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Functions of exogenous strigolactone application and strigolactone biosynthesis genes *GhMAX3*/*GhMAX4b* in response to drought tolerance in cotton (*Gossypium hirsutum* L.)

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Abstract

Background Drought stress markedly constrains plant growth and diminishes crop productivity. Strigolactones (SLs) exert a beneficial influence on plant resilience to drought conditions. Nevertheless, the specific function of SLs in modulating cotton's response to drought stress remains to be elucidated.

Results In this study, we assess the impact of exogenous SL (*rac*-GR24) administration at various concentrations (0, 1, 5, 10, 20 μM) on cotton growth during drought stress. The findings reveal that cotton seedlings treated with 5 μM exogenous SL exhibit optimal mitigation of growth suppression induced by drought stress. Treatment with 5 μM exogenous SL under drought stress conditions enhances drought tolerance in cotton seedlings by augmenting photosynthetic efficiency, facilitating stomatal closure, diminishing reactive oxygen species (ROS) generation, alleviating membrane lipid peroxidation, enhancing the activity of antioxidant enzymes, elevating the levels of osmoregulatory compounds, and upregulating the expression of drought-responsive genes. The suppression of cotton SL biosynthesis genes, *MORE AXILLARY GROWTH 3* (*GhMAX3*) and *GhMAX4b*, impairs the drought tolerance of cotton. Conversely, overexpression of *GhMAX3* and *GhMAX4b* in respective *Arabidopsis* mutants ameliorates the drought-sensitive phenotype in these mutants.

Conclusion These observations underscore that SLs significantly bolster cotton's resistance to drought stress.

Keywords Strigolactones, Cotton, Drought, *GhMAX3*, *GhMAX4b*

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Background

Strigolactones (SLs), an identified class of phytohormones synthesized in plant roots, were originally described as seed germination stimulants for root-parasitic plants, notably *Striga* and *Orobanche* [1]. Moreover, SLs function as rhizospheric signals, facilitating symbiotic associations between arbuscular mycorrhizal (AM) fungi and their host plants [2]. Beyond their initial characterization, SLs play pivotal roles in orchestrating a multitude of plant growth and developmental processes, including the modulation of shoot branching [3, 4], root architecture refinement [5, 6], hypocotyl elongation [7], secondary growth enhancement [8], and leaf senescence regulation [9, 10]. Additionally, they are integral in mediating plant responses to biotic stressors [11–13] and abiotic stressors, encompassing salinity stress [14], drought stress [15–17], and phosphate scarcity [18, 19].

Genes implicated in the biosynthesis and signaling pathways of SLs have been delineated via the study of mutants exhibiting high branching in various species, including the *more axillary growth* (*max*) mutants (*max3*, *max4*, *max1*, *d14*, and *max2*) in *Arabidopsis thaliana* (L.) Heynh. [20–24], high-tillering *dwarf* (*d27*, *d17*, *d10*, *d14*, *d3*, and *d53*) mutants in *Oryza sativa* L. [25–30], *decreased apical dominance* (*dad*) mutants (*dad3*, *dad1*, and *dad2*) in *Petunia* × *hybrida* hort. ex Vilm. [9, 31, 32], and *ramosus* (*rms*) mutants (*rms5*, *rms1*, *rms3*, and *rms4*) in *Pisum sativum* L. [33–35]. SLs, categorized as carotenoid-derived terpenoid lactones, are produced through the action of the *β*-carotene isomerase D27 (encoded by *D27*) and carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8, encoded by *MAX3*/*D17*/*DAD3*/*RMS5* and *MAX4*/*D10*/*DAD1*/*RMS1*, respectively), facilitating the transformation of carotenoids into carlactone [36]. *MAX1*, encoded by a cytochrome P450 monooxygenase, catalyzes the conversion of carlactone into biologically active SLs [37]. The F-box protein encoded by *MAX2*/*D3*/*RMS4*, part of the SCF ubiquitin ligase complex, is pivotal for substrate recognition and proteasome-mediated proteolysis. D14/DAD2/RMS3, an α/β-fold hydrolase, binds and hydrolyzes SLs and interacts with MAX2/D3/RMS4 in an SL-dependent manner, instigating the ubiquitination and subsequent degradation of repressors D53/SUPPRESSOR OF MAX2 1-LIKE6/7/8 (SMXL6/7/8), thus facilitating gene de-repression [38]. Loss-of-function mutations in genes related to SL biosynthesis lead to diminished SL production and increased branching, a phenotype reversible by the synthetic SL analog *rac*-GR24. Conversely, mutations in SL signaling genes also result in increased branching, yet this phenotype is not amendable with *rac*-GR24 supplementation [38].

Drought stress, a prevalent environmental challenge, detrimentally affects plant survival and yield. To counteract drought stress, plants have developed sophisticated defensive strategies encompassing morphological adaptations, membrane integrity preservation, photosynthetic activity, stomatal regulation, reactive oxygen species (ROS) detoxification, hormonal modulation, and stress protein activation [39, 40]. SLs significantly contribute to drought stress resilience in plants. Studies have demonstrated that plants treated with *rac*-GR24, a synthetic SL analog, exhibit enhanced drought tolerance through the modulation of electrolyte leakage, stomatal conductance, antioxidant enzyme activity, and malondialdehyde (MDA) levels [41, 42]. Furthermore, SL-deficient and SL-response mutants (*max3*, *max4*, and *max2*) in *Arabidopsis* show increased susceptibility to drought stress compared to wild-type [14]. The *Arabidopsis* SL receptor *d14* mutant, which is hypersensitive to drought, displayed increased stomatal aperture and reduced anthocyanin accumulation under drought conditions [15]. Conversely, the *Arabidopsis smxl6*,*7*,*8* triple mutant, more drought-resistant than WT, exhibited heightened sensitivity to abscisic acid (ABA) and increased anthocyanin levels under drought stress [43].

Cotton, a paramount fiber and oilseed crop, holds substantial economic value and is extensively cultivated globally. Drought deleteriously impacts all growth stages of cotton, leading to a marked reduction in yield and deterioration of fiber quality [44]. While the positive regulation of plant drought stress response by SLs has been documented, their specific roles in cotton's drought stress response remain to be elucidated. The primary objective of this study was to investigate the role of SLs in enhancing drought resistance in cotton. Specifically, the research aimed to: evaluate the effects of exogenous SL (*rac*-GR24) application on various physiological and biochemical parameters associated with drought tolerance in cotton; and elucidate the role of SL biosynthesis genes *GhMAX3* and *GhMAX4b* in the regulation of drought response mechanisms in cotton. This study demonstrates that treating cotton seedlings with 5 μM exogenous SL (*rac*-GR24) bolsters drought resistance by modulating photosynthesis, stomatal closure, ROS metabolism, osmoregulation, and the expression of drought-responsive genes. Conversely, diminishing endogenous SL levels through the silencing of cotton SL biosynthesis genes weakens drought resistance. Furthermore, overexpression of these SL biosynthesis genes in the corresponding *Arabidopsis* mutants mitigated the drought-sensitive phenotype in these mutants. These results furnish novel insights into the mechanisms underlying cotton's drought resistance.

Methods

Plant materials, growth conditions and stress treatment

Gossypium hirsutum L. cv. SF06 seeds were employed in this study, which were selected and bred by our laboratory and are currently kept in the laboratory. The cotton seeds were sown in a mixture of vermiculite and peat in plastic pots at 25 °C. The cotton seedlings were grown in a greenhouse maintained at 25 °C, with a photoperiod of 16 h light/8 hours dark, a light intensity of 3,300 lx, and a relative humidity of 70%. Samples of roots, stems, and leaves were harvested from cotton seedlings aged 21 days. The cotton seedlings grown for 21 days were then subjected to drought conditions by immersing them in Hoagland's solution containing 20% (v/v) polyethylene glycol (PEG) 6000. Root tissues were collected at intervals of 0, 3, 6, 12, and 24 h post-treatment initiation. Subsequently, all samples were immediately frozen in liquid nitrogen and preserved at −80 °C for subsequent total RNA extraction.

Arabidopsis thaliana (L.) Heynh. mutants *max3* (SALK_023975C) and *max4* (SALK_072750C) were acquired from *AraShare* (https://www.arashare.cn/index/). Wild-type (Col-0 ecotype, preserved by our laboratory), mutant, and transgenic *Arabidopsis* lines were cultivated in a controlled growth chamber, maintained at 22 °C with a 16-hour light/8-hour dark photoperiod.

