

# Enhancing cotton tolerance to cold and drought stress: A protective role of SikCOR413PM1 gene overexpression

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# Abstract

This study explores the potential role of the *SikCOR413PM1* gene, isolated from *Saussurea involucrata* (Kar Kir.), in enhancing cotton (*Gossypium hirsutum*) tolerance to cold and drought stress through transgenic methods. We found that overexpressing *SikCOR413PM1* in cotton plants led to increased relative water content, reduced malondialdehyde content and relative conductivity, and elevated proline and soluble sugar levels as osmotic regulators. These findings suggest that *SikCOR413PM1* can minimize cell membrane damage and boost plant stability under challenging conditions. Additionally, overexpression of this gene upregulated antioxidant enzyme-related genes in transgenic cotton plants, resulting in enhanced enzyme activity, lowered peroxide content, and reduced oxidative stress. *SikCOR413PM1* overexpression also modulated the expression of stress-related genes (*GhDREB1A*, *GhDREB1B*, *GhDREB1C*, *GhERF2*, *GhNAC3*, and *GhRD22*). In field trials, transgenic cotton plants with *SikCOR413PM1* overexpression displayed higher yields and increased environmental resistance. Our study concludes that *SikCOR413PM1* can regulate stress-related genes, osmotic adjustment factors, and peroxide content while preserving cell membrane stability and improving cotton tolerance to low-temperature and drought conditions.

## Background

During their growth and development, plants are most commonly affected by abiotic stress factors such as cold, drought, and salt stress [1]. Previous studies have found that exposure of plants to a certain degree of abiotic stress in advance can improve their ability to better adapt to subsequent stress [2, 3].

To cope with environmental changes, plants have evolved various regulatory mechanisms under abiotic stress, including osmotic and oxidative stress, for self-protection. Stress can lead to increases in reactive oxygen species (ROS) in plants [4, 5, 6]. However, excessive ROS accumulation can cause oxidative damage to cells, inhibiting plant physiological processes [7, 8]. Under normal growth conditions, plants generally maintain a relatively low level of ROS, and ROS scavenging and production are usually balanced [9].

The main ROS components are  $O_2^-$  and  $H_2O_2$ , which are important signal transduction molecules mediating multiple stress tolerance [10].  $H_2O_2$  triggers the regulation of the plant antioxidant system through ABA and BRs to achieve cold stress regulation and improve cold tolerance [11, 12, 13, 14]. Numerous studies have shown that the antioxidant mechanism of inducing ROS scavenging is essential in resisting various stresses [15, 16, 17].

Plant responses to cold stress involve multiple levels of regulation, including metabolic changes such as the accumulation of sugars and amino acids and the C-receptor binding factor (CBF)/dehydration response element binding factor (DREB) pathway [18, 19]. CBF proteins can bind to C-receptor (CRT) cis-elements and activate the transcription of downstream cold-regulated (COR) genes to improve cold tolerance [12, 20, 21, 22].

The CBF transcription factor acts as a positive regulator of cold signals. The CBF-encoded domain binds to the conserved C-repeat/dehydration response element (CRT/DRE) motif on the COR gene promoter to activate the transcription and expression of the COR gene [12, 23, 24, 25]. Numerous studies have demonstrated that the expression of the COR gene is positively correlated with the cold resistance of plants [26, 27, 28, 29].

The *COR413* family encodes plant-specific multi-transmembrane proteins [30]. Based on predicted intracellular localization, the COR413 protein family can be divided into two subclasses: chloroplast *COR413-TM/IM* (thylakoid membrane/intima) and plasma membrane *COR413-PM* [30]. Some studies have shown that *COR413* family genes can improve plant stress resistance. For example, in *Arabidopsis*, *AtCOR413IM* can stabilize the chloroplast membrane under low-temperature stress [31, 32], while overexpression of *COR413-TM1* in rice can enhance drought tolerance [33]. The gene encoding tomato chloroplast *COR413* (*SICOR413IM1*) also improves the drought tolerance of tobacco plants [34].

*COR413-PM*, located in the plasma membrane, can also improve plant stress resistance. Overexpression of *SikCOR413PM1* isolated from *Saussurea involucreata* (Kar Kir.) enhanced the tolerance of transgenic tobacco to cold and drought stress [35]. Zhou et al. (2018) [36] found that overexpression of *PsCOR413PM2* increased *Arabidopsis* tolerance to osmotic stress and improved its cold tolerance. Similarly, Zhang et al. (2021) [37] found that *LeCOR413PM2* improved the cold tolerance of transgenic tomato [37]. Overexpression of the plasma membrane *COR413PM* gene isolated from drought-resistant plants in *Arabidopsis thaliana* can enhance tolerance to drought and low temperatures.

Cotton is one of the most important agricultural products in the world, and has been cultivated in over 80 countries according to statistics [38]. It is mainly grown in arid regions such as tropical and subtropical areas, but has poor tolerance to drought and cold stress [39, 40]. The cotton industry has been severely affected by drought, leading to a rapid decline in fiber production [41]. Furthermore, a combination of two or more abiotic stresses can cause much greater yield loss than one single stress [42]. Therefore, developing crops with tolerance to multiple stresses has significant practical significance for agricultural production. Many scientists have studied stress-related genes and used transgenic technology to improve plant stress resistance [43].

In our previous study, we have isolated the *SikCOR413PM1* gene from *Saussurea involucreata* (Kar Kir.), whose overexpression was found to improve the stress resistance of transgenic tobacco [35]. This study further explored the role of *SikCOR413PM1* gene overexpression in cotton. The results showed that *SikCOR413PM1* cotton exhibited stronger stress tolerance compared to the wild-type in both greenhouse and field trials. The findings provide a theoretical and experimental basis for future research on this gene and the application of new cotton varieties.

## Results

### Acquisition and identification of transgenic cotton plants with the *SikCOR431PM1* gene

To investigate the influence of the *SikCOR431PM1* gene on the cold and drought tolerance of cotton, we integrated the *SikCOR431PM1* gene into cotton using the pollen tube channel method to obtain *SikCOR431PM1* transgenic cotton. Ten transgenic cotton lines were screened and analyzed by qRT-PCR (Fig. 1A). The relative expression levels of OE-4, OE-5, and OE-7 were found to be higher and were further analyzed by RT-PCR. The results were consistent with those of qRT-PCR (Fig. 1B). Therefore, OE-4, OE-5, and OE-7 were selected to conduct further gene function research.

