induced control group were sampled upon showing the embryogenic phenotype. Total RNA was extracted from all samples using the Biospin Plant Total RNA Extraction Kit (DNA-free)(BioFlux, China) for subsequent use.

1.2.3. Quantitative Real-time PCR (qRT-PCR)

cDNA was synthesized as template with *TransScript*[®] First-Strand cDNA Synthesis SuperMix kit (Cat# AT301-02, TransGen Biotech, China) and qRT-PCR was performed with the TransGen Biotech qRT-PCR Kit (Cat#AQ211-01, TransGen Biotech, China), following the manufacturer's instructions. The gene sequences of the cotton *GbJMJ25* gene (GB_A12G1572), the reactive oxygen species scavenging-related gene *GbSOD1* (GB_A13G2043), and the apoptosis inhibitory gene *GbBI-1* (GB_A03G0096) were obtained by searching the cotton database website (<https://www.cottongen.org/>). Fluorescent quantitative primers (*GbJMJ25*-qF/R; *GbSOD1*-qF/R; *GbBI-1*-qF/R) were designed using Oligo 7 software, with the cotton *UBQ7* gene (*UBQ7*-F/R) used as the reference gene (Table S1). Relative expression levels of the target gene were calculated using the $2^{-\Delta\Delta C_t}$ method. Three biological replicates were analyzed for each sample. Data were statistically analyzed and visualized using GraphPad Prism 8.0.1 software.

1.2.4. Construction of Plant Expression Vectors

The fusion expression vector pCAMBIA1304::GbJMJ25::GFP was constructed using seamless cloning with inserted *Nco* I and *Spe* I restriction sites. The silencing vector pTRV2::GbJMJ25 was constructed via seamless cloning using *Eco*R I and *Bam*H I sites, targeting a 1124-bp specific fragment of *GbJMJ25*. The antisense expression vector pTA7002::GbJMJ25 was constructed using seamless cloning with *Xho* I and *Spe* I sites. The resulting recombinant vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 via the freeze-thaw method for subsequent applications.

1.2.5. Subcellular Localization

Agrobacterium strains carrying pCAMBIA1304::GbJMJ25::GFP were infiltrated into leaves of 30-day-old *Nicotiana benthamiana* plants, following a reported procedure (Bian et al., 2023). After 48~72 hours of cultivation, GFP signals were observed using a confocal laser scanning microscope.

1.2.6. Cytological Identification and Physiological Index Measurement

Primary embryogenic callus was dissociated in a pre-warmed (55°C) dissociation solution (95% ethanol:hydrochloric acid = 1:1) in a water bath for 5~10 min, according to Yang (2021). The solution was discarded, and the tissue was rinsed 3~5 times with sterile water. Samples were mounted on slides, stained with modified carbol fuchsin for 3~5 min, rinsed, dried, and gently pressed under a coverslip to disperse cells. Observations and imaging were performed under an optical microscope. Simultaneously, primary embryogenic callus or the second true leaf was immersed in BCIP/NBT staining solution (Cat#SK2030, Coolaber Biotech, China), Trypan Blue staining solution (Cat#SL7121, Coolaber Biotech, China) or DAB staining solution (Cat#SK1815L, Coolaber Biotech, China) for 3~5 min or 2~3 min, respectively. Staining solutions were removed with a pipette, and residual stain was washed off with sterile water. Tissue samples were then mounted for direct photography or observation and documentation under a light microscope.

For the determination of ROS-related physiological indices, 0.1 g of plant leaves, embryogenic cell tissue, or non-embryogenic callus tissue was weighed and homogenized in 1 mL of extraction buffer using an ice bath. The homogenate was centrifuged at 8000 × g and 4 °C for 10 min, and the supernatant was kept on ice until analysis. Assays were performed according to the instructions of Superoxide Dismutase (SOD) Activity Assay Kit (Cat: BC0170, Solarbio, China), Malondialdehyde (MDA) Content Assay Kit (Cat: BC0020, Solarbio, China), and the Anthocyanin Content Assay Kit (Cat#BC1380, Boxbio, China). For chlorophyll quantification, samples were decolorized by soaking in a mixture of acetone: absolute ethanol (2:1, v/v). The absorbance of the extract was measured at 663 nm and 645 nm using a microplate reader, and chlorophyll content was calculated with the following formula: **Total Chlorophyll Content (mg/g) = 8.02 × OD₆₆₃ + 20.2 × OD₆₄₅**.

1.2.7. *Agrobacterium*-Mediated Infection of Cotton Tissues

For the VIGS system in plants, following Tian (2024), an *Agrobacterium* culture containing pTRV1 was mixed 1:1 with cultures containing either pTRV2, pTRV2::CLA1, or pTRV2::GbJMJ25. The mixture was infiltrated into cotyledons of cotton seedlings at the two-true-leaf stage. Plants exhibiting the albino phenotype (pTRV2::CLA1) were photographed 15 days post-inoculation. For callus infection, the same bacterial strains were used. Infection conditions were as follows: bacterial cultures were adjusted to 0.5 at OD₆₀₀, vacuum pressure was set at 0.06 MPa, and infected cells were washed once with sterile water followed by twice with 0.2 mg/mL cefotaxime solution. When pTRV2::CLA1-infected callus turned albino from its original yellowish-green color, it was transferred to medium containing NAA for embryogenic cell induction. For hypocotyl infection, performed as described by Tuerhong (2024), *Agrobacterium* was activated to 0.5 at OD₆₀₀ and resuspended in a solution containing 10 mmol/L MgCl₂, 10 mmol/L MES, and 200 μmol/L acetosyringone. The suspension was kept at room temperature in the dark for 3 h, subjected to vacuum infiltration at 0.04 MPa for 5 min, followed by ultrasonic treatment at 40 kHz for 45 s. After co-cultivation in the dark on MS medium for 1 day, tissues were transferred to medium with or without DEX for continued culture and observation.