Exogenous SL (*rac*-GR24) treatment under drought stress

Uniformly healthy, 4-week-old cotton seedlings were utilized for this experiment. The seedlings underwent drought treatment, entailing 18 days without water. The study comprised six treatments: (1) well-watered (control); (2) drought stress with 0 μM *rac*-GR24 foliar application (using distilled water with equivalent volumes of acetone and Tween 20); (3) drought stress with 1 μM *rac*-GR24 foliar application; (4) drought stress with 5 μM *rac*-GR24 foliar application; (5) drought stress with 10 μM *rac*-GR24 foliar application; (6) drought stress with 20 μM *rac*-GR24 foliar application. The synthetic SL analog, *rac*-GR24 (Chiralix, Netherlands, catalog number: CX23880), was solubilized in acetone and then diluted with distilled water. The foliar application of *rac*-GR24 was performed using a hand sprayer until the solution visibly dripped from the leaves. The seedlings were sprayed with *rac*-GR24 twice weekly, for a total of five times during the drought treatment. To enhance the foliar absorption of *rac*-GR24, two drops of the surfactant Tween 20 were added to every 100 mL of the solution. Control-treated seedlings were consistently watered. In brief, four-week-old cotton seedlings were exposed to drought stress namely water-deficit treatment for 18 days and were treated bi-weekly with varying concentrations of exogenous SL (*rac*-GR24) (0, 1, 5, 10, and 20 μM). Normally watered seedlings served as controls. Cotton leaves were collected, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent total RNA extraction. Each treatment group was systematically replicated three times, encompassing 36 plants per replicate.

Physiological parameters of cotton seedlings were assessed, encompassing plant height, shoot fresh weight, leaf relative water content (LRWC), chlorophyll fluorescence parameter (Fv/Fm), chlorophyll content, stomatal closure, ROS (hydrogen peroxide [H_2O_2] and superoxide anion [$O_2^{\bullet-}$]) levels, malondialdehyde (MDA) content, activities of antioxidant enzymes (superoxide dismutase [SOD], peroxidase [POD] and catalase [CAT]), proline, soluble sugar, and soluble protein concentrations. Additionally, the expression levels of drought stress-responsive genes in the cotton seedlings were analyzed. Each experimental setup was supported by a minimum of three biological replicates.

Measurement of plant height, shoot fresh weight and leaf relative water content

Plant height and shoot fresh weight were quantified using a ruler and an analytical balance, respectively. LRWC was determined following the method described by Turner [45].

Measurement of chlorophyll fluorescence parameter Fv/Fm, chlorophyll concentration and stomatal closure

Chlorophyll fluorescence parameter Fv/Fm (variable fluorescence/maximum fluorescence) was analyzed using a Multi-Function Plant Efficiency Analyser at room temperature. Chlorophyll concentration was measured spectrophotometrically based on the previously described method [46]. Total chlorophyll content (C_T) was calculated as follows: $C_a = 13.95\ A_{665} - 6.88\ A_{649}$; $C_b = 24.96\ A_{649} - 7.32\ A_{665}$; total chlorophyll ($C_T$) = $C_a + C_b$. Stomatal closure was assessed by the ratio of stomatal length to width, following the method described by Zhang et al. [47]. Stomatal apertures were observed and imaged in cotton leaves under a microscope, and the images were analyzed using ImageJ software to quantify stomatal length and width. The ratio of stomatal length to width was quantified for more than 50 stomata per sample.

Measurement of ROS and MDA contents

H_2O_2 and $O_2^{\bullet-}$ levels were determined using the methods previously described [48, 49]. Lipid peroxidation and membrane integrity were assessed by measuring the MDA content, following the protocol outlined by Shi et al. [50].

Measurement of antioxidant enzyme activities

SOD, POD and CAT activities were determined respectively based on the method described by Beauchamp et al. [51]., Kochba et al. [52]. and Beers et al. [53].

Measurement of proline, soluble sugar and soluble protein contents

Proline content was determined as described by Abrahám et al. [54]. Soluble sugar and soluble protein contents were determined respectively based on the previously described method [55, 56].

RNA extraction and qRT-PCR analysis

Total RNA was extracted from 100 mg of cotton tissue and *Arabidopsis* leaf samples using the RNA-prep Pure Plant Kit (Polysaccharides & Polyphenolics-rich, DP441) from Tiangen (Beijing, China), according to the manufacturer's instructions. RNA concentration and purity were assessed using 1.5% agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed using HiScript® II Q RT SuperMix for qPCR with gDNA wiper (Vazyme, Nanjing, China). Quantitative RT-PCR was conducted with SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China), and relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [57], with data analysis in Excel. *GhUBQ7* (NCBI accession: DQ116441) served as the internal reference gene [58]. Gene-specific primers, used for qRT-PCR and designed via Primer Premier 5.0 [59], are listed in Table S1.

Phylogenetic, protein domain and gene structure analysis

The genome annotation file of *G. hirsutum*, JGI, was obtained from CottonGen [60]. The protein sequences for *Arabidopsis* AtMAX3 (AT2G44990.2) and AtMAX4 (AT4G32810.2) were retrieved from TAIR [61]. The BLAST algorithm for proteins (BLASTP) [62] was used to search MAX3 and MAX4 proteins from the *G. hirsutum* genome annotation file. Sequence alignment for *G. hirsutum* and *Arabidopsis* MAX3 and MAX4 proteins was executed using ClustalW [63]. The phylogenetic tree was constructed with MEGA 6.0 employing the Neighbor-Joining (NJ) method and 1000 bootstrap replicates [64]. Protein domain analysis for MAX3 and MAX4 in both *G. hirsutum* and *Arabidopsis* was performed using the Pfam database [65] and SMART [66], with visualizations generated through IBS software [67]. The intron/exon structures of the *MAX3* and *MAX4* genes in both species were delineated using GSDS 2.0 [68], based on their coding sequences (CDSs) and genomic sequences.

Gene cloning and vector construction

For the virus-induced gene silencing (VIGS) experiments, gene fragments of *GhMAX3*, *GhMAX4a* and *GhMAX4b* were devised using the SGN VIGS Tool [69]. These specific cDNA fragments were PCR-amplified from the cotton cDNA library employing Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China) and subsequently cloned into the pTRV2 vector at the BamHI restriction site via ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China), resulting in the recombinant plasmids TRV2:*GhMAX3*, TRV2:*GhMAX4a*, and TRV2:*GhMAX4b*.

In the transformation assays for *Arabidopsis*, the CDSs of *GhMAX3* and *GhMAX4b* were retrieved from CottonGen [60] and amplified from the cotton cDNA library via PCR. These amplified fragments were cloned into the pRI 201-AN-GUS vector at the Nde I and Sac I restriction sites, generating the recombinant plasmids pRI 201-AN-*GhMAX3* (35S::*GhMAX3*) and pRI 201-AN-*GhMAX4b* (35S::*GhMAX4b*), respectively.

The recombinant plasmids were individually transformed into *Escherichia coli* DH5α cells and confirmed through Sanger sequencing. Subsequently, the positively recombinant plasmids determined by sequencing, were transformed into *Agrobacterium tumefaciens* strain GV3101. The primers employed for vector construction are detailed in Table S1.

Agrobacterium-mediated VIGS in cotton and drought treatment

Agrobacterium-mediated VIGS assays in cotton were conducted following the protocol established by Gao et al. [70]. pTRV1 vectors were combined with pTRV2 vectors harboring the target genes or the empty vector pTRV2:00 at a 1:1 ratio for co-infiltration into 10-day-old cotton cotyledons. TRV2:*GhCLA* served as the positive control, while TRV2:00 functioned as the negative control. Two weeks post-*Agrobacterium* infiltration, the bleaching phenotype in the positive controls became prominent. Gene silencing efficiency was evaluated by quantifying the expression levels of *GhMAX3*, *GhMAX4a* and *GhMAX4b* genes via qRT-PCR, utilizing RNA extracted from the roots of 4-week-old both control (TRV2:00) and gene-silenced (TRV2:*GhMAX3*, TRV2:*GhMAX4a*, and TRV2:*GhMAX4b*) cotton plants.

For the drought treatment, 4-week-old control (TRV2:00) and silenced cotton plants were subjected to water deprivation for 12 days. As a reference, normally watered cotton plants served as the controls. Cotton leaves were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent total RNA extraction. The LRWC, Fv/Fm, chlorophyll content, stomatal closure, levels of ROS and MDA, activities of antioxidant enzymes, proline concentration, soluble sugars, soluble proteins, and the expression of drought-responsive genes were assessed in both control and drought-treated plants. The experimental setup included at least three biological replicates, each with 18 plants.

Germination assay of *Orobanche cumana* wallr. seeds

The *O. cumana* seeds were provided by Professor Yanbing Lin at the College of Life Sciences, Northwest A&F

University. The germination assay for *O. cumana* was conducted following the protocols outlined by Xi et al. and Yi et al. [13, 71]. Root samples (100 mg each) from control (TRV2:00) and silenced cotton plants (TRV2:*GhMAX3*, TRV2:*GhMAX4a*, and TRV2:*GhMAX4b*) were pulverized and sonicated in 1.5 mL of water for 30 min to prepare aqueous extracts. The extracts were then centrifuged, and the supernatants were immediately used to assess the germination of *O. cumana* seeds. Each extract solution (20 µL) was applied directly to *O. cumana* seeds placed on 8-mm disks of glass fiber filter paper (GFFP, Whatman, GE Healthcare UK Ltd., Buckingham shire, UK) within Petri dishes. The treated seeds were incubated in darkness at 25 °C, and their germination rates were evaluated after 8 days. A five-micromolar *rac*-GR24 solution and deionized water served as positive and negative controls, respectively, for assessing *O. cumana* seed germination. The number of germinated seeds was quantified using a stereomicroscope. The experimental design included at least three biological replicates, each containing 50 seeds.