### **Growth of cotton seedlings overexpressing *SikCOR413PM1* gene under low-temperature and drought stress conditions**

Cotton seedlings were exposed to low temperature and 20% PEG6000 stress. Under normal conditions (25°C), the WT and transgenic lines grew well without treatment. However, after exposure to 4°C, the leaves of WT cotton seedlings showed severe wilting, and most of them became necrotic and damaged due to the low temperature. In contrast, the leaves of transgenic lines showed much less severe wilting and still had certain vitality (Fig. 2A). After treatment with 20% PEG6000, the leaves of some WT lines exhibited noticeable wilting and curling, while those of transgenic cotton showed only weak wilting. This pattern persisted for 15 days, during which time the WT lines exhibited more significant wilting and withering than the transgenic lines. After 20 days of treatment, some WT lines exhibited even more severe growth retardation and leaf wilting, whereas the transgenic lines showed less severe leaf wilting (Fig. 2B).

To further clarify whether overexpression of the *SikCOR413PM1* gene can improve cell protection mediated by low-temperature and drought stress in cotton seedlings, we measured the survival rates of WT and transgenic lines (OE-4, OE-5, and OE-7) under low-temperature and 20% PEG6000 stress to evaluate their survival under these conditions. Under normal conditions, the survival rate of each cotton line was 100%. However, after exposure to 4°C, the survival rates of the WT and transgenic lines decreased to 23.3% (WT), 65.6% (OE-4), 73.3% (OE-5), and 80% (OE-7), respectively. After treatment with 20% PEG6000, the survival rates of the WT and transgenic lines were reduced to 33.3% (WT), 61.1% (OE-4), 53.3% (OE-5), and 68.9% (OE-7), respectively. Compared to the WT, the transgenic lines exhibited higher viability (Fig. 2C).

Under normal conditions, there was no significant difference in average fresh weight and dry weight between the WT and transgenic lines. However, after exposure to low temperatures, the wet weight of transgenic lines OE-4, OE-5, and OE-7 was 23.6%, 36.6%, and 41.3% higher than that of the WT, respectively (Fig. 2D), while the dry weight was 23.7%, 24.1%, and 28.1% higher (Fig. 2E). Under 20% PEG6000 stress, the fresh weight and dry weight were lower than in the untreated control group. Nevertheless, the OE-4, OE-5, and OE-7 lines exhibited 56.0%, 50.9%, and 72.5% higher fresh weight than the WT, and 56.5%, 44.7%, and 63.5% higher dry weight than the WT. These results demonstrate that overexpression of *SikCOR413PM1* enhances the tolerance of transgenic cotton to low temperatures and drought at the seedling stage.

### **Overexpression of the *SikCOR413PM1* gene enhanced the cold and drought tolerance of cotton seedlings.**

The WT and transgenic cotton plants grown for 70 days exhibited robust growth at room temperature (25°C) without treatment. However, after 24 h of exposure to 4°C, the leaves at the top of the WT plants showed wilting, whereas there was no significant change in the transgenic plants. After 48 h of exposure to 4°C, the WT plants exhibited severe wilting at the top growth point, while the transgenic plants showed only slight leaf wilting and drooping (Fig. 3A). After 14 days of drought treatment, the WT plants showed obvious signs of wilting, and the leaves near the base became loose and yellow. In contrast, the leaves of transgenic cotton showed less severe wilting. After 21 days of drought treatment, compared to the transgenic plants, the WT plants exhibited more severe growth retardation and leaf wilting and shedding, while the leaves of transgenic plants did not fall off, and the stems of all transgenic plants remained full throughout the treatment process (Fig. 3B).

Under normal conditions, there was no significant difference in average fresh weight and dry weight between the WT and transgenic plants. However, after exposure to low temperatures, the wet weights of transgenic plants OE-4, OE-5, and OE-7 were 19.4%, 24.2%, and 25.5% heavier than those of WT plants (Fig. 3C), and dry weights were 28.3%, 36.9%, and 41.1% heavier (Fig. 3D). Under drought stress, the fresh weight and dry weight were lower than in the untreated control group. Nevertheless, the fresh weight of transgenic plants OE-4, OE-5, and OE-7 was 30.1%, 37.2%, and 42.6% heavier than that of the WT, and the dry weight was 40.6%, 50.6%, and 60.4% heavier than that of the WT. These findings demonstrate that overexpression of the *SikCOR413PM1* gene enhances the cold and drought resistance of transgenic cotton seedlings.

**Figure 3 Phenotype analysis of WT and *SikCOR413PM1* -expressing cotton plants under cold and drought stress.**(A) Analysis of phenotype 70-d-old WT and transgenic plants OE-4, OE-5, and OE-7 under 4°C for 24 h and 48 h, respectively; (B) Analysis of phenotype 70-d-old WT and transgenic plants OE-4, OE-5, and OE-7 after drought treatment 14 and 21 days, respectively; (C) Fresh weight ; (D) Dry weight. The data represent the average of three independent biological replicates. Bars represent SDs. \*P < 0.05 and \*\*P < 0.01 indicate significant differences relative to WT cotton plants.

### **Overexpression of *SikCOR413PM1* alleviated cell membrane damage and accumulated more osmoregulatory substances.**

To further elucidate the possible physiological mechanism through which *SikCOR413PM1* overexpression improves cell protection under low-temperature and drought stress in cotton, we assessed the malondialdehyde (MDA) content and relative conductivity of WT and transgenic plants (OE-4, OE-5, and OE-7) to evaluate their physiological response to low-temperature and drought stress conditions. Compared to that of the WT plants, the MDA content in the leaves of transgenic plants was significantly reduced (Fig. 4A). Under 4°C stress treatment, the MDA content of transgenic cotton was 31.7% (OE-4), 31.6% (OE-5), and 36.9% (OE-7) lower than that of WT cotton, respectively. Under drought treatment, the MDA content of transgenic cotton was 26.3% (OE-4), 27.5% (OE-5), and 35.8% (OE-7) lower than that of WT cotton, respectively (Fig. 4B). There was no significant difference in relative conductivity between transgenic lines and WT under normal conditions. However, after exposure to 4°C, the relative electrolyte

leakage (REL) of the WT and transgenic plants increased by 49.9% (WT), 36.3% (OE-4), 33.9% (OE-5), and 39.4% (OE-7), respectively. After drought treatment, the REL of the WT and transgenic plants increased by 46.6% (WT), 33.2% (OE-4), 29.5% (OE-5), and 36.8% (OE-7), respectively.