1.2.8. Physiological Assays and Data Analysis

Assays for Superoxide Dismutase (SOD) activity, Malondialdehyde (MDA) content, and Anthocyanin content were performed strictly according to the respective manufacturer's instructions (Solarbio Life Science, Beijing, China; Kits BC0170, BC0020, and BC1380). For chlorophyll content, approximately 1 g of tissue was immersed in an acetone: absolute ethanol (2:1) mixture for decolorization. Absorbance of the extract was measured at 663 nm and 645 nm using a microplate reader. Total chlorophyll content (mg/g) was calculated using the formula: $(8.02 \times A_{663}) + (20.2 \times A_{645})$. Three biological replicates were analyzed per treatment. Data processing was performed using Excel software to calculate means and standard errors. Statistical significance was assessed using SPSS software.

Results

2.1. A Comparative Analysis of Embryogenic and Non-embryogenic Callus Implicates Cellular Redox State Regulation in Cotton Somatic Embryogenesis

To elucidate the cytological basis of primary embryogenic callus formation, we conducted a systematic comparative analysis between embryogenic callus (EC) and non-embryogenic callus (NEC). Primary embryogenic callus cells originate from within black-brown non-embryogenic callus, a transformation process likely profoundly influenced by the cellular physiological state. Cytological observations revealed significant morphological differences between the two: as shown in Fig. 1A, embryogenic callus cells were small in volume with large and distinct nuclei, exhibiting typical embryogenic cell characteristics. In contrast, non-embryogenic callus cells were larger with less distinct nuclear structures. Further viability assays showed that NEC had a higher proportion of dead cells and lower overall cell viability compared to EC (Fig. 1A). Consistent with this, the expression level of the anti-apoptotic gene *Bcl-1* was significantly higher in EC than in NEC (Fig. 1B), providing molecular evidence for their viability differences. Comparison of oxidative stress status revealed noticeable hydrogen peroxide accumulation in NEC (indicated by light brown DAB staining), whereas EC showed only weak staining signals (Fig. 1A). Concurrently, EC demonstrated higher expression levels of the peroxide-scavenging gene *SOD1* (Fig. 1B), along with significantly increased superoxide dismutase activity and anthocyanin content, while malondialdehyde content was lower than in NEC (Fig. 1C). This series of results collectively indicates that embryogenic callus possesses stronger antioxidant capacity than non-embryogenic callus. Particularly noteworthy is that the expression of the *GbJMJ25* gene was significantly lower in EC than in NEC (Fig. 1B). Considering the aforementioned differences in cell viability and redox status, this expression pattern suggests that *GbJMJ25* may play an important role in the regulatory network governing primary embryogenic cell initiation. Its downregulated expression might help maintain an appropriate cellular redox balance, thereby promoting the establishment of the embryogenic state.

2.2. *GbJMJ25* Encodes a Conserved Nuclear Protein with RING and JmjC Domains

To elucidate the molecular characteristics and potential function of the *GbJMJ25* gene, we conducted analyses of its encoded protein sequence, including domain identification, phylogenetic relationship analysis, and subcellular localization studies. The *GbJMJ25* protein consists of 1055 amino acids. Structural analysis revealed that it contains a typical RING domain (located at amino acids 292-377),

which is often associated with E3 ubiquitin ligase activity, and a JmjC domain (located at amino acids 710-1012) (Fig. 2A). The JmjC domain is a characteristic functional domain of the histone demethylase family, suggesting that *GbJMJ25* may be involved in epigenetic regulation. Phylogenetic analysis indicated that *GbJMJ25* is most closely related to its homologs in other *Gossypium* species (*GhJMJ25* from *G. hirsutum*, *GrJMJ25* from *G. raimondii*, and *GaJMJ29* from *G. arboreum*), suggesting that this gene is relatively conserved during cotton evolution. Further clustering analysis with the *Arabidopsis thaliana* JMJ gene family showed that *GbJMJ25* groups closely with *AtJMJ25* within the same clade that includes *AtJMJ24/26/27/28/29*; this clade belongs to the KDM3/JHDM2 subfamily (Fig. 2B). This result, from an evolutionary perspective, supports the notion that *GbJMJ25* may function as a histone demethylase. To determine the subcellular distribution of this protein, we constructed a *GbJMJ25::GFP* fusion expression vector and performed transient transformation of epidermal cells from *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated infiltration. Observations using confocal laser scanning microscopy revealed that in cells transfected with the empty vector control (CK), GFP signals were distributed in both the cell membrane and the nucleus. In contrast, in cells transfected with *GbJMJ25::GFP*, GFP signals were specifically localized to the nucleus (Fig. 2C). This result directly confirms that the *GbJMJ25* protein is a nuclear-localized protein, which is consistent with its bioinformatically predicted function as a histone demethylase. This provides key evidence for its role in chromatin modification and gene expression regulation within the nucleus.

2.3. Expression Pattern Analysis Implicates *GbJMJ25* in Cotton Growth, Development, and Stress Responses

To investigate the physiological function of *GbJMJ25*, we analyzed the 2000-bp promoter sequence upstream of the start codon (ATG). Using the PlantCARE tool, we predicted the presence of multiple cis-regulatory elements in this promoter region. These elements include light-responsive components (such as ACE, AE-box, and G-Box), phytohormone-related elements (ABRE and TGA-element), stress-responsive elements (LTR, TC-rich repeats, and ARE), as well as development-associated elements (O2-site, HD-Zip 1, and GCN4_motif) (Fig. 3A). Expression profiling revealed that the transcript levels of *GbJMJ25* were highest during the non-embryogenic callus stage of somatic embryo development. Furthermore, its expression in roots was significantly higher than in stems and leaves (Fig. 3B). Following treatment with IAA or PEG-6000 (which simulates drought stress), the expression of *GbJMJ25* increased over time, peaking at 24 hours post-treatment. Under ABA treatment, its expression initially decreased before rising again, also reaching a maximum at 24 hours (Fig. 3B). These findings suggest that *GbJMJ25* is involved in regulating cotton growth, development, and stress responses.