Transformation of *GhMAX3* and *GhMAX4b* into corresponding *Arabidopsis* mutants and drought treatment

Agrobacterium tumefaciens strain GV3101 harboring the recombinant plasmids pRI 201-AN-*GhMAX3* (35S::*GhMAX3*) and pRI 201-AN-*GhMAX4b* (35S::*GhMAX4b*) was utilized for *Arabidopsis* transformation via the floral dip method [72]. The transformed seeds were grown on half-strength Murashige and Skoog (MS) medium supplemented with 50 mg/L kanamycin. After two weeks, seedlings with normal, healthy green cotyledons were transferred into vermiculite in plastic boxes. DNA was extracted from the independent transgenic lines to confirm the integration of *GhMAX3* and *GhMAX4b* through PCR. *AtACTIN2* (AT3G18780) served as the internal control. The expression levels of *GhMAX3* and *GhMAX4b* in these three-week-old transgenic *Arabidopsis* lines were quantified using qRT-PCR. The T3 generation of these transgenic lines was selected for subsequent experiments. The primers used are detailed in Table S1.

Seeds of wild-type (WT), mutant, and transgenic *Arabidopsis* were germinated on the half-strength MS medium. After two weeks, only the healthy and morphologically uniform plants were transplanted into vermiculite in plastic boxes. For the drought treatment, three-week-old *Arabidopsis* seedlings were subjected to water deprivation for 21 days, while normally watered seedlings served as the controls. The LRWC and chlorophyll concentration in *Arabidopsis* leaves were assessed in both the control and drought-treated plants. The experiment was conducted with at least three biological replicates, each comprising 60 *Arabidopsis* plants.

Statistical analysis

All experiments were conducted with a minimum of three biological replicates. Statistical analysis was carried out using analyses of variance (ANOVAs) via DPS software [73]. Significant differences among samples were identified and subsequently compared using Tukey's test, with a significance level set at $p<0.05$ [74].

Results

Exogenous SL affects cotton growth under drought stress

Drought stress curtailed cotton growth compared to the controls (Fig. 1A). Notably, under drought conditions, the 1, 5, and 10 µM SL treatments mitigated growth inhibition in cotton seedlings, with the 5 µM SL treatment significantly reversing the drought-induced growth suppression (Fig. 1A). The results indicated that a 5 µM SL concentration is optimal for countering growth inhibition in drought-stressed cotton seedlings. Furthermore, plant height was significantly reduced under drought stress compared to controls by about 52% with the 0 µM SL treatment, 32% with the 1 µM SL treatment, 21% with the 5 µM SL treatment, 43% with the 10 µM SL treatment, and 54% with the 20 µM SL treatment (Fig. 1B), indicating that the 5 µM SL treatment is particularly effective in alleviating height reduction under drought stress. Similarly, shoot fresh weight decreased significantly under drought stress compared to controls by about 79% with the 0 µM SL treatment, 59% with the 1 µM SL treatment, 45% with the 5 µM SL treatment, 70% with the 10 µM SL treatment, and 79% with the 20 µM SL treatment (Fig. 1C). Additionally, LRWC was significantly reduced under drought stress compared to controls by about 66% with the 0 µM SL treatment, 40% with the 1 µM SL treatment, 27% with the 5 µM SL treatment, 52% with the 10 µM SL treatment, and 66% with the 20 µM SL treatment (Fig. 1D). Therefore, we selected the 5 µM SL concentration for subsequent experiments.

Exogenous SL affects photosynthesis under drought stress

To elucidate the protective effect of exogenous SL on cotton photosynthesis under drought conditions, we assessed the chlorophyll fluorescence parameter Fv/Fm and chlorophyll concentration. Both Fv/Fm and chlorophyll levels were significantly reduced by about 32% and 45%, respectively, under drought stress with 0 µM SL treatment compared to control plants (Fig. 2A, B). The exogenous treatment with 5 µM SL under drought conditions resulted in an increase in Fv/Fm and chlorophyll levels by about 21% and 33%, respectively, compared to the 0 µM SL treatment (Fig. 2A, B).

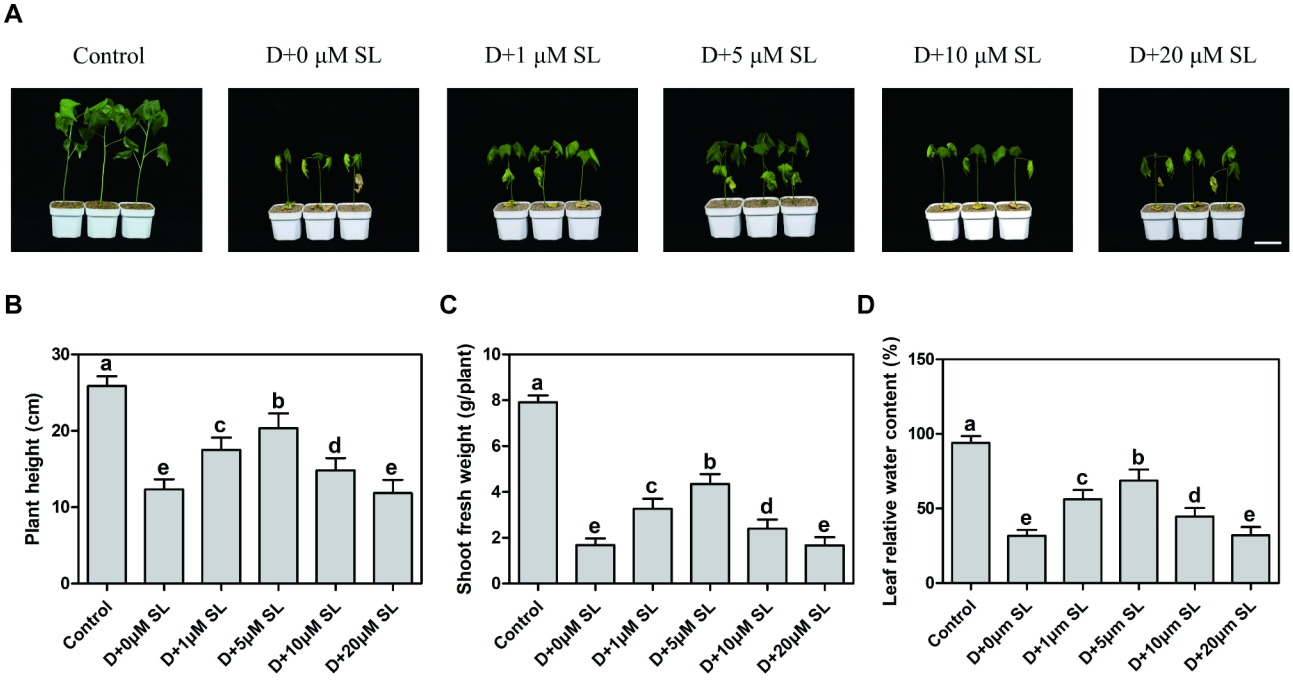

Fig. 1 The effects of exogenous SL (*rac*-GR24) with different concentrations (0, 1, 5, 10 and 20 μM) on the growth of cotton seedlings under drought stress. **(A)** cotton seedlings growth status, **(B)** plant height, **(C)** shoot fresh weight, **(D)** leaf relative water content. The 4-week-old cotton seedlings were subjected to normal water (control), drought stress with 0 μM SL (D + 0 μM SL), 1 μM SL (D + 1 μM SL), 5 μM SL (D + 5 μM SL), 10 μM SL (D + 10 μM SL), and 20 μM SL (D + 20 μM SL), respectively. The cotton seedlings were subjected to drought stress namely water-deficit treatment for 18 days. Exogenous SL was applied bi-weekly using a hand sprayer until the solution visibly dripped from the leaves. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test. Scale bar, 6 cm