Figures 4C and D (Fig. 4) show that before stress, the contents of proline and soluble sugar were low in both WT and *SikCOR413PM1* transgenic plants, and there was no significant difference between them. However, the contents of these two osmolytes significantly increased after exposure to low temperature and drought stress. Under 4°C stress, the proline and soluble sugar contents of transgenic lines were 43.1% and 32.0% (OE-4), 40.8% and 38.6% (OE-5), and 55.1% and 42.9% (OE-7) higher than those of WT lines, respectively. After drought treatment, the proline and soluble sugar contents of transgenic lines were 41.8% and 36.9% (OE-4), 42.7% and 45.8% (OE-5), and 47.2% and 47.9% (OE-7) higher than those of the WT, respectively. In summary, overexpression of the *SikCOR413PM1* gene in cotton can maintain the integrity of the cell membrane and promote the accumulation of osmotic protective agents under stress conditions.

### **Overexpression of the *SikCOR413PM1* gene maintained high antioxidant enzyme activity and expression analysis of related genes.**

In this study, we detected the contents of  $O_2^-$  and  $H_2O_2$ , which can reflect the response of plants to stress in both WT and transgenic lines. The results showed that there was no significant difference among all lines under normal conditions. However, after exposure to low temperature and drought stress, there were significant differences between these lines. Compared to those in the WT lines, the contents of  $O_2^-$  and  $H_2O_2$  in transgenic lines were significantly reduced (Fig. 5A and B).

We also measured the activity levels of the antioxidant enzymes SOD, POD, CAT, and GST. Under normal conditions, there was no significant difference between the transgenic lines and the WT. However, under low-temperature and drought stress conditions, each stress treatment enhanced the antioxidant enzyme activity of all plants, and the transgenic lines had higher antioxidant enzyme activities than the WT lines. Consistently, the expression levels of the *GhSOD*, *GhPOD*, *GhCAT*, and *GhGST* coding genes in the transgenic lines were also significantly higher than those in the WT lines after stress treatment (Fig. 5C-J). In summary, overexpression of the *SikCOR413PM1* gene increased the expression of antioxidant enzyme genes and enhanced the activity of corresponding antioxidant enzymes, thereby regulating the amount of ROS to minimize the effect of oxidative stress.

**Figure 5 Analysis of ROS and antioxidant enzyme activity and expression of related genes in WT and transgenic *SikCOR413PM1* plants under cold and drought stress.** (A)  $O_2^-$  contents; (B)  $H_2O_2$  contents; (C) SOD activity; (D) Expression level of *GhSOD*; (E) POD activity; (F) Expression level of *GhPOD*; (G) CAT activity; (H) Expression level of *GhCAT*; (I) GST activity; (J) Expression level of *GhGST*. Data for the WT are means of three replicates, whereas data for *SikCOR413PM1* transgenic plants are means of three different lines. Bars represent SDs. \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant differences relative to WT cotton plants.

## Analysis of stress-related gene expression in *SikCOR413PM1* overexpressing plants

We analyzed the expression levels of several stress-related genes, including *GhDREB1A*, *GhDREB1B*, *GhDREB1C*, *GhERF2*, *GhNAC3*, and *GhRD22*. Under normal conditions, there was no significant difference in the expression levels of *GhDREB1A*, *GhDREB1B*, and *GhDREB1C* between the WT and transgenic lines. However, after low-temperature and drought stress treatment, the expression levels of these genes in transgenic lines were significantly higher than those in the WT lines. Under low-temperature stress, the expression of *GhDREB1A*, *GhDREB1B*, and *GhDREB1C* genes in transgenic lines was 16.7, 9.9, and 8.6 times (OE-4), 16.7, 9.5, and 9.1 times (OE-5), and 18.7, 13.1, and 10.2 times (OE-7) higher than those in the WT, respectively. Under drought stress, the expression of these genes in transgenic lines was 9.6, 17.7, and 5.5 times (OE-4), 9.1, 19.3, and 4.9 times (OE-5), and 11.0, 21.7, and 6.6 times (OE-7) higher than those in the WT, respectively (Fig. 6A-C). Under low-temperature stress, there was no significant difference in *GhRD22* expression between transgenic and WT cotton. Under drought stress, the expression of *GhRD22* was significantly upregulated and was 11.2-fold (OE-4), 13.1-fold (OE-5), and 15.1-fold (OE-7) higher than that of the WT, respectively (Fig. 6D). Additionally, the expression of the *GhERF2* gene in WT and transgenic plants was significantly different under stress treatment. The expression of the *GhERF2* gene in transgenic plants was 9.1 and 11.7 times (OE-4), 8.1 and 13.4 times (OE-5), and 13.8 and 15.9 times (OE-7) higher than that in the WT under low-temperature and drought stress, respectively (Fig. 6E). Under drought stress, there was no significant difference in the expression of the *GhNAC3* gene between transgenic and WT cotton, but after cold stress treatment, the expression levels increased in each plant. The expression levels of transgenic lines OE-4, OE-5, and OE-7 were 10.7, 12.9, and 14.5 times higher than those of the WT (Fig. 6F). These results indicate that overexpression of *SikCOR413PM1* helps increase the expression level of stress response genes and enhances the low-temperature and drought tolerance of cotton.

## Agronomic traits of *SikCOR413PM1* overexpressing cotton in the field

The plant height of transgenic cotton overexpressing *SikCOR413PM1* increased slightly but not significantly, and the number of fruit branches per plant and bolls per plant increased compared with that of the WT (Table.S1). The *SikCOR413PM1* transgenic lines had eight to nine fruit branches per plant, and the WT plants had only seven fruit branches. In addition, compared with WT plants, *SikCOR413PM1* transgenic lines had an average of one or two bolls per plant. Although there was no significant difference in boll weight and lint weight between *SikCOR413PM1* transgenic lines and WT plants, the fiber yield of *SikCOR413PM1* transgenic cotton lines was moderately increased. The seed cotton yield and lint yield of *SikCOR413PM1* cotton were significantly increased. This may result from multiple tolerance activities of *SikCOR413PM1*, which effectively reduce the yield loss caused by abiotic stresses such as low temperature and drought.