2.4. Silencing *GbJMJ25* Enhances Drought Tolerance in Cotton Seedlings

Previous studies have indicated that α -ketoglutarate dehydrogenase (KGDH), regulated by reactive oxygen species (ROS), can inhibit the activity of histone demethylase (JMJ) enzymes by competitively consuming their common substrate, α -ketoglutarate. This mechanism is involved in the cellular ROS stress response (Huang et al., 2023). Our earlier analysis revealed that the promoter region of *GbJMJ25* contains abscisic acid (ABA)-responsive elements, suggesting a potential role for this gene in drought response. To test this hypothesis, we specifically silenced *GbJMJ25* in *G. barbadense* using virus-induced

gene silencing (VIGS). 2 weeks post-inoculation, positive control seedlings injected with pTRV2::CLA1 exhibited an albino phenotype (Fig. 4A), confirming the effective operation of the VIGS system. Concurrently, the expression level of **GbJMJ25** was significantly reduced in plants injected with pTRV2::GbJMJ25 compared to those injected with the empty vector control pTRV2 (Fig. 4B), verifying successful gene silencing. Under well-watered conditions, no obvious phenotypic differences were observed between the silenced plants and the controls (Fig. 4E). However, after 20 days of drought treatment, control plants showed severe leaf wilting, with a final survival rate of only 37.7%. In contrast, **GbJMJ25**-silenced plants achieved a survival rate of 65% (Fig. 4C). Further physiological assays revealed that under drought stress, the accumulation of O_2^- , H_2O_2 , and malondialdehyde (MDA) in control leaves was significantly higher than in silenced plants (Fig. 4D). Correspondingly, silenced plants maintained higher levels of superoxide dismutase (SOD) activity and anthocyanin content (Fig. 4F). These results collectively demonstrate that silencing **GbJMJ25** enhances drought tolerance in cotton. The underlying mechanism is likely associated with an overall improvement in the plant's antioxidant capacity. On one hand, the reduction in **GbJMJ25** expression may indirectly affect the substrate competition between KGDH and JMJ enzymes, thereby modulating the metabolic flux of α -ketoglutarate. On the other hand, its silencing may directly or indirectly activate intracellular ROS scavenging systems, maintaining redox homeostasis and ultimately mitigating oxidative damage caused by drought stress.

2.5. Silencing **GbJMJ25** Promotes Somatic Embryogenesis in Cotton Callus

To investigate the direct role of **GbJMJ25** in cotton somatic embryogenesis, we employed *Agrobacterium*-mediated VIGS on cotton non-embryogenic callus. After culturing the infiltrated callus on phytohormone-free medium for approximately 40 days, compared to the pTRV2 empty vector control, callus treated with pTRV2::CLA1 (a magnesium chelatase subunit) exhibited obvious whitening and a significant reduction in chlorophyll content, indicating the effective operation of the VIGS system at the callus level (Fig. 5A). Gene expression analysis further confirmed that the expression of both the **CLA1** gene in pTRV2::CLA1-treated cells and **GbJMJ25** in pTRV2::GbJMJ25-treated cells was significantly suppressed (Fig. 5B), successfully establishing the gene silencing system within the callus. On hormone-free medium, **GbJMJ25**-silenced callus did not show obvious phenotypic differences compared to the control group. However, a striking divergence emerged when they were transferred to medium supplemented with 0.3 mM naphthaleneacetic acid (NAA), an exogenous auxin. After approximately 50 days of culture, the **GbJMJ25**-silenced group produced abundant embryogenic cells, achieving a somatic embryogenesis frequency of about 45.71%, whereas the frequency in the control group was only 4.17% (Fig. 5A). This result clearly demonstrates that silencing **GbJMJ25** effectively promotes the initiation of embryogenic cells, but this promotive effect depends on exogenous auxin signaling. To further dissect the underlying mechanism, we examined the expression of related antioxidant genes and corresponding physiological indicators. We found that the expression levels of the peroxide-scavenging genes **GbSOD1** and **GbCAT** in **GbJMJ25**-silenced embryogenic callus were not only significantly higher than in the control callus but also higher than in the primary embryogenic callus that had not undergone *Agrobacterium* infiltration (Fig. 5B). Consistently, the **GbJMJ25**-silenced embryogenic

callus exhibited higher superoxide dismutase (SOD) and catalase (CAT) activities, higher anthocyanin content, and lower malondialdehyde (MDA) levels (Fig. 5C). Together, these data lead to the conclusion that silencing ***GbJMJ25*** enhances the cellular peroxidase system, thereby creating an intracellular environment with lower reactive oxygen species (ROS) levels. This low oxidative stress state may facilitate exogenous auxin-induced cell fate reprogramming by reducing oxidative damage, altering the epigenetic modifications or signaling of key developmental genes, ultimately significantly promoting the occurrence of cotton somatic embryogenesis.

2.6. Repressing *GbJMJ25* Expression Promotes Plant Regeneration in Cotton

To apply the molecular mechanism mediated by *GbJMJ25* to cotton genetic transformation in practice, the research team utilized antisense RNA technology to specifically inhibit the expression of *GbJMJ25*. A key 1124-bp fragment of the *GbJMJ25* gene was selected to construct an antisense expression vector (Fig. 6A). Gene silencing of Poplar JMJ25 leads to abnormal synthesis and deposition of anthocyanins (Fan et al., 2018). To consist with it, On medium without the chemical inducer DEX, embryogenic cells remained light yellow with no significant morphological changes. However, on induction medium supplemented with DEX, some cells gradually turned dark red (Fig. 6B). Further biochemical assays confirmed that DEX-induced cells accumulated significantly higher levels of anthocyanins (Fig. 6C), indicating effective interference with the function of endogenous *GbJMJ25*. Subsequently, the constructed *GbJMJ25* antisense expression vector was introduced into cotton hypocotyl explants. After approximately 50 days of culture, somatic embryogenic cells first appeared on hypocotyls cultured on DEX-containing medium, a process that occurred about 20 days earlier than on medium without DEX. Statistical results showed that the somatic embryogenesis frequency reached 35.31% under DEX induction conditions, significantly higher than the 5.52% observed in the control medium (Fig. 6D). The resulting cotyledonary somatic embryos developed normally, with clear and intact morphological structures (Fig. 6E). This enhancement in regenerative capacity was closely associated with an improved cellular redox state (Fig. 6F and 6G). The study suggests that inhibiting *GbJMJ25* expression activates reactive oxygen species (ROS) scavenging pathways, effectively alleviating oxidative stress. This creates a more favorable cellular environment for somatic embryogenesis, significantly accelerating the formation of somatic embryos in cotton.