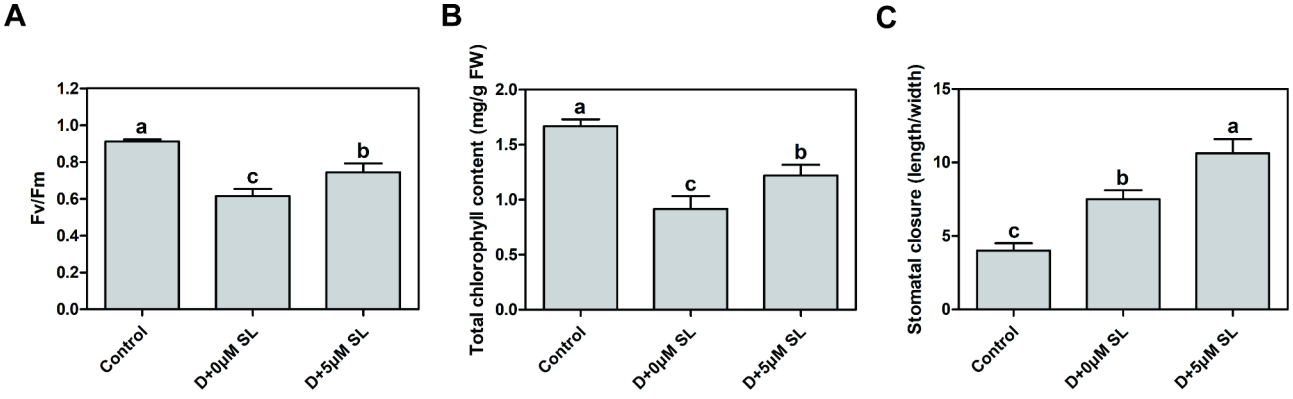

Fig. 2 The effects of 5 μM exogenous SL on **(A)** Fv/Fm, **(B)** total chlorophyll content, **(C)** stomatal closure (length/width) in cotton seedlings leaves under drought stress. The 4-week-old cotton seedlings were subjected to normal water (control), drought stress with 0 μM SL treatment (D + 0 μM SL), drought stress with 5 μM SL treatment (D + 5 μM SL), respectively. The leaves of cotton seedlings were harvested and analyzed after 18 days of treatment. Exogenous SL was applied bi-weekly using a hand sprayer until the solution visibly dripped from the leaves. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test

Exogenous SL affects stomatal closure under drought stress

Stomata play a pivotal role in plant photosynthesis and transpiration [75], prompting us to evaluate stomatal closure, defined as the length-to-width ratio. Stomatal closure was increased significantly by about 88% under drought stress with 0 μM SL treatment compared to control plants (Fig. 2C). The exogenous treatment with 5 μM SL notably enhanced stomatal closure by about 42% under drought stress, in contrast to the 0 μM SL treatment (Fig. 2C).

Exogenous SL affects ROS metabolism under drought stress

Under drought stress, plants accumulate high levels of ROS such as H_2O_2 and $O_2^{\bullet -}$, leading to oxidative stress.

MDA, an indicator of membrane lipid peroxidation and resultant oxidative damage, signifies the extent of cellular injury [76]. We quantified the levels of H_2O_2, $O_2^{\bullet-}$, and MDA in cotton leaves, as illustrated in Fig. 3A-C. These compounds increased significantly in cotton leaves with 0 μM SL treatment under drought stress by about 325%, 228%, and 80%, respectively, compared to the controls (Fig. 3A-C). Conversely, leaves from cotton seedlings treated with 5 μM exogenous SL reduced the H_2O_2, $O_2^{\bullet-}$, and MDA contents by about 37%, 35%, and 23%, respectively, under drought stress compared to those treated with 0 μM SL (Fig. 3A-C).

Plants deploy their enzymatic antioxidant system, comprising SOD, POD, and CAT, to mitigate oxidative damage from excess ROS induced by drought stress [76]. The impact of 5 μM exogenous SL on the activities of SOD, POD, and CAT in cotton leaves under drought stress is depicted in Fig. 3D-F. The activities of these enzymes in drought-stressed cotton leaves with 0 μM SL treatment increased significantly by about 87%, 132%, and 159%, respectively, compared to the controls (Fig. 3D-F). Notably, the exogenous treatment with 5 μM SL further increased the enzymatic activities of SOD, POD, and CAT in the cotton leaves by about 30%, 28%, and 33%, respectively, compared to the 0 μM SL treatment under drought stress (Fig. 3D-F).

Exogenous SL affects osmotic adjustment substance content under drought stress

Under drought stress, plants augment the accumulation of osmotic adjustment substances, including proline, soluble sugars, and soluble proteins, to mitigate water loss and counteract growth inhibition [77]. Figure 4 illustrates the influence of 5 μM exogenous SL on the levels of proline, soluble sugars, and soluble proteins in cotton leaves under such conditions. Compared with control plants, the proline, soluble sugars, and soluble proteins contents in cotton leaves with 0 μM SL treatment under drought stress increased by about 364%, 54%, and 82%, respectively (Fig. 4). Under drought stress, 5 μM SL treatment increased the proline, soluble sugars, and soluble proteins contents in cotton leaves by about 74%, 33%, and 32%, respectively, compared with 0 μM SL treatment (Fig. 4).

Exogenous SL affects the expression levels of drought-responsive genes

To investigate the role of SLs in modulating the drought stress response, we assessed the expression levels of the

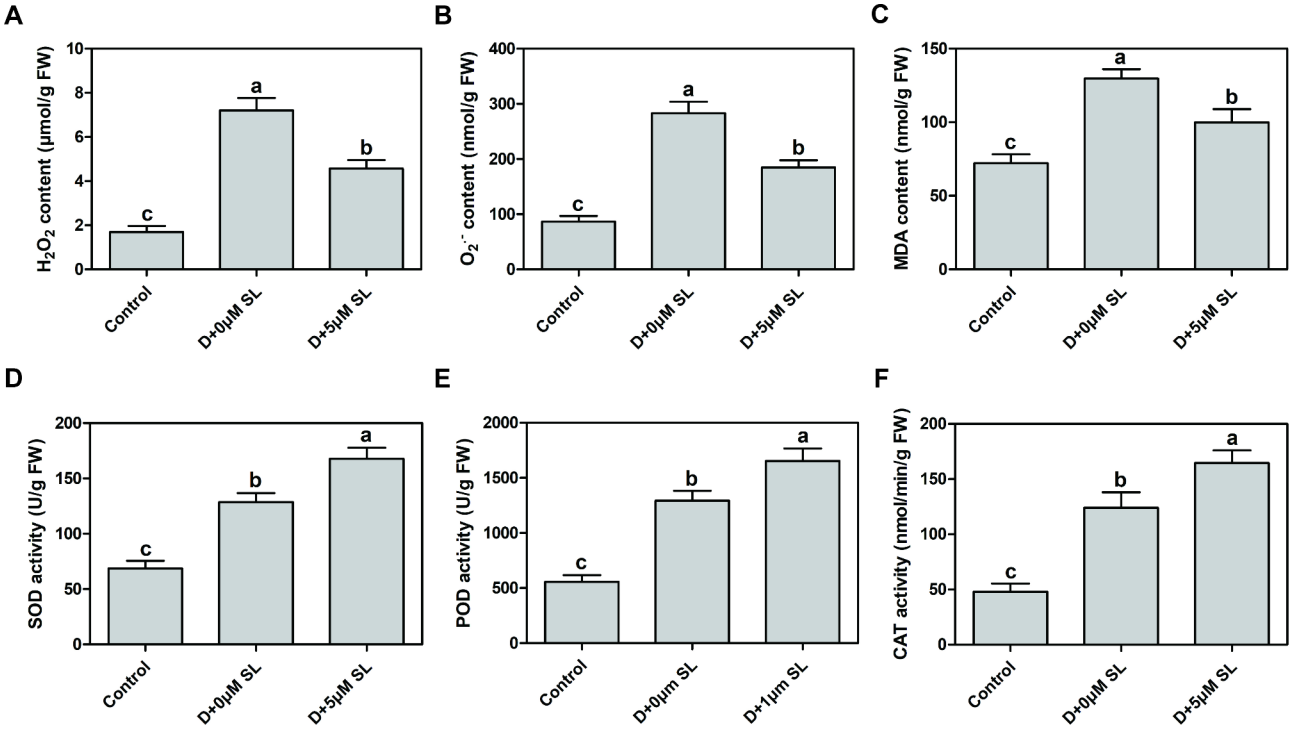

Fig. 3 The effects of 5 μM exogenous SL on **(A)** H_2O_2 content, **(B)** $O_2^{\bullet-}$ content, **(C)** MDA content, **(D)** SOD activity, **(E)** POD activity, **(F)** CAT activity in cotton seedlings leaves under drought stress. The 4-week-old cotton seedlings were subjected to normal water (control), drought stress with 0 μM SL treatment (D + 0 μM SL), drought stress with 5 μM SL treatment (D + 5 μM SL), respectively. The leaves of cotton seedlings were harvested and analyzed after 18 days of treatment. Exogenous SL was applied bi-weekly using a hand sprayer until the solution visibly dripped from the leaves. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test