In addition to the greenhouse experiments for low-temperature and drought resistance, we also evaluated the drought resistance of cotton plants overexpressing *SikCOR413PM1* in the field (Fig. 7). The single boll weight, single boll lint weight, and lint percentage did not significantly differ between OE-4 and OE-7 lines,

but the plant height, fruit branch number per plant, boll number per plant, and seed cotton yield of transgenic OE-4 and OE-7 lines were significantly higher than those of the WT. Our results indicate that overexpression of *SikCOR413PM1* can improve drought tolerance and yield of cotton under both normal and drought stress conditions in the field.

## Methods

### Plant materials and plant growth conditions

The cotton material (*Gossypium hirsutum*) used in this study was the seed of Xinjiang upland cotton Xinluzao 17, that was provided by Li Baocheng, the vice president of Xinjiang Academy of Agriculture and Reclamation Sciences. The tissue culture conditions for wild-type (WT) and transgenic cotton were a relative humidity of 60–70%, a photoperiod of 16h light and 8h dark cycle, and an average temperature of 25°C. The growth conditions for seedlings were in plastic pots containing a composite matrix of equal parts nutrient soil, vermiculite, and perlite (1:1:1), and a culture chamber with a light intensity of 500  $\mu\text{mol}^{-2} \text{s}^{-1}$  at a temperature range of 22–28°C and relative humidity of 60–70%.

#### Real-time quantitative PCR for *SikCOR413PM1* expression analysis

Total RNA was isolated using the RNAiso Plus kit (TaKaRa) and on-column DNase I treatment, following the product instructions. The first-strand cDNA was synthesized from the total RNA using oligo(dT) primers and PrimeScript® RTase (TaKaRa). The expression patterns of the *SikCOR413PM1* gene and related stress response genes were determined by quantitative RT-PCR using the LightCycler 480 instrument (Roche Diagnostics, Switzerland) and SYBR® Premix Ex Taq™ (TaKaRa, China) following the supplier's instructions and MIQE guidelines. Each reaction mixture (10  $\mu\text{L}$ ) contained 50 ng cDNA, 5  $\mu\text{L}$  2× SYBR® Green MasterMix reagent (Applied Biosystems), and 0.2  $\mu\text{M}$  corresponding gene-specific primers (Table.S2). The qRT-PCR reaction program included pre-denaturation at 95°C for 60 s, denaturation at 95°C for 15 s, annealing at 61°C for 30 s, 40 cycles, extension at 72°C for 30 s, and pre-denaturation at 52°C for 40 s. The *GhHis3* gene (AF024716) was used as the reference gene. Each analysis was repeated three times, and the relative gene expression was calculated using the  $\Delta\Delta\text{Ct}$  method [43].

### Acquisition of transgenic cotton

In our previous studies, we have identified and sequenced the *SikCOR413PM1* gene. Transgenic cotton plants containing the *SikCOR413PM1* gene were obtained using the pollen tube pathway method, and the receptors without the exogenous gene were confirmed through molecular detection before the transformation. The receptor plants were transformed at the flowering stage until the cotton bolls opened. The seed cotton from the cotton bolls was harvested and labeled, and the seeds were separated into T0 seeds based on the treatment. The T0 seeds were planted in isolated and protected experimental fields during the second year. When the true leaves of the seedlings had expanded, kanamycin at a concentration of 2 g/L was used for screening. After the screening, the DNA of the plants to be tested was

extracted for PCR detection, and the total RNA of the positive plants identified by PCR was extracted for qRT-PCR identification. The positive cotton plants screened and identified were routinely managed and grown to blossom and produce bolls. The transgenic cotton seeds were collected and sown on 1/2 MS medium containing kanamycin (80 µg/mL) for screening. The resistant seedlings were transferred to soil for growth, and the seeds were collected after maturation for continued screening up to the T4 generation as experimental materials.

## **Plant stress treatments**

WT and *SikCOR413PM1* overexpression transgenic cotton seedlings at 20 days old were subjected to 4°C treatment for 24, 48, and 72 h to observe the phenotype and were treated with 20% PEG6000 for 10, 15, and 20 days to observe the phenotype and to count the survival rate, wet weight, and dry weight. The 70-day-old WT and *SikCOR413PM1* overexpression transgenic cotton seedlings were treated with low temperatures at 4°C for 24 and 48 h, respectively. Phenotypic changes were recorded, and cotton leaves in the same position were collected after 48 h of treatment. After 14 and 21 days of drought treatment, phenotypic observation was carried out, and samples were collected after 21 days to determine the fresh and dry weights. All collected samples were used for subsequent physiological index detection, enzyme activity detection, and related gene expression analysis.

## **Determination of physiological and biochemical indexes**

The relative water content of leaves was measured using the weighing method [45]. The malondialdehyde content [46], relative conductivity [47], free proline, and soluble sugar [48] were determined using a spectrophotometer (UV-160A, Shimadzu Scientific Instrument, Japan).

## **Determination of antioxidant enzyme activity and active oxygen content**

The activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione s-transferase (GST) was determined after preparing the enzyme solution, following the instructions of the detection kit (Nanjing Jiancheng Bioengineering Institute). The quantitative determination of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> was performed using the method of Benikhlef et al (2013)[49]. Each experimental plant was treated with at least three plants per treatment, and each index was repeated three times.

## **Investigation of agronomic traits**

The plant height, root length, and stem circumference of the selected WT and transgenic plants were measured using a tape and electronic vernier caliper. The plant height was measured from the root neck to the growth point, and the stem diameter was measured at 1 cm above the first leaf. The fresh weight and dry weight were measured using an electronic weighing scale.

## **Investigation on agronomic traits of cotton under field drought stress**

Independent T0 transgenic plants were grown until maturity in the plot. Inbred line seeds (T1) were harvested and sown in the experimental field. Based on the results of PCR detection of the kanamycin resistance gene, T2 seeds were screened from positive T1 lines and transgenic homozygous plants. The homozygous transgenic lines were selected for selfing to obtain the T3 generation. This selection continued until the T4 progeny before the agronomic traits of the transgenic homozygous lines were stable. The performance of T4 homozygous transgenic and WT cotton lines was evaluated during the normal growing season of cotton in both normal and drought-prone areas.