Discussion

Reactive oxygen species (ROS), generated during aerobic metabolism, act as pivotal signaling molecules that determine cell fate and promote proliferation. An appropriate spatiotemporal distribution of ROS is essential for callus proliferation and differentiation, critically regulating the transition to embryogenic cells (Zhu et al., 2024; Yuan et al., 2023). In cotton, stress responses activated during somatic embryogenesis (SE) are known to fine-tune ROS homeostasis, often through crosstalk with phytohormone signaling pathways such as auxin (Zhou et al., 2016). Our findings align with this paradigm: we observed a distinct redox status between non-embryogenic callus (NEC) and embryogenic cells (EC). NEC exhibited higher H₂O₂ accumulation and oxidative damage, whereas EC displayed a more reductive environment characterized by lower H₂O₂ and MDA levels, coupled with elevated antioxidant

capacity (higher SOD activity and anthocyanin content) (Fig. 1). This stark contrast reinforces the concept that the precise temporal regulation of intracellular ROS is a key determinant of cell fate during SE, potentially serving as a metabolic switch that licenses cellular reprogramming.

The transcriptomic identification of ***GbJMJ25*** as a gene significantly downregulated during the NEC-to-EC transition positions it as a potential upstream regulator of this redox switch. As a histone demethylase belonging to the KDM3/JHDM2 subfamily, *GbJMJ25*'s activity is inherently linked to cellular metabolism through its dependence on α -ketoglutarate (α -KG), a product of the tricarboxylic acid (TCA) cycle. Intriguingly, α -ketoglutarate dehydrogenase (KGDH), the enzyme that produces α -KG, also functions as a redox sensor (McLain et al., 2011). This establishes a plausible **“metabolism-epigenetics” feedback loop**: under oxidative stress, KGDH activity may be modulated, leading to competitive consumption or altered availability of α -KG, thereby inhibiting JMJ demethylase activity (Huang et al., 2023). Our results functionally substantiate this model in the context of SE. Silencing *GbJMJ25* mimicked a low- α -KG-availability or inhibited-JMJ-activity state, resulting in enhanced peroxidase accumulation and a maintained low intracellular ROS level (Figs. 4D, 4F, 5C, 6G). This suggests that ***GbJMJ25* normally acts as a brake on the antioxidant system**, and its downregulation—whether developmentally programmed or experimentally induced—releases this brake, facilitating the establishment of the low-ROS environment conducive to embryogenic transition.

The pivotal question then becomes: how does the *GbJMJ25*-mediated low-ROS state promote embryogenesis? We propose a two-pronged mechanism. First, a reduced oxidative environment likely **potentiates auxin signaling**. ROS and auxin pathways are known to interact antagonistically; high ROS can oxidize and inhibit key components of auxin signaling (Roy et al., 2025). The low-ROS state in *GbJMJ25*-silenced cells may thus sensitize the tissue to auxin, explaining why the profound pro-embryogenic effect of silencing was strictly dependent on exogenous auxin application (Figs. 5A, 6D). Second, by altering histone methylation landscapes (likely H3K9me2/me3 given its subfamily), *GbJMJ25* downregulation may directly **derepress genes essential for stress acclimation and totipotency**. This epigenetic reprogramming, coupled with permissive auxin signaling, could synergistically activate the core transcriptional network for SE initiation. Our observation that silencing *GbJMJ25* also enhanced anthocyanin accumulation—a common stress-protectant response—supports its role in orchestrating a broader stress-adaptive transcriptome (Figs. 4F, 6G).

From a practical perspective, our study transitions *GbJMJ25* from a differential expression signature to a **validated biotechnological target**. While VIGS proved its functional necessity (Fig. 5A), the stable downregulation achieved via an inducible antisense RNA system (Fig. 6) offers a translatable tool. The significant increase in embryogenic callus frequency from 5.52% to 35.31% is not merely statistically significant but **agronomically relevant**, potentially transforming the efficiency of transformation pipelines for recalcitrant *G. barbadense* cultivars. This approach, which modulates an endogenous epigenetic regulator to enhance cellular competence, may be more effective and genotype-flexible than empirical medium optimization alone.

Nevertheless, our study opens several avenues for future research. First, identifying the **direct target genes** of *GbJMJ25* through techniques like ChIP-seq is crucial to elucidate the specific transcriptional cascades it controls. Second, evaluating the **generality of this mechanism** across diverse cotton genotypes with varying embryogenic capacities will determine its broad applicability. Third, exploring the **interplay between *GbJMJ25* and other epigenetic modifiers** (e.g., DNA methyltransferases) during SE could provide a more holistic view of the epigenetic reprogramming landscape.

Conclusion

This study delineated a novel pathway in which the histone demethylase ***GbJMJ25*** acts as a critical link between oxidative stress perception and cellular reprogramming in cotton. By negatively regulating antioxidant defenses, *GbJMJ25* maintains a level of ROS that appears to suppress embryogenic potential. Its downregulation, through a putative metabolism-epigenetics axis, reshapes the redox and epigenetic landscape to create a permissive state for auxin-induced somatic embryogenesis. This mechanistic insight provides a strong rationale for targeting *GbJMJ25* to overcome genotype-dependent regeneration barriers, offering a promising strategy to advance the molecular breeding of elite cotton varieties.

Declarations

Author contributions X. W conducted the experiments, collected and interpreted the data, and wrote the manuscript. M. T, Y.L and X.S collected and analysis the data. X.Z designed the experiments and revised the manuscript , All authors have read and agreed to the published version of the manuscript.

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Data availability The authors confirm that the data will be available on request.

Ethical approval Not applicable.

Conflict of interest The authors have no conflict of interest to declare.