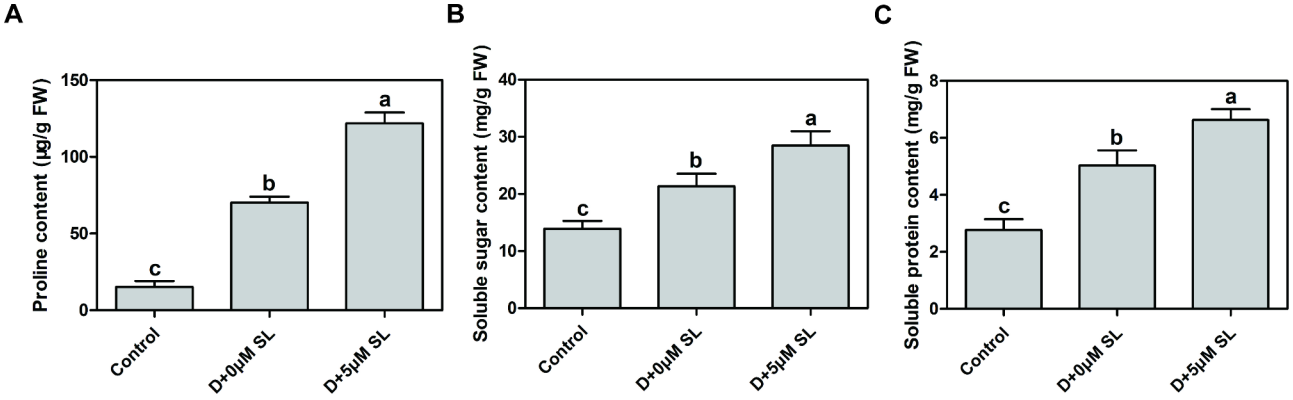

Fig. 4 The effects of 5 μM exogenous SL on **(A)** proline content, **(B)** soluble sugar content, **(C)** soluble protein content in cotton seedlings leaves under drought stress. The 4-week-old cotton seedlings were subjected to normal water (control), drought stress with 0 μM SL treatment (D + 0 μM SL), drought stress with 5 μM SL treatment (D + 5 μM SL), respectively. The leaves of cotton seedlings were harvested and analyzed after 18 days of treatment. Exogenous SL was applied bi-weekly using a hand sprayer until the solution visibly dripped from the leaves. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test

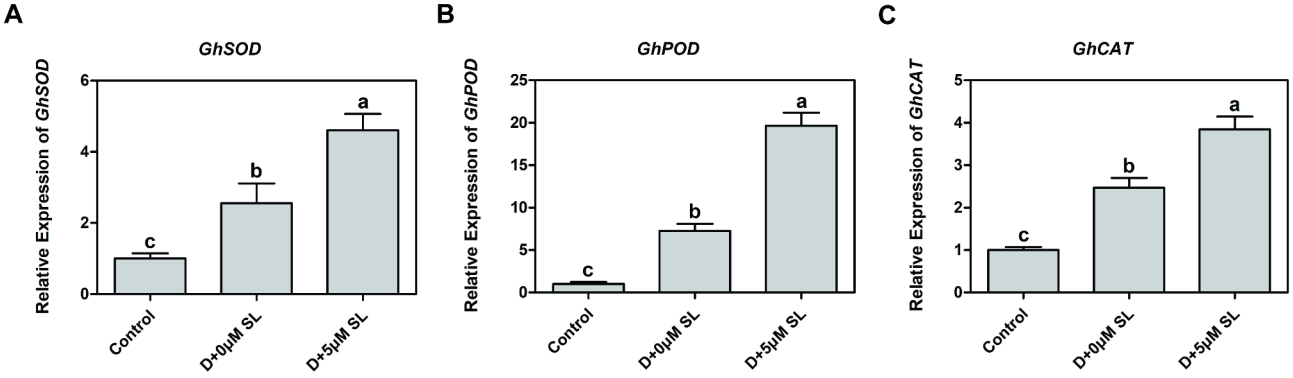

Fig. 5 The effects of 5 μM exogenous SL on the expression levels of drought stress-responsive genes in cotton seedlings leaves under drought stress. **(A)** the relative expression level of *GhSOD*, **(B)** the relative expression level of *GhPOD*, **(C)** the relative expression level of *GhCAT*. The 4-week-old cotton seedlings were subjected to normal water (control), drought stress with 0 μM SL treatment (D + 0 μM SL), drought stress with 5 μM SL treatment (D + 5 μM SL), respectively. The leaves of cotton seedlings were harvested and analyzed after 18 days of treatment. Exogenous SL was applied bi-weekly using a hand sprayer until the solution visibly dripped from the leaves. *GhUBQ7* was used as the internal control. The relative expression levels of *GhSOD*, *GhPOD* and *GhCAT* in normal water (control) plants were used as references and set to a value of 1. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test

drought-responsive genes *GhSOD*, *GhPOD*, *GhCAT*, *GhP5CS*, *GhRD22*, and *GhDREB2* using qRT-PCR. Figs. 5 and S6B-D display the impact of 5 μM exogenous SL on the expression of these genes in cotton leaves under drought stress. Relative to control plants, the expression of *GhSOD*, *GhPOD*, *GhCAT*, *GhP5CS*, *GhRD22*, and *GhDREB2* genes in drought-stressed cotton leaves with 0 μM SL treatment was significantly elevated by about 156%, 627%, 147%, 143%, 271%, and 264%, respectively (Figs. 5 and S6B-D). Furthermore, under drought stress, 5 μM SL treatment increased the *GhSOD*, *GhPOD*, *GhCAT*, *GhP5CS*, *GhRD22*, and *GhDREB2* expression in cotton leaves by about 80%, 170%, 56%, 99%, 103%, and 192%, respectively, compared with 0 μM SL treatment (Figs. 5 and S6B-D).

Identification and characterization of *GhMAX3* and *GhMAX4* in cotton

The SL biosynthesis genes in *Arabidopsis*, *AtMAX3* and *AtMAX4*, have been shown to enhance *Arabidopsis* drought resistance [14]. Utilizing the protein sequences of AtMAX3 (AT2G44990.2) and AtMAX4 (AT4G32810.2) as references, BLASTP searches were conducted to find their homologs in upland cotton. This analysis revealed two homologs of AtMAX3, GhMAX3-A12 (Gohir.A12G096200) and GhMAX3-D12 (Gohir.D12G098900), and four homologs of AtMAX4, GhMAX4a-A06 (Gohir.A06G092800), GhMAX4a-D06 (Gohir.D06G092400), GhMAX4b-A07 (Gohir.A07G022500), and GhMAX4b-D07 (Gohir.D07G025900). All identified MAX3 and MAX4 in both *Arabidopsis* and upland cotton contain the conserved RPE65 domain (pfam03055), and intron/

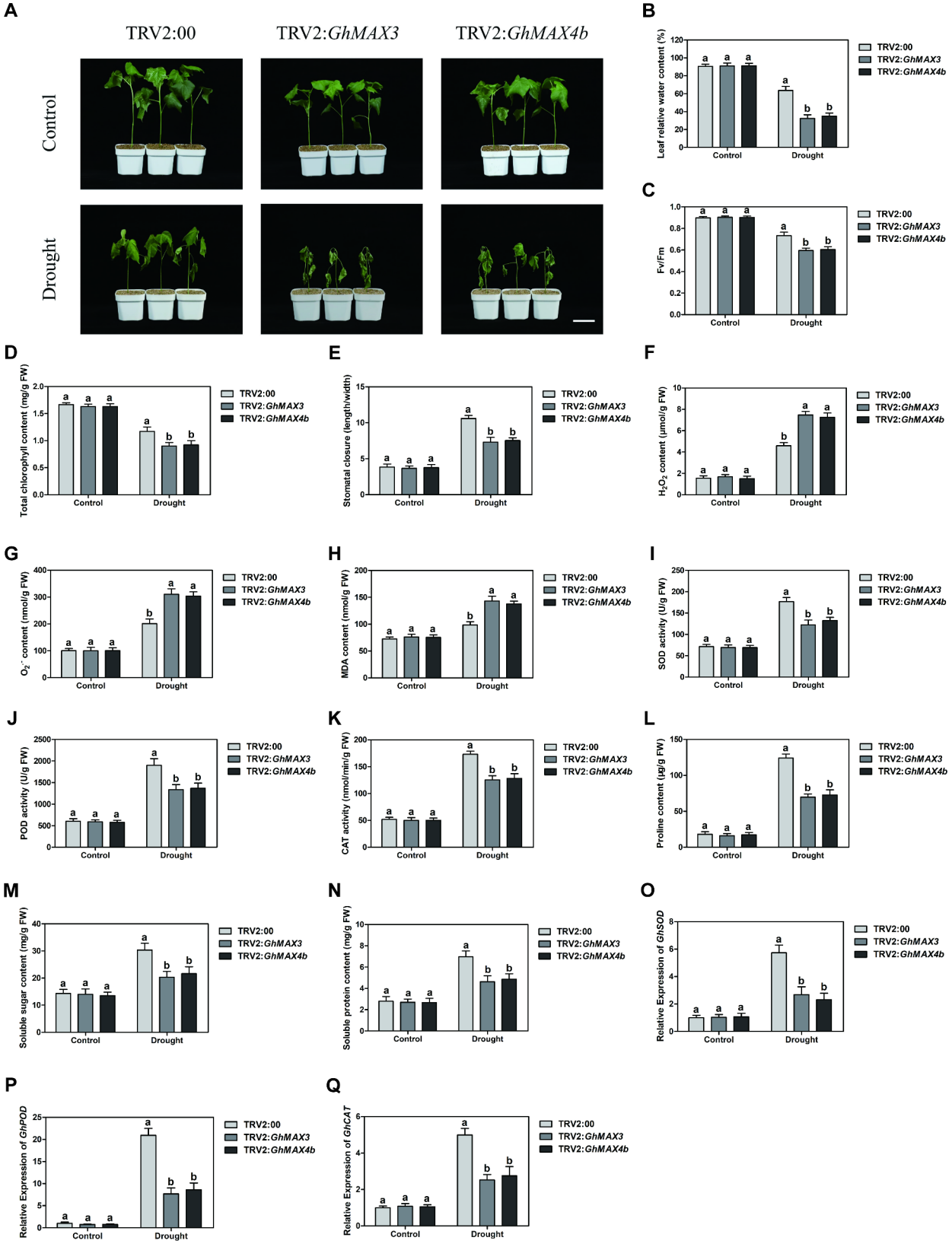

Fig. 6 (See legend on next page.)