The field traits under normal environmental conditions were studied in the experimental field of the Agricultural Biotechnology Key Laboratory of Shihezi University in Shihezi City, Xinjiang. One month after germination, when the plants had matured, irrigation was stopped for drought stress testing. In all field traits, plant growth followed a randomized complete block design with three replicates. Each transgenic cotton was planted in a test plot of about 10.25 m<sup>2</sup>, with 240 plants per plot, and repeated three times. The plant spacing was 12.5 cm, and the row spacing was 30 cm. From sowing to harvest, all recommended plant protection measures were taken. Fifty plants were randomly selected from each replicate for important agronomic traits, including plant height, fruit branch number per plant, boll number per plant, boll weight, lint weight per boll, seed cotton yield per plant, lint yield per plant, lint percentage, seed cotton yield, and lint yield per plot. The plant height was determined as the height of the main stem at the boll opening stage. The vegetative branches and fruit branches of the main stem were artificially separated, and the number of fruit branches per plant was measured. Bolls per plant were collected and counted. All naturally opened bolls were collected from a plot and dried in an oven at 37°C. One hundred bolls were randomly selected for weight measurement. To determine the actual yield of cotton, all bolls before and after frost were collected in a small area and treated according to the above method.

## Statistical analysis

GraphPad Prism 7.0 and SPSS software (version 11.0; SPSS, Chicago, IL, USA) were used for statistical analysis. The pictures were drawn using CorelDRAW X4 SP2 drawing software. The data were expressed as the mean  $\pm$  standard deviation of three repeated experiments. The expression level at 0 h (control time point) was defined as 1.0. Dunnett's multiple comparison test was used to determine the difference between WT and transgenic lines. All transgenic overexpression lines were statistically compared with WT lines, in which \*P < 0.05 represents a significant difference, and \*\*P < 0.01 represents an extremely significant difference.

## Discussion

Plants commonly exhibit physiological adaptation to water shortage, accumulation of various osmotic regulators and antioxidants, and production of ROS in response to drought and cold stress [22, 50]. Abiotic stresses are known to cause water deficits in plants [22, 51, 52]. Under cold and drought conditions, plants maintain cell water by accumulating osmotic solutes, regulating osmotic potential, and adjusting osmotic pressure [53, 54]. Plants often initiate defense mechanisms by increasing the

concentration of sugars, proline, amino acids, and other substances in cells, reducing cell osmotic potential, and absorbing water from external sources [55].

In this study, the content of soluble sugar and proline increased under cold and drought stress, particularly in transgenic lines (Fig. 4C and Fig. 4D). Generally, the stronger the osmotic adjustment ability, the higher the tolerance and adaptability to osmotic stress. This finding suggests that overexpression of the *SikCOR413PM1* gene enhances the environmental adaptability of cotton by improving the osmotic adjustment ability through accumulation of osmotic adjustment substances, thus improving cold and drought tolerance.

Under cold stress, plants stabilize cell membranes to prevent freezing-induced dehydration [56]. Environmental changes can affect cell membrane function, and the plasma membrane is the primary site of freezing injury [57, 58, 59]. Some studies have shown that certain soluble sugars and proline can stabilize proteins and cell structures, enhance cell membrane integrity, and improve frost resistance [60, 61, 62, 63]. In cotton, soluble sugars and proline protect cell membranes from oxidative damage caused by various abiotic stresses [64]. Proline, the most common osmotic solute found in many organisms, prevents water loss, reduces mechanical damage, and enhances stress tolerance [61, 65, 66]. The overexpression of the *SikCOR413PM1* gene in plants resulted in a greater accumulation of soluble sugars and proline than in WT plants in cold and drought environments (Fig. 4C-D). We speculate that this accumulation is related to improvement of cell membrane stability.

The cell membrane is a primary target of adverse stress, and maintaining its integrity and stability under abiotic stress conditions is a critical for plant environmental stress tolerance [67]. Under low-temperature stress, the structure and function of plant cell membranes change, and membrane lipid peroxidation occurs, leading to increases in ROS, cell membrane permeability, and relative conductivity [68, 69]. The content of the membrane lipid peroxidation product, malondialdehyde (MDA), can reflect the degree of membrane lipid peroxidation and cell damage [68]. Relative conductivity indicates plasma membrane damage caused by freezing [69]. Both can be used to evaluate the cold tolerance of plants.

In comparison to that of WT plants, the MDA content in the leaves of transgenic plants was significantly lower (Fig. 4A), but there was no significant difference in relative conductivity between transgenic and WT plants. Both MDA content and relative conductivity had different degrees of improvement after post-treatment at 4°C and drought treatment under normal conditions (Fig. 4B). Therefore, this result further shows that lower MDA content and higher relative conductivity contribute to the stress tolerance of cotton by reducing the degree of plasma membrane damage. The overexpression of the *SikCOR413PM1* gene in cotton can reduce the degree of plant plasma membrane damage. Plants can enhance freezing tolerance (FT) by improving antioxidant mechanisms [52, 70]. Thus, the decrease in MDA content in *SikCOR413PM1* transgenic cotton also indicates a reduction in membrane lipid peroxidation and improved stress resistance of plants.

Several studies have investigated whether COR genes can be induced in response to water shortage and cold stress. Most *Arabidopsis* COR genes are induced in response to water shortage [71, 72]. Chen et al.

(2011)[73] speculated that the *COR25* gene is also induced by low temperature and drought to protect *Brassica napus* cells from dehydration, given that overexpression of the *SikCOR413PM1* gene maintains cell membrane stability. Therefore, we speculate that the *SikCOR413PM1* gene may also be induced by drought and cold stress. In summary, this study demonstrates that the transgenic *SikCOR413PM1* gene protects cell membranes and reduces damage by affecting various osmotic regulators, MDA, and relative conductivity in cotton, thereby regulating plant tolerance to cold and drought stress.