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Figures

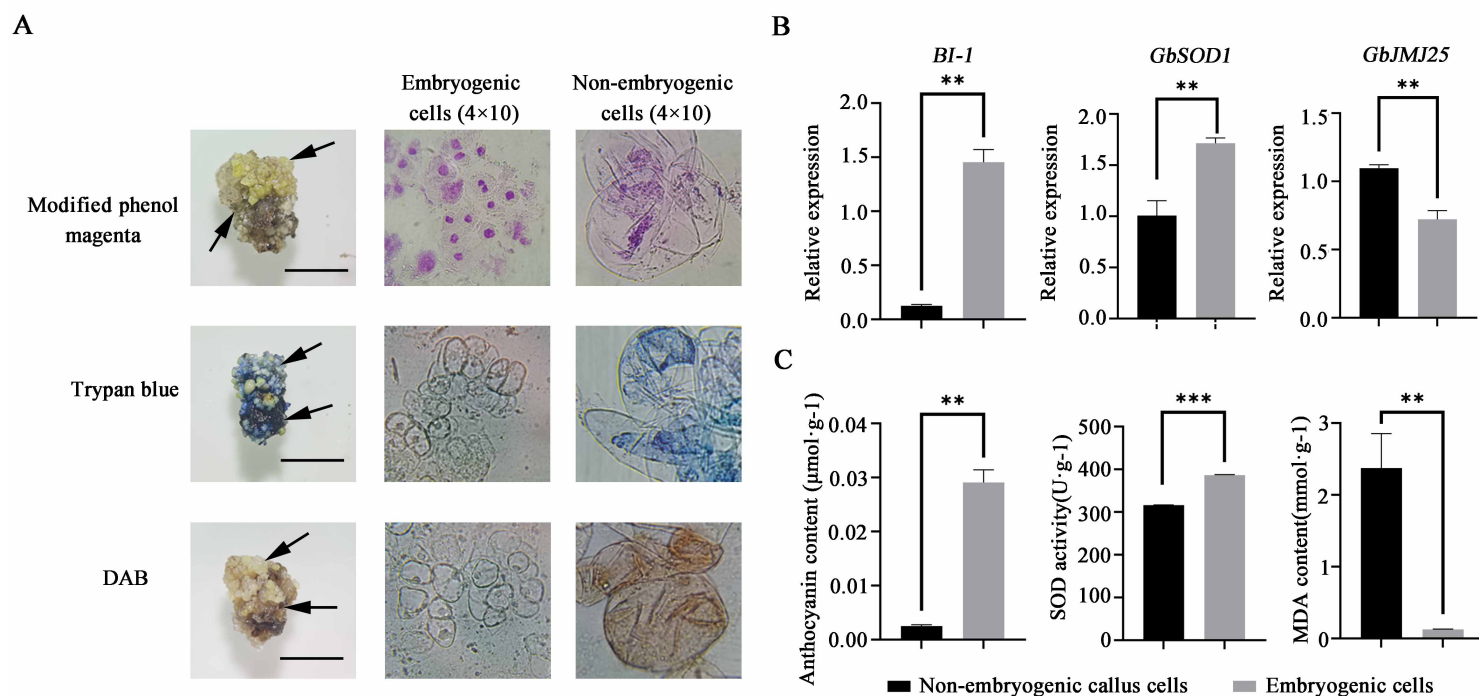


Figure 1

Cytological identification and physiological assays of primary embryogenic callus in cotton

A. Viability and redox state of primary embryogenic cells. Scale bar: 1 cm. B. Relative expression levels of *BI-1*, *GbSOD1*, and *GbJM25* in cotton primary embryogenic cells. C. Determination of ROS-scavenging related physiological indices in cotton primary embryogenic cells. Note: **, $P < 0.01$; ***, $P < 0.001$.