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Fig. 6 Silencing the cotton SLs biosynthesis genes of *GhMAX3* and *GhMAX4b* individually using VIGS technology decreased cotton drought resistance. **(A)** Phenotype of TRV2:00, TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants under normal water treatment (control) and water-deficit treatment (drought treatment) for 12 days. (**B**-**Q**) LRWC, Fv/Fm, total chlorophyll content, stomata closure (length/width), H_2O_2 content, $O_2^{\bullet-}$ content, MDA content, SOD activity, POD activity, CAT activity, proline content, soluble sugars content, soluble proteins content, the relative expression level of *GhSOD*, the relative expression level of *GhPOD*, and the relative expression level of *GhCAT* in TRV2:00, TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants under normal water treatment and water-deficit treatment. *GhUBQ7* was used as the internal control. The relative expression levels of *GhSOD*, *GhPOD* and *GhCAT* in TRV2:00 plants under normal water treatment were used as references and set to a value of 1. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test. Scale bar, 6 cm

exon structures of the homologs exhibit high similarity, as depicted in Fig. S1.

The expression patterns of cotton SL biosynthesis genes, *GhMAX3*, *GhMAX4a* and *GhMAX4b*, were examined across various organs: roots, stems, and leaves. *GhMAX3* and *GhMAX4b* exhibited high expression levels in roots, whereas *GhMAX4a* was predominantly expressed in stems (Fig. S2A). Initially identified in cotton root exudates, SLs were later recognized as a novel class of plant hormones, known to facilitate the seed germination of root parasitic plants like *Striga* and *Orobanche* [1, 3, 4]. Given that SLs are primarily synthesized in plant roots, we focused on analyzing the relative expression levels of *GhMAX3*, *GhMAX4a* and *GhMAX4b* in cotton roots subjected to varying durations of PEG-induced drought stress (0, 3, 6, 12, and 24 h). *GhMAX3* expression in cotton roots was down-regulated at 3 h and up-regulated at 6, 12, and 24 h post-PEG treatment (Fig. S2B). *GhMAX4a* expression was down-regulated at 3 h and up-regulated at 24 h under PEG treatment (Fig. S2B). *GhMAX4b* expression significantly increased at 6 h following PEG exposure compared to the initial time point (Fig. S2B).

Silencing *GhMAX3* or *GhMAX4b* in cotton decreased drought resistance

To elucidate the role of *GhMAX3*, *GhMAX4a* and *GhMAX4b* in cotton's drought resistance, we employed VIGS to specifically reduce the expression of these genes. The empty vector TRV2:00 and the TRV2:*GhCLA* served as the negative and positive controls, respectively. The leaf albino phenotype in TRV2:*GhCLA* plants became visible 14 days post-*Agrobacterium*-mediated injection (Fig. S3A). In the roots of TRV2:*GhMAX3*, TRV2:*GhMAX4a*, and TRV2:*GhMAX4b* plants, the expression of *GhMAX3*, *GhMAX4a* and *GhMAX4b* was significantly lower than in TRV2:00 plants (Fig. S3B). To verify whether the reduction in SL content in these silenced plants, we utilized the *O. cumana* seed germination assay, a bioassay indicative of SL presence in root extracts [13, 78]. The germination percentages of *O. cumana* seeds were reduced by treatment with the extracts from TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants, but not with that from TRV2:*GhMAX4a* plants, relative to that of TRV2:00 plants (Fig. S3C). These findings suggest that silencing *GhMAX3* and *GhMAX4b* effectively reduced SL levels in these plants. Thus, we selected the *GhMAX3* and *GhMAX4b* genes for further investigation into their contribution to cotton's drought resistance.

For the drought treatment, four-week-old TRV2:00, TRV2:*GhMAX3*, and TRV2:*GhMAX4b* cotton plants were subjected to water deprivation for 12 days, while normally watered plants served as controls. Under drought stress, TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants displayed more pronounced wilting compared to TRV2:00 plants (Fig. 6A). Correspondingly, the LRWC, Fv/Fm, and chlorophyll concentration in TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants were lower than in TRV2:00 plants under drought stress (Fig. 6B-D). Stomatal closure in TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants was also reduced compared to TRV2:00 plants under drought stress (Fig. 6E). The levels of H_2O_2, $O_2^{\bullet-}$, and MDA were higher in TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants than in TRV2:00 plants under drought stress (Fig. 6F-H). The activities of SOD, POD, and CAT were lower in TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants compared to TRV2:00 plants under drought stress (Fig. 6I-K). Moreover, the contents of proline, soluble sugars, and soluble proteins were significantly lower in TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants compared to TRV2:00 plants under drought stress (Fig. 6L-N). Additionally, the expression levels of *GhSOD*, *GhPOD*, *GhCAT*, *GhP5CS*, *GhRD22*, and *GhDREB2* were significantly down-regulated in TRV2:*GhMAX3* and TRV2:*GhMAX4b* plants relative to TRV2:00 plants under drought conditions (Figs. 6O-Q and S7B-D). These findings demonstrate that silencing of TRV2:*GhMAX3* and TRV2:*GhMAX4b* in cotton reduces drought resistance.

Overexpressing *GhMAX3* and *GhMAX4b* in corresponding *Arabidopsis* mutants rescued the drought-sensitive phenotype of mutants

Arabidopsis SL-biosynthetic mutants (*max3* and *max4*) demonstrated hypersensitivity to drought stress [14]. To elucidate the function of *GhMAX3* and *GhMAX4b* in drought stress response, these cotton SL biosynthesis genes were individually transformed into *Arabidopsis max3* and *max4* mutants. PCR analysis confirmed the presence of *GhMAX3* and *GhMAX4b* in the transgenic *Arabidopsis* lines, but not in the WT and mutants (Fig. S4A). qRT-PCR revealed that the expression levels of

GhMAX3 and *GhMAX4b* in the transgenic lines were significantly higher than in WT and mutants (Fig. S4B). These findings demonstrate the successful integration of *GhMAX3* and *GhMAX4b* into the *Arabidopsis* genome, facilitating further analysis of their roles in drought stress response.

For the drought treatment, three-week-old *Arabidopsis* seedlings underwent 21 days without water, while normally watered seedlings served as controls. It was observed that under normal conditions, the *Arabidopsis* mutants (*max3* and *max4*) had more rosette leaves compared to WT, but this phenotype was restored to WT levels in the transgenic lines (35S::*GhMAX3* and 35S::*GhMAX4b*) (Fig. 7A). Post-drought treatment, the leaves of the *Arabidopsis* mutants displayed severe dehydration and wilting, in contrast to both the WT and the transgenic lines (Fig. 7A). While the LRWC and chlorophyll concentration showed no significant difference between WT and transgenic lines under drought conditions, these parameters were markedly reduced in the mutants compared to both WT and transgenic lines (Fig. 7B, C). These findings indicate that overexpression of *GhMAX3* and *GhMAX4b* in the respective *Arabidopsis* mutants effectively mitigated the drought-sensitive phenotype of these mutants.

Discussion

Drought stress significantly impairs crop growth and substantially reduces yield by various mechanisms. While SLs have been reported to enhance drought resistance in plants, their specific roles in the cotton drought response are not well understood. This study aimed to elucidate the mechanisms through which SLs modulate the response of cotton to drought stress.