Currently, research on *COR413* family genes is limited, but studies have shown that overexpression of the *LeCOR413PM2* gene located in the plasma membrane may enhance the tolerance of transgenic tomato to cold stress by protecting the membrane from cold damage [37]. Previous studies have also shown that the overexpression of the *SikCOR413PM1* gene, which is located on the plasma membrane, improves drought and cold stress in tobacco and cotton by protecting cell membrane stability [35]. It has been predicted that *COR413* family proteins located in the plasma membrane (*COR413-PM*) have a film-forming function [71]. Therefore, we speculate that *COR413* family proteins located in the plasma membrane may play a role in maintaining cell membrane stability, which requires further investigation.

Under water shortage and extreme temperature stress, plants may experience cell dehydration, damage to photosynthesis, and oxidative damage to membranes and other cellular components [22]. Abiotic stresses can interfere with photosynthetic electron transport and produce ROS, which can cause serious damage to chloroplasts [74, 75, 76, 53]. The primary source of ROS production is organelles with high oxidative activity, such as mitochondria, chloroplasts, and peroxisomes, which generate ROS such as  $O_2^-$  and  $H_2O_2$ , leading to oxidative damage of proteins, DNA, and lipids [6, 8, 75]. Therefore, regulating ROS can protect cells from oxidative damage [78, 79].

The ROS signal is an adaptive regulation of plants to the stressful environment [53]. Plants have established an efficient ROS scavenging system that reduces ROS-induced damage through antioxidant enzymes and antioxidants [80]. Cell-protective enzymes mainly include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase. Overexpression of these genes often significantly improves the ability of plants to scavenge ROS and thus improves their tolerance to abiotic stress [81, 82, 83, 84].

In this study, the content of  $O_2^-$  and  $H_2O_2$ , which can reflect the response of plants to stress, was detected in WT and transgenic lines. Compared with that in WT lines, the content of  $O_2^-$  and  $H_2O_2$  in transgenic lines was significantly reduced (Fig. 5A and B), indicating that overexpression of the *SikCOR413PM1* gene enhances plant ability to scavenge ROS. Moreover, the enzyme activity level of ROS scavenger antioxidant enzymes showed that all stress treatments enhanced the activity of antioxidant enzymes, particularly in the transgenic lines. The expression levels of *GhSOD*, *GhPOD*, *GhCAT*, and *GhGST* coding genes in transgenic lines were also significantly higher than those in WT lines after stress treatment (Fig. 5C–J). Therefore, the enhancement of ROS scavenging ability is closely related to ROS scavenger antioxidant enzymes. The overexpression of the *SikCOR413PM1* gene increases the expression level of genes corresponding to antioxidant enzymes in transgenic cotton, thereby enhancing the activity of

corresponding antioxidant enzymes that can regulate the amount of ROS to reduce oxidative stress. Therefore, the *SikCOR413PM1* gene maintains high antioxidant enzyme activity and related gene expression, reducing oxidative stress in plants.

Previous studies have shown that cold-induced changes in antioxidant enzyme activity in the leaves of several cereal varieties are related to frost resistance [85, 86, 87]. Overexpression of SOD in tobacco and APX in tomato increased their tolerance to low temperature [88, 89]. The antioxidant enzyme defense system is closely related to plant frost resistance [75, 80, 90]. Cold acclimation induces the accumulation of ROS, activating the antioxidant enzyme defense system [91]. By increasing the activities of enzymes that scavenge ROS, such as guaiacol peroxidase, superoxide dismutase (SOD), and catalase (CAT), the contents of H<sub>2</sub>O<sub>2</sub> and MDA were reduced before the drought, and the homeostasis of ROS was better maintained [92]. In the study of Chen et al (2015)[93], down-regulation of *GbMYb5* resulted in a higher oxidative stress rate under drought conditions due to decreases in the activities of POD, SOD, and CAT enzymes. This further illustrates that the *SikCOR413PM1* gene can reduce the oxidative stress of plants and improve their cold and drought resistance.

Proline protects cells from increased stress-related ROS levels [53]. In a previous study, transgenic wheat accumulated higher proline than WT plants and showed less membrane lipid peroxidation during drought, indicating that proline plays a role in reducing ROS damage during drought [94]. Cold acclimation induces rapid proline accumulation by changing the activity of related enzymes in the proline metabolic pathway, thereby improving frost resistance [95]. In this study, proline was used as an osmotic regulator to improve the cold and drought tolerance of cotton. The proline content of transgenic plants under drought and cold stress was higher than that of the WT plants. Based on research and analysis, we speculate that overexpression of the *SikCOR413PM1* gene may increase proline accumulation, and proline can reduce the impact of ROS on cells for protection.

DREB family transcription factors have been shown to play an important role in cold adaptation-induced FT, and are transcription factors that induce the expression of cold and dehydration stress-regulated genes in plants [96, 97, 98]. The expression of *DREB* genes is enhanced under cold and drought conditions [99]. *DREB1A*, *DREB1B*, and *DREB1C* expression is strongly induced by cold stress [98]. The expression of the *DREB1* gene can partly explain the resistance of plants to stress.

In this study, the expression levels of *GhDREB1A*, *GhDREB1B*, and *GhDREB1C* in transgenic lines were significantly higher than those in WT lines after low-temperature and drought stress treatment (Fig. 6A–C). The expression levels of *GhRD22*, *GhERF2*, and *GhNAC3* also increased under the two stresses (Fig. 6D–F). These results indicate that *GhDREB1A*, *GhDREB1B*, and *GhDREB1C* are strongly induced by drought and cold, and the expression of the above five stress-related genes was increased in transgenic *SikCOR413PM1* cotton plants compared with in WT. Therefore, overexpression of *SikCOR413PM1* helps increase the expression level of stress response genes. The increase in the expression of the transgenic cotton *DREB1* gene indicates that it has stronger tolerance than the WT under cold and drought conditions. In Arabidopsis, the DREB1 protein activates *COR* genes, such as *COR15a* [100], *COR6.6* [101],

and *COR47* [102], and its protein plays a functional role in enhancing tolerance to cold and drought stress. Therefore, it can be speculated that stress increases the expression of the *DREB1* gene, which may be related to the activation of the *COR413* gene. In summary, the overexpression of *SikCOR413PM1* promotes the expression level of stress genes in drought and cold environments, thereby improving plant stress resistance.