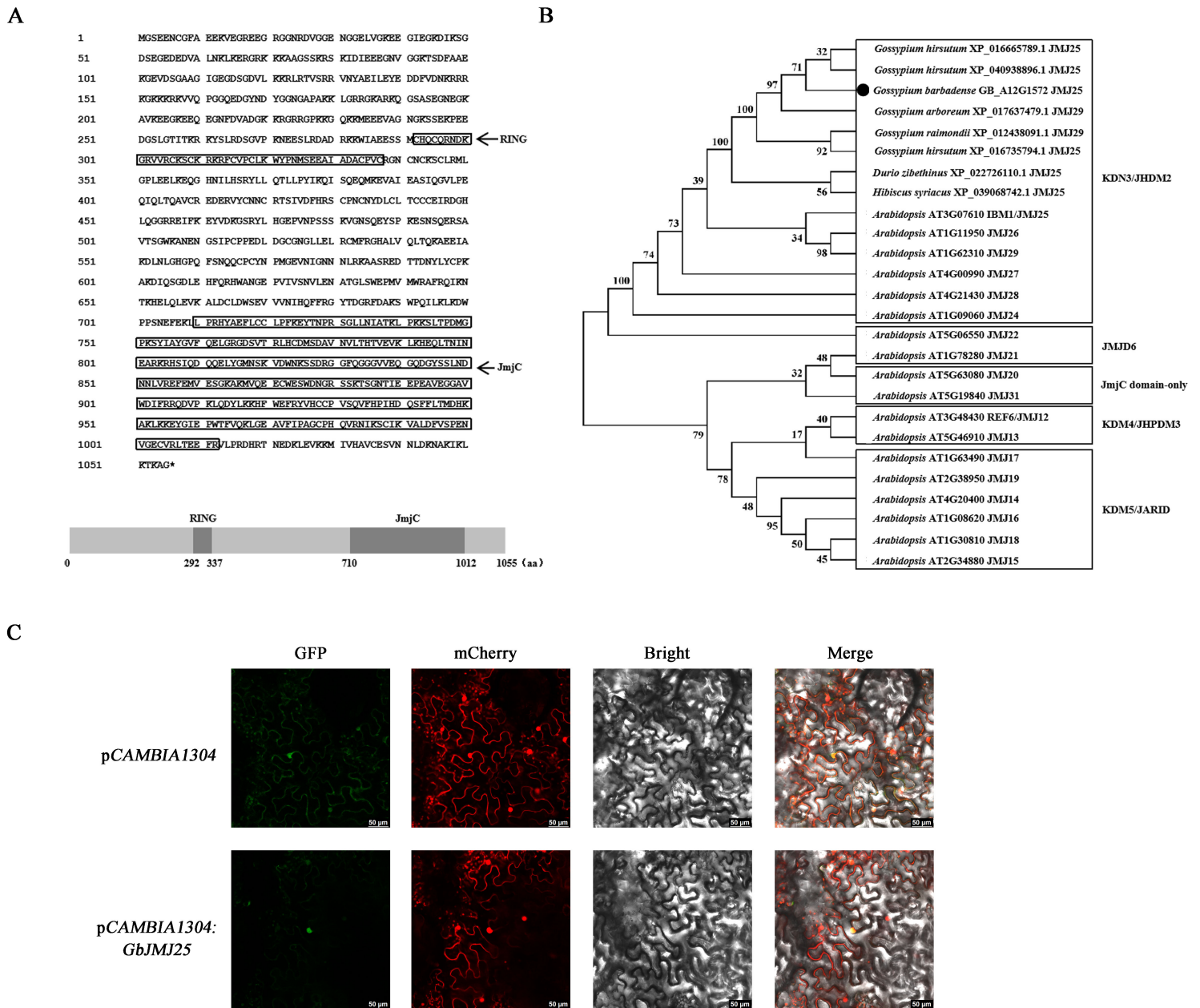


Figure 2

Bioinformatics analysis and subcellular localization of *GbJMj25*

A. Conserved domains in the *GbJMj25* amino acid sequence. B. Phylogenetic tree of the *GbJMj25* gene. C. Subcellular localization of the *GbJMj25* protein.

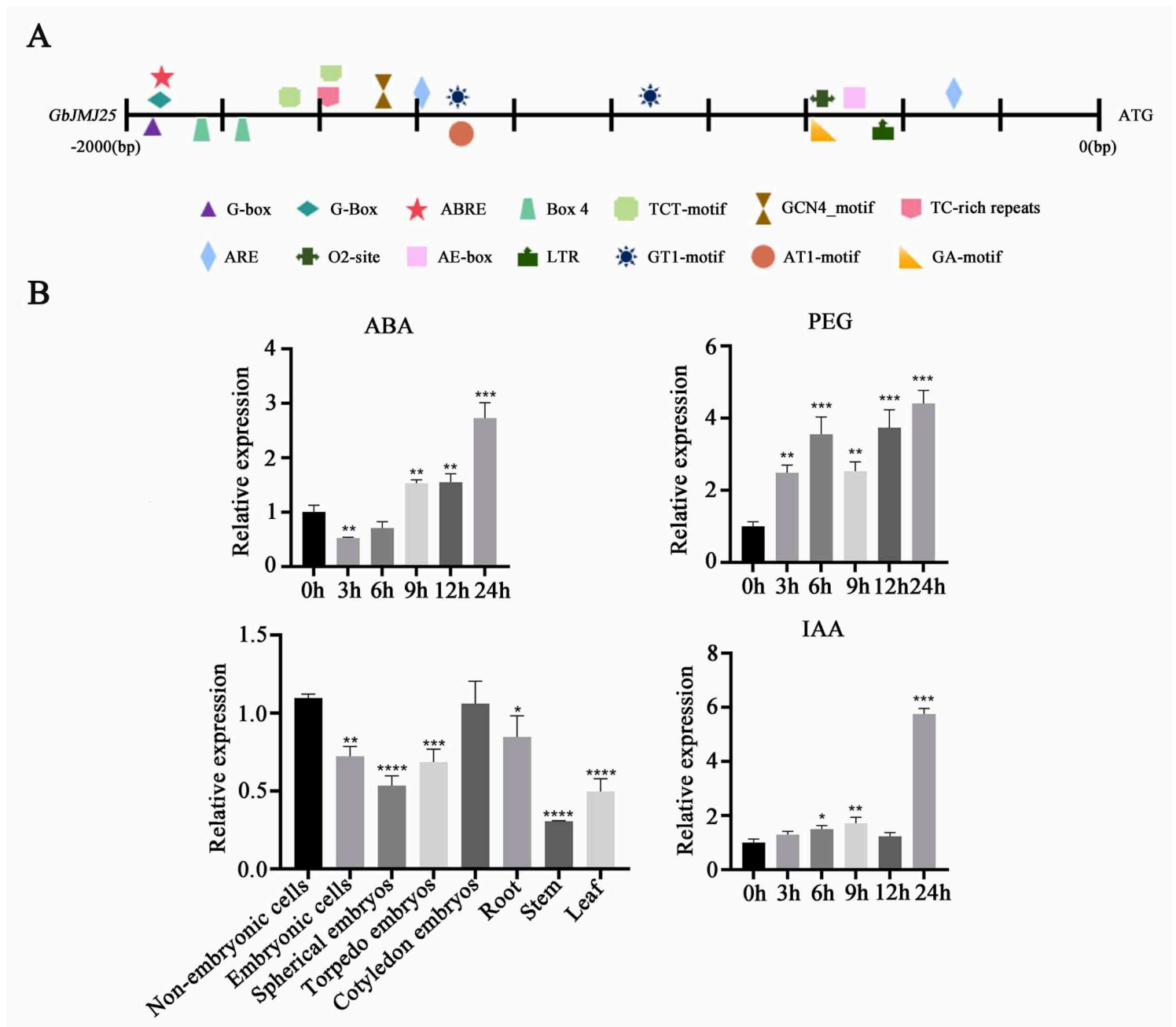


Figure 3

Promoter and expression pattern analysis of **GbJMJ25**

A. Identification of cis-acting elements in the *GbJMJ25* promoter. **B.** Expression pattern analysis of *GbJMJ25*. Note: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