SLs enhance cotton resistance to drought stress

Drought stress profoundly affects plant growth and significantly reduces crop yields. Exogenous application of SL (*rac*-GR24) has been documented to mitigate growth inhibition under drought conditions and to enhance drought resistance in plants [41, 79]. In this study, four-week-old cotton seedlings underwent water-deficit treatment for 18 days and received bi-weekly sprays of exogenous SL (*rac*-GR24) at varying concentrations (0, 1, 5, 10, and 20 μM). Normally watered seedlings served as controls. Growth parameters were observed to decrease under drought stress compared to the control condition

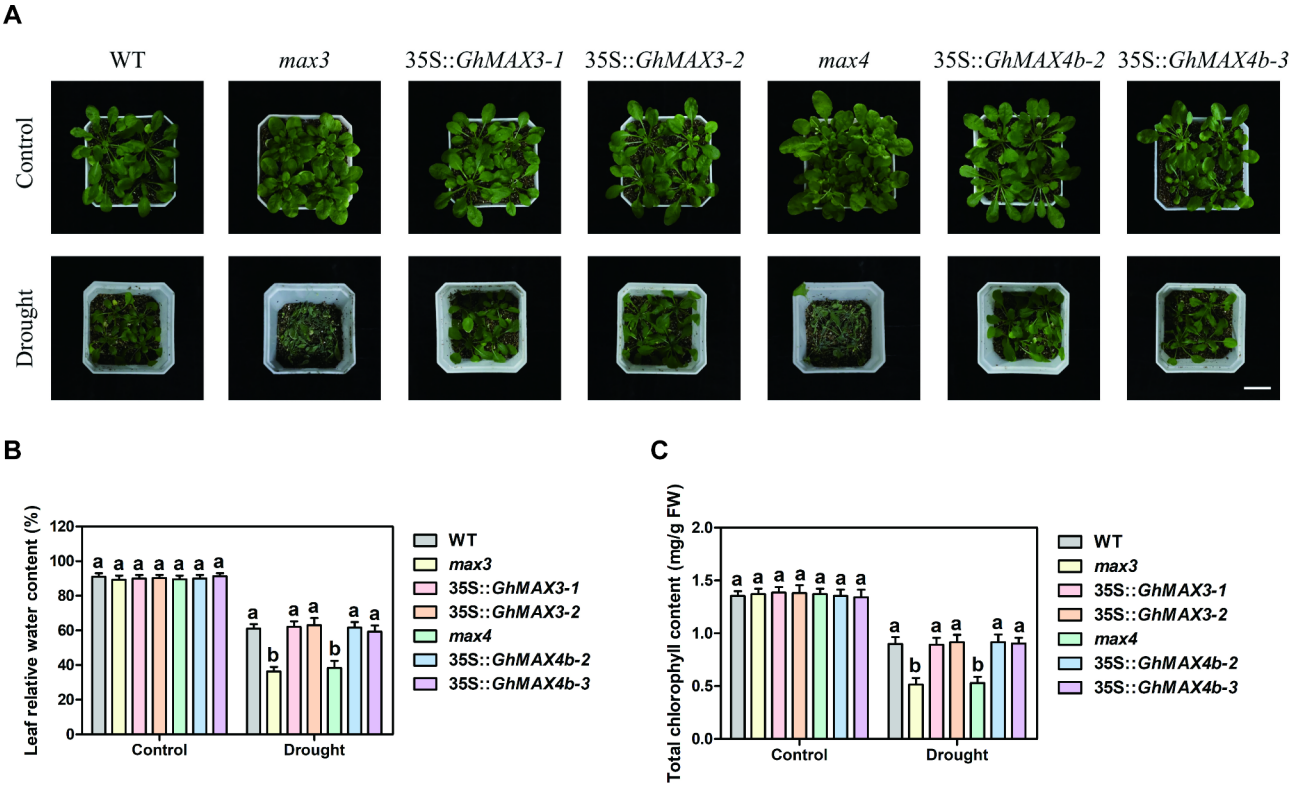

Fig. 7 Overexpressing the cotton SLs biosynthesis genes *GhMAX3* and *GhMAX4b* in corresponding *Arabidopsis* mutants rescued the drought-sensitive phenotype of *Arabidopsis* mutants. **(A)** Phenotype of WT, mutant (*max3* and *max4*) and transgenic lines (35S::*GhMAX3* and 35S::*GhMAX4b*) under normal water treatment (control) and water-deficit treatment (drought treatment) for 21 days. (**B**-**C**) LRWC and total chlorophyll content in WT, mutant (*max3* and *max4*) and transgenic lines (35S::*GhMAX3* and 35S::*GhMAX4b*) under normal water treatment and water-deficit treatment. The values indicate means ± SD, $n = 3$. Different letters represent significant differences ($p < 0.05$) according to Tukey's test. Scale bar, 2 cm

(Fig. 1). However, the growth inhibition caused by drought stress was significantly alleviated by the 5 μM SL application, suggesting that this concentration optimally counteracts drought-induced growth reduction and enhances drought resistance in cotton. Additionally, silencing SL synthesis genes (*GhMAX3* and *GhMAX4b*) in cotton through VIGS technology increased the cotton's drought sensitivity (Fig. 6). Previous reports have identified SL as a crucial modulator in the drought response of *Arabidopsis*, with SL-biosynthetic mutants (*max3* and *max4*) displaying hypersensitivity to drought conditions [14]. Overexpression of cotton SL biosynthesis genes (*GhMAX3* and *GhMAX4b*) in these *Arabidopsis* mutants ameliorated their drought-sensitive phenotypes (Fig. 7). The findings of this study confirm that SLs play a significant role in mitigating drought-induced growth suppression and in boosting drought resistance in cotton. The regulation of *GhMAX3* and *GhMAX4b* has not been extensively studied in cotton. Understanding the regulatory mechanisms governing these genes is crucial for elucidating their roles in cotton's response to drought stress. Future studies that focus on the upstream regulatory elements and transcription factors influencing the expression of *GhMAX3* and *GhMAX4b* under drought conditions in cotton would be particularly interesting.

Diverse mechanisms of SLs-mediated cotton resistance to drought stress

Drought stress impairs the structure and function of plant photosynthetic organs, affecting both the photochemical and dark reactions of photosynthesis [80]. The chlorophyll fluorescence parameter Fv/Fm, indicative of the maximal photochemical efficiency of photosystem II (PSII), is particularly sensitive to drought stress [81]. Drought stress leads to a decline in the photosynthetic rate of leaves, subsequently impacting plant growth and productivity. In our study, both Fv/Fm and total chlorophyll content decreased under drought conditions; however, these reductions were significantly mitigated by the application of 5 μM exogenous SL (Fig. 2A, B), echoing findings in grapevine subjected to drought stress [41]. Under normal conditions, no significant difference was observed in Fv/Fm and total chlorophyll content between control (TRV2:00) and silenced plants (TRV2:*GhMAX3* and TRV2:*GhMAX4b*). However, under drought stress, both Fv/Fm and total chlorophyll content decreased in silenced plants compared to control plants (Fig. 6C, D). These findings suggest that SLs play a crucial role in enhancing photosynthetic efficiency under drought stress by preserving the integrity of cotton chlorophyll.

Stomata, formed by a pair of guard cells, are critical for gas exchange between the leaf interior and the atmosphere, regulating both CO_2 uptake for photosynthesis and water vapor loss during transpiration [82]. Stomatal closure, a key physiological adaptation to drought stress, effectively minimizes plant water loss [83]. However, this water conservation through stomatal closure compromises photosynthesis, growth, and yield due to the restricted CO_2 entry and transpiration [75]. In our study, a 5 μM exogenous SL application significantly enhanced stomatal closure under drought stress compared to the application of 0 μM SL (Figs. 2C, S6A), a finding echoed in grapevine subjected to similar stress [41]. Additionally, under normal conditions, stomatal closure did not differ significantly between the control (TRV2:00) and silenced plants (TRV2:*GhMAX3* and TRV2:*GhMAX4b*). However, under drought conditions, the stomatal closure in silenced plants was less than in control plants, indicating a higher rate of water conservation in the latter (Figs. 6E, S7A). These observations suggest that SLs modulate cotton's stomatal closure, effectively reducing water loss during drought stress.

Numerous studies have shown that drought stress disrupts the balance of ROS metabolism by influencing the production and removal of ROS, including H_2O_2 and $O_2^{\bullet-}$. Excessive ROS accumulation can lead to membrane lipid peroxidation, causing metabolic disorders. MDA, a product of lipid peroxidation, reflects the level of lipid peroxidation and the extent of membrane damage in plant cells. Drought stress tends to increase ROS production, leading to a significant rise in MDA levels within plant cells. SOD, POD, and CAT are crucial antioxidant enzymes that effectively remove excess ROS, such as H_2O_2 and $O_2^{\bullet-}$, from plant cells [76]. In our study, the foliar application of 5 μM exogenous SL under drought stress significantly reduced ROS (H_2O_2 and $O_2^{\bullet-}$) and MDA accumulation while increasing the activities of antioxidant enzymes (SOD, POD, and CAT) in cotton leaves, in contrast to the 0 μM SL application (Fig. 3). This is in line with observations in winter wheat under drought stress [79]. Furthermore, under normal conditions, no significant difference was noted in ROS and MDA levels or antioxidant enzyme activities between control (TRV2:00) and silenced plants (TRV2:*GhMAX3* and TRV2:*GhMAX4b*). However, under drought conditions, increased ROS and MDA contents, along with decreased antioxidant enzyme activities, were observed in silenced plants compared to control plants (Fig. 6F-K). These findings suggest that SLs can modulate oxidative balance in cotton under drought stress, preventing excessive ROS accumulation and thereby reducing membrane lipid peroxidation damage.