Overexpression of the *SikCOR413PM1* gene in cotton was found to have positive effects on cotton growth under greenhouse conditions. To investigate its actual impact on cotton production, the effect of this gene on transgenic cotton was studied in the field. The results showed that transgenic cotton had significant increases in both seed cotton yield and lint yield, making it more resistant to yield loss under stress. Although the growth status of the two lines did not differ significantly under normal conditions, transgenic OE-4 and OE-7 lines had significantly better growth under drought conditions than the WT (Fig. 7). Moreover, the number of fruit branches per plant, number of bolls per plant, and seed cotton yield were also significantly higher in transgenic cotton (Table.S1). These results suggest that overexpression of the *SikCOR413PM1* gene can improve the drought tolerance and yield of cotton. The findings provide a theoretical foundation for developing cotton varieties and demonstrate the potential for transgenic cotton to improve environmental adaptability and yield.

## Abbreviations

***ABA***: Absciscic Acid

***BRs***: Epibrassinolide

***CAT***: Catalase

***CBF***: C-receptor binding factor

***COR***: Cold-regulated

***CRT***: C-receptor

***DREB***: Dehydration response element binding factor

***GST***: Glutathione S-transferase

***POD***: Peroxidase

***qRT-PCR***: Quantitative Real-Time PCR

***REL***: Relative electrical conductivity

***ROS***: Reactive oxygen species

***SOD***: Superoxide Dismutase

## Declarations

### Availability of data and materials

We submitted the gene sequence at NCBI, and since the gene is not publicly available, only the GenBank accession number of the nucleotide sequence provided by NCBI is available: BankIt2706231

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### Contributions

Mei Wang and Lepeng Wang were the experimental designers and study executors of the study, completed the data analysis, and wrote the first draft of the manuscript. Xiangxue Yu and Jingyi Zhao

carried out the experimental results and data analysis. Zhijia Tian, Xiaohong Liu, and Guoping Wang participated in the experimental data inspection and data investigation. Xinyong Guo and Li Zhang were the project planners and principals, guiding the paper's writing and revision. All authors read and agreed to the final text.

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### **Ethics declarations**

### **Ethics approval and consent to participate**

The plant material *Saussurea involucrata* (Kar Kir.) used in this study was obtained from the Key Laboratory of Agricultural Biotechnology, College of Life Sciences, Shihezi University, Xinjiang, China. All the *Saussurea involucrata* (Kar Kir.) used in this experiment were laboratory histoculture seedlings and were not collected from endangered materials or species. All the procedures were followed in accordance with the relevant guidelines.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests.

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## Figures

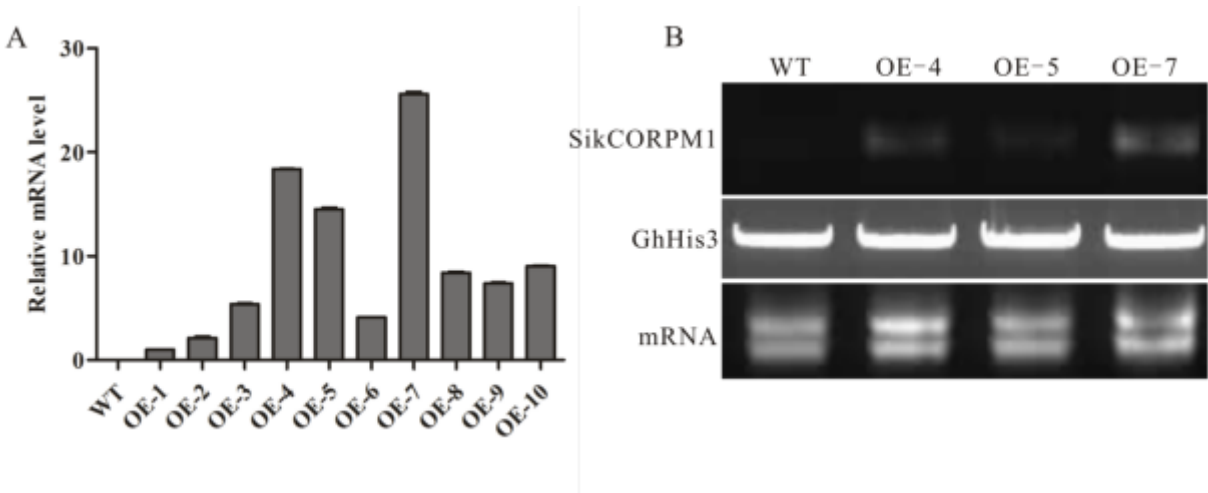
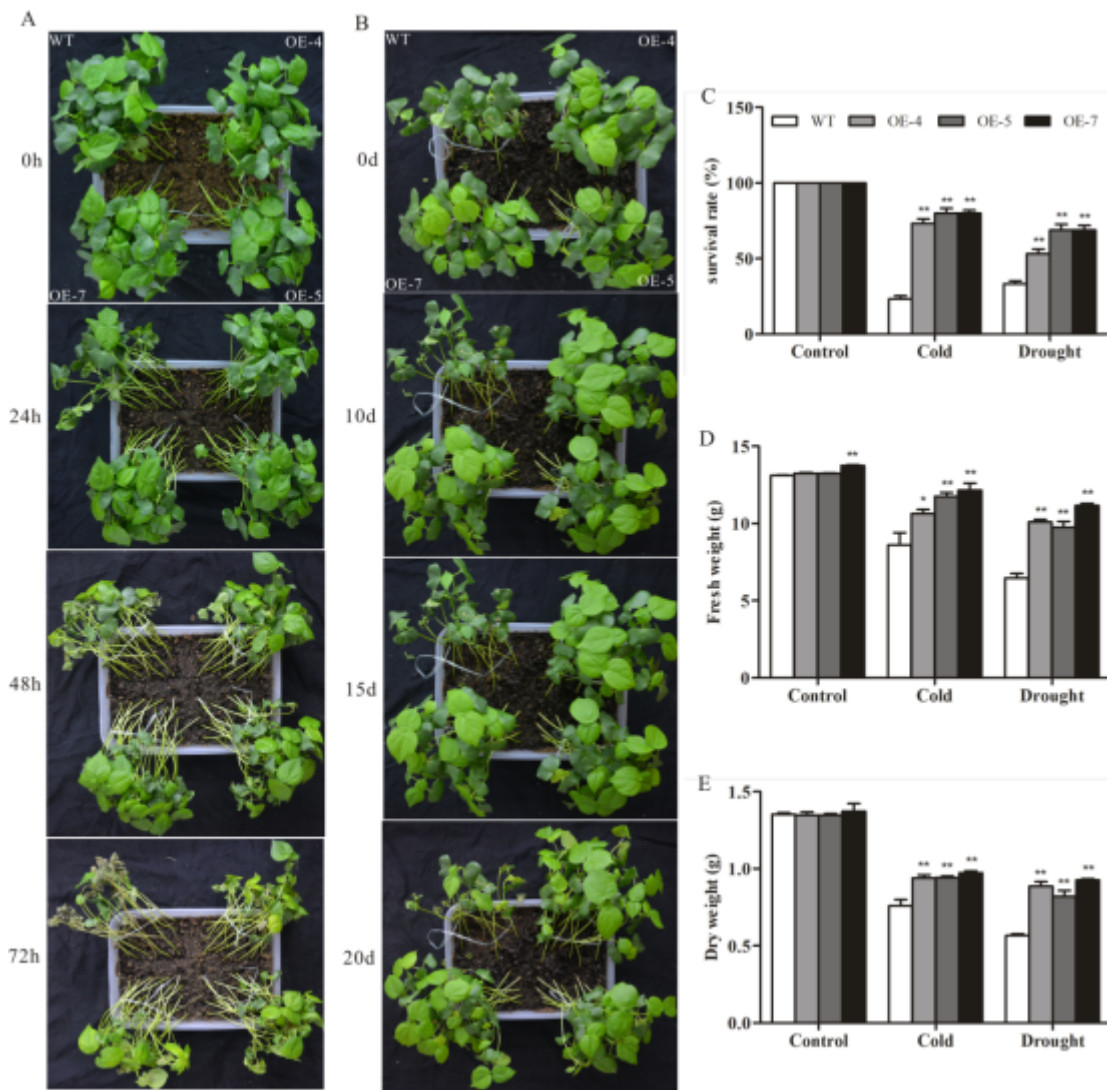