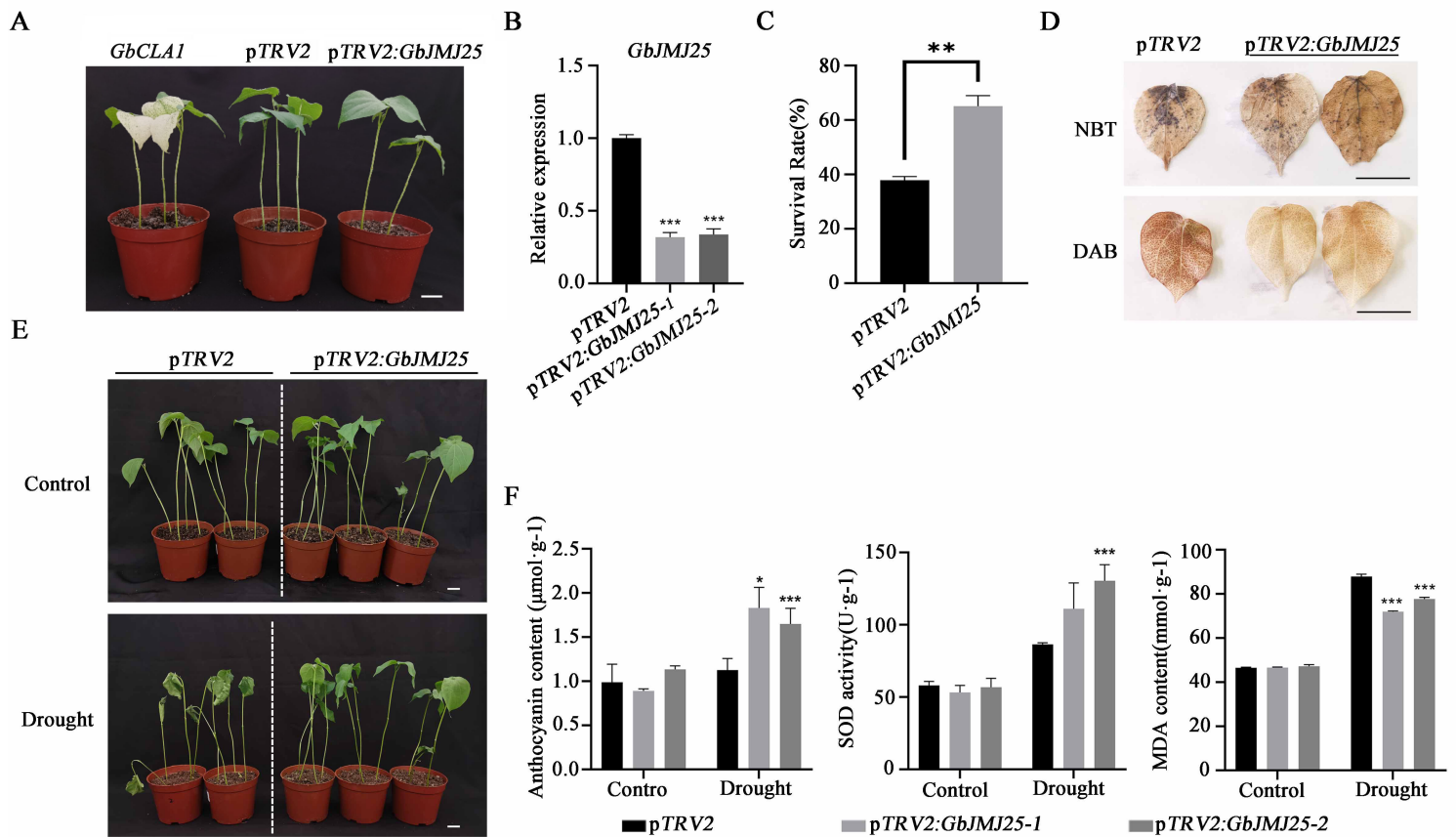


Figure 4

GbJMJ25 deficiency confers drought tolerance in cotton through enhanced ROS- scavenging ability

A. Phenotype of *GbJMJ25*-silenced cotton plants generated by VIGS. Scale bar: 2 cm. B. Relative expression level of *GbJMJ25* in silenced plants. C. Plant survival rate after drought treatment. D. Histochemical detection of superoxide and hydrogen peroxide by NBT and DAB staining after drought stress. Scale bar: 1 cm. E. Representative phenotypes before and after drought stress. F. Assays of ROS-scavenging related physiological parameters before and after drought stress. Note: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