Osmotic regulation is a crucial self-defense mechanism in plants under stress. Substances like proline, soluble sugars, and soluble proteins, are involved in osmotic regulation, enhance cellular osmotic pressure, mitigate water loss, alleviate growth inhibition caused by stress, and bolster plant stress resistance [77]. Drought stress has been reported to

increase the content of osmotic adjustment substances [84–87]. In our study, the levels of osmotic adjustment substances, including proline, soluble sugars, and soluble proteins, were significantly increased under drought stress. Furthermore, this increase was notably enhanced by the application of 5 μM exogenous SL under drought stress (Fig. 4), echoing findings in wheat and alfalfa [88, 89]. Under normal conditions, there was no significant difference in the content of osmotic adjustment substances between control (TRV2:00) and silenced plants (TRV2:*GhMAX3* and TRV2:*GhMAX4b*). Yet, under drought stress, the levels of these substances were lower in silenced plants compared to control plants (Fig. 6L-N). These findings suggest that SLs play a crucial role in regulating cotton's osmotic balance, thereby minimizing the detrimental effects of drought stress.

The genes *SOD*, *POD*, and *CAT* encode crucial antioxidant enzymes that efficiently eliminate excessive ROS from plant cells, thereby playing a vital role in the plant's response to drought stress [76, 90, 91]. In this study, the foliar application of 5 μM exogenous SL significantly enhanced the expression levels of the *GhSOD*, *GhPOD*, and *GhCAT* genes compared to the 0 μM SL treatment under drought conditions (Fig. 5). Additionally, under normal conditions, no significant difference was observed in the expression levels of these genes between control (TRV2:00) and silenced plants (TRV2:*GhMAX3* and TRV2:*GhMAX4b*). Yet, under drought stress, the expression levels of these genes were lower in silenced plants than in control plants (Fig. 6O-Q). These findings align with the observed activities of SOD, POD, and CAT enzymes and the levels of ROS (Figs. 3 and 6F-K). *GhP5CS*, *GhRD22*, and *GhDREB2* were used to evaluate the drought resistance of cotton [92, 93]. In this study, the foliar application of 5 μM exogenous SL significantly enhanced the expression levels of the *GhP5CS*, *GhRD22*, and *GhDREB2* genes compared to the 0 μM SL treatment under drought conditions (Fig. S6B-D). Additionally, under drought stress, the expression levels of these genes were lower in silenced plants (TRV2:*GhMAX3* and TRV2:*GhMAX4b*) than in control plants (TRV2:00) (Fig. S7B-D). These findings indicate that SLs may play a critical role in enhancing cotton's drought resistance by regulating the expression of these drought stress-responsive genes.

In conclusion, SLs significantly bolster cotton's resistance to drought. This enhancement is achieved through the modulation of various physiological processes and molecular pathways, including photosynthesis, stomatal closure, ROS metabolism, osmotic adjustment, and the regulation of drought stress-responsive gene expression (Fig. 8). These multifaceted roles underscore the integral function of SLs in improving the drought resilience of cotton.

SLs inhibit shoot branching

SLs play a crucial role in regulating shoot branching. Research has shown that SLs inhibit bud outgrowth, as demonstrated in experiments with highly branched mutants, such as *ccd8* (involved in SL biosynthesis) and *max2* (involved in SL signaling). In these studies, applying GR24 to buds restored the branching of *ccd8* mutants to WT levels, but it had no effect on *max2* mutants [3, 4]. These findings highlight SLs as a promising target for modifying plant architecture to enhance productivity. SL-deficient mutants (*max3* and *max4*) in *Arabidopsis* exhibit increased branching [23]. In this study, we observed that under normal conditions, the *Arabidopsis* mutants (*max3* and *max4*) developed more rosette leaves than the WT, but

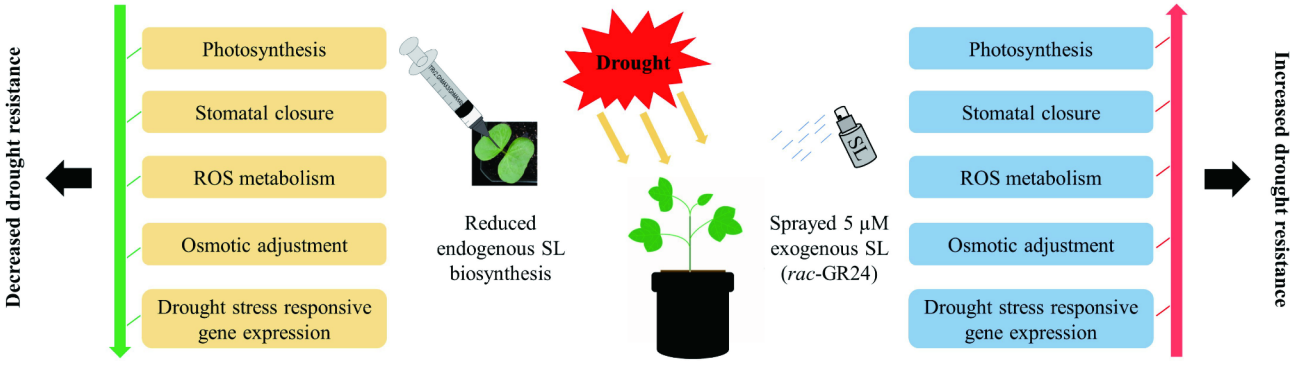

Fig. 8 A proposed model for the function of SLs in enhancing cotton drought resistance by regulating photosynthesis, stomatal closure, ROS metabolism, osmotic adjustment, and drought stress-responsive gene expression. Foliar application of 5 μM exogenous SL (*rac*-GR24) improves the photosynthetic capacity, facilitates stomatal closure to minimize water loss, and reduces ROS production, thus decreasing membrane lipid peroxidation and cellular damage. *rac*-GR24 also boost the activities of antioxidant enzymes like SOD, POD, and CAT, which help in detoxifying excess ROS. Additionally, they increase the levels of osmotic regulatory substances such as proline, soluble sugars, and soluble proteins, aiding in water retention and cellular osmoregulation. This is complemented by the upregulation of drought stress-responsive genes, enhancing the plant's ability to withstand drought conditions. Conversely, silencing the cotton SLs biosynthesis genes *GhMAX3* and *GhMAX4b* through VIGS technology leads to adverse effects: reduced photosynthetic capacity and stomatal closure, increased ROS production and membrane lipid peroxidation, decreased antioxidant enzyme activities, lowered osmotic regulatory substance levels, and diminished expression of drought stress-responsive genes, ultimately decreasing cotton's drought resistance.

this phenotype was restored to WT levels in the transgenic lines (35S::*GhMAX3* and 35S::*GhMAX4b*) during vegetative growth (Fig. 7A). In cotton, Yi et al. reported that SLs positively influence verticillium wilt resistance [13]. Additionally, Wen et al. demonstrated that SLs contribute to cotton fiber elongation and secondary cell wall thickening [94]. However, the role of SLs in regulating cotton shoot branching has yet to be fully elucidated. Investigating how SLs regulate cotton shoot branching presents an intriguing area for future research.

Conclusions

This study comprehensively demonstrates the pivotal role of SLs in enhancing drought resistance in cotton, providing significant insights into both physiological enhancements and genetic regulations. Our results reveal that the application of exogenous SL (*rac*-GR24) at a concentration of 5 μM optimizes several key mechanisms that underpin drought resistance. These include the enhancement of photosynthetic capacity, effective stomatal closure to reduce water loss, and a reduction in oxidative stress through decreased ROS production and lipid peroxidation. Furthermore, *rac*-GR24 elevates the activities of critical antioxidant enzymes such as SOD, POD, and CAT, and boosts the levels of osmotic regulatory substances like proline, soluble sugars, and soluble proteins, which are vital for maintaining cellular function under drought conditions. Importantly, the silencing of cotton SL biosynthesis genes, *GhMAX3* and *GhMAX4b*, through VIGS technology highlighted their essential roles in regulating these drought resistance pathways, as their inhibition led to a significant decrease in the plant's drought tolerance. This was evidenced by the diminished photosynthetic efficiency, reduced stomatal regulation, increased oxidative damage, decreased osmotic regulatory substances contents, and lower expression of drought-responsive genes. Our research underscores the potential of modulating SL pathways as a novel strategy for improving drought resilience in cotton, which could have profound implications for sustainable agriculture and crop production under changing climatic conditions.

Abbreviations

SL	Strigolactone
MAX	More axillary growth
AM	Arbuscular mycorrhizal
PEG	Polyethylene glycol
LRWC	Leaf relative water content
Fv/Fm	variable fluorescence/maximum fluorescence
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
$O_2^{\bullet-}$	Superoxide anion
MDA	Malondialdehyde
SOD	Superoxide dismutase
POD	Peroxidase
CAT	Catalase
BLASTP	BLAST algorithm for proteins
VIGS	Virus-induced gene silencing
ANOVAs	Analyses of variance

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05726-w.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8
Supplementary Material 9

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Author contributions

JD performed the experiments and wrote the original manuscript; CD performed the experiments. HC, HF and RP performed the data curation. FS reviewed and edited the manuscript. WW designed the experiments and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability

All supporting data for this manuscript are included in the manuscript and its additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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