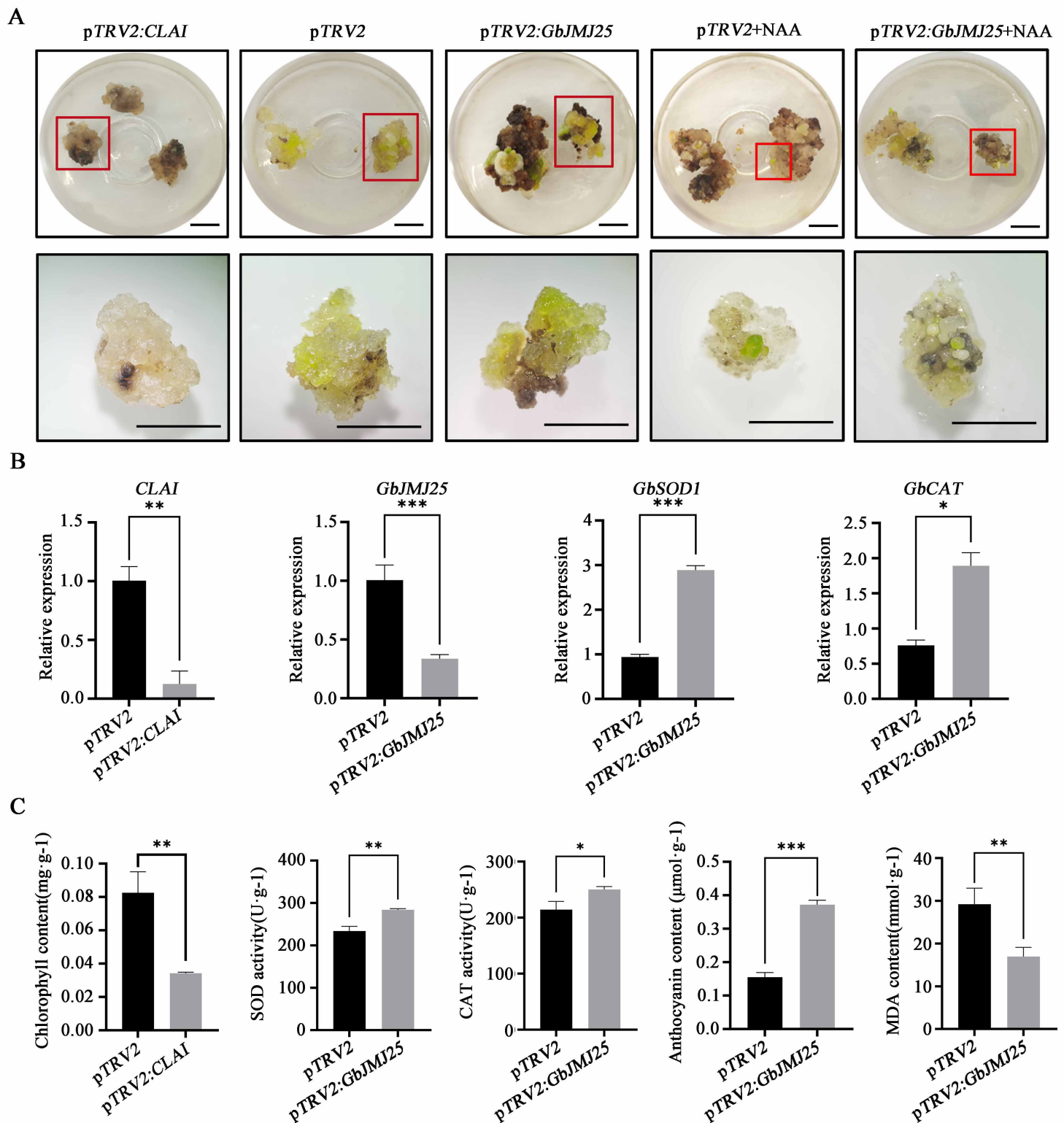


Figure 5

***GbJMJ25* silencing promotes embryogenesis in cotton callus via VIGS**

A. Induction of embryogenic cells and somatic embryos from VIGS-mediated *GbJMJ25*-silenced cotton callus on NAA-containing medium. Scale bar: 1 cm. **B.** Expression analysis of *CLAI*, *GbJMJ25*, *GbSOD1*, and *GbCAT* genes in the obtained embryogenic callus. **C.** Detection of chlorophyll content and ROS-

scavenging related physiological indices in the obtained embryogenic callus. Note * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

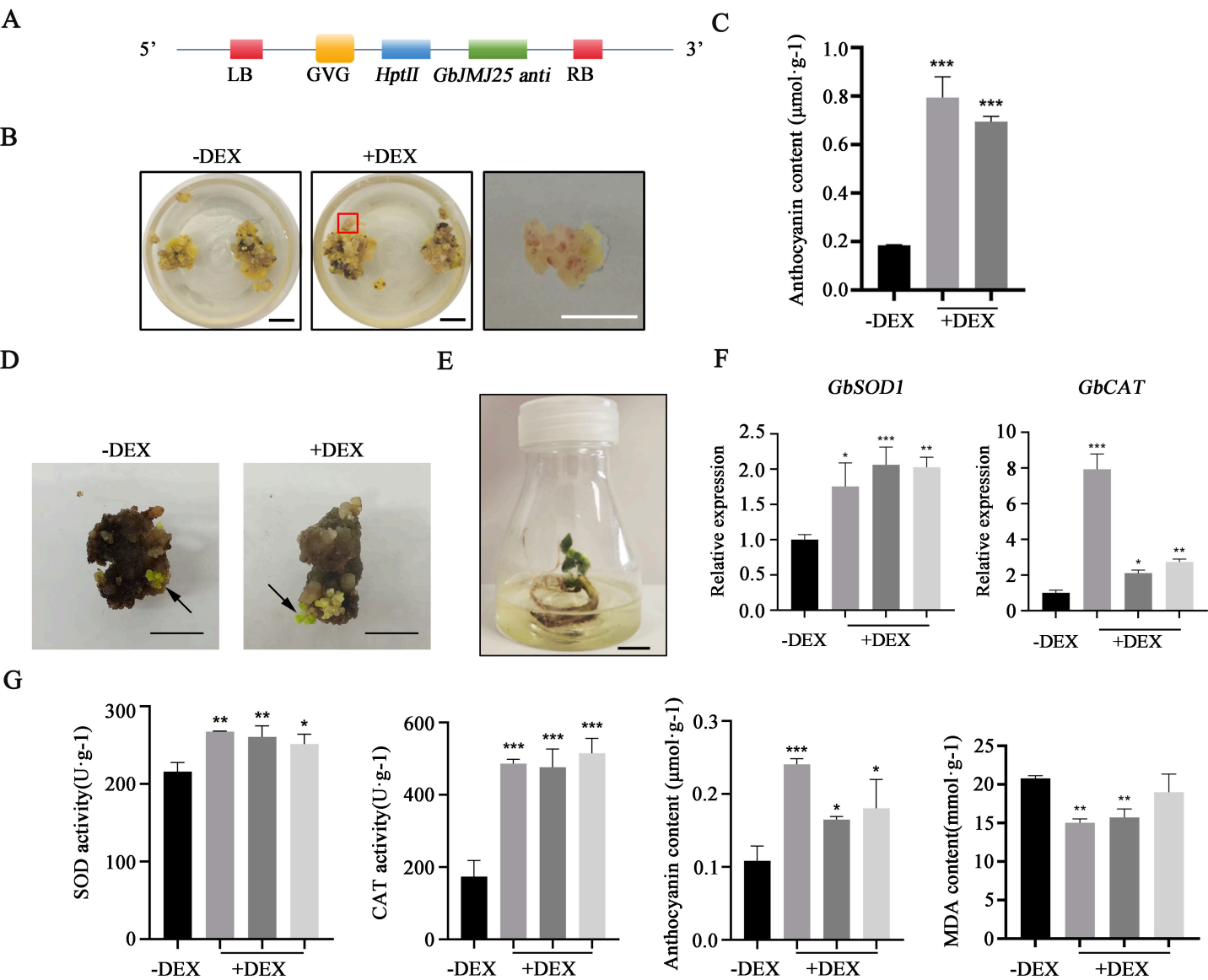


Figure 6

GbJMJ25 suppression promotes cotton somatic embryogenesis

Schematic diagram of the *GbJMJ25* antisense recombinant vector. **B**. Accumulation of red pigment in DEX-induced transgenic embryogenic cells. **C**. Detection of anthocyanin content in transgenic embryogenic cells. **D**. Induction of primary embryonic callus and somatic embryos from transgenic cotton hypocotyls on DEX-containing medium. **E**. Regenerated cotton plants obtained via somatic embryogenesis. **F**. Expression analysis of reactive oxygen species (ROS)-scavenging genes in the induced primary embryonic callus. **G**. Measurement of ROS-related physiological indices in the induced primary embryonic callus. Note * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

Supplementary Files

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