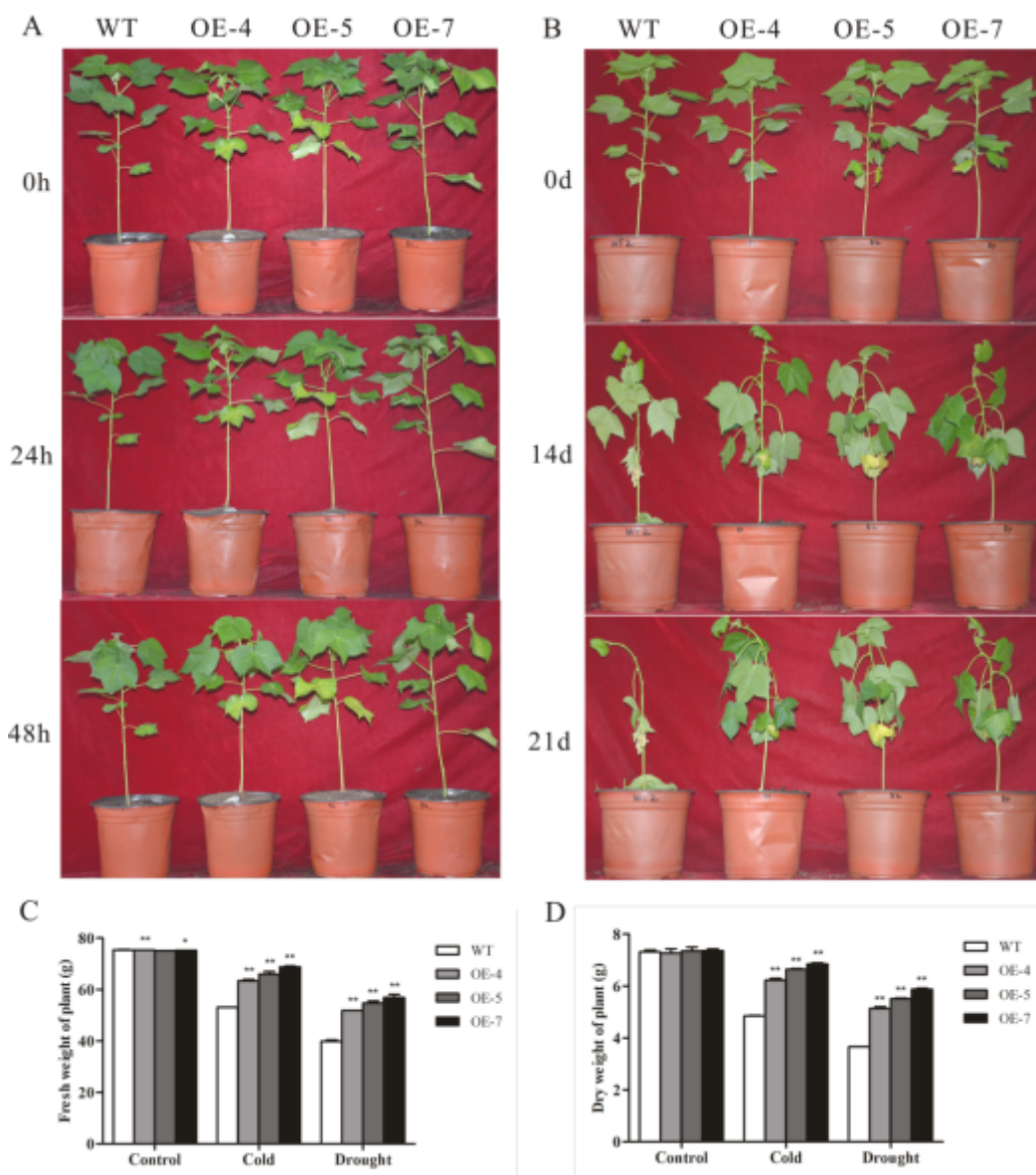
Figure 1

**Expression levels of *SikCOR413PM1* in WT and transgenic cotton lines.** (A) Quantitative real-time PCR analysis of relative expression of *SikCOR413PM1* in WT and ten transgenic lines; (B) Semi-quantitative PCR analyses of *SikCOR413PM1* expression in WT and three transgenic lines (OE-4, OE-5 and OE-7).



**Figure 2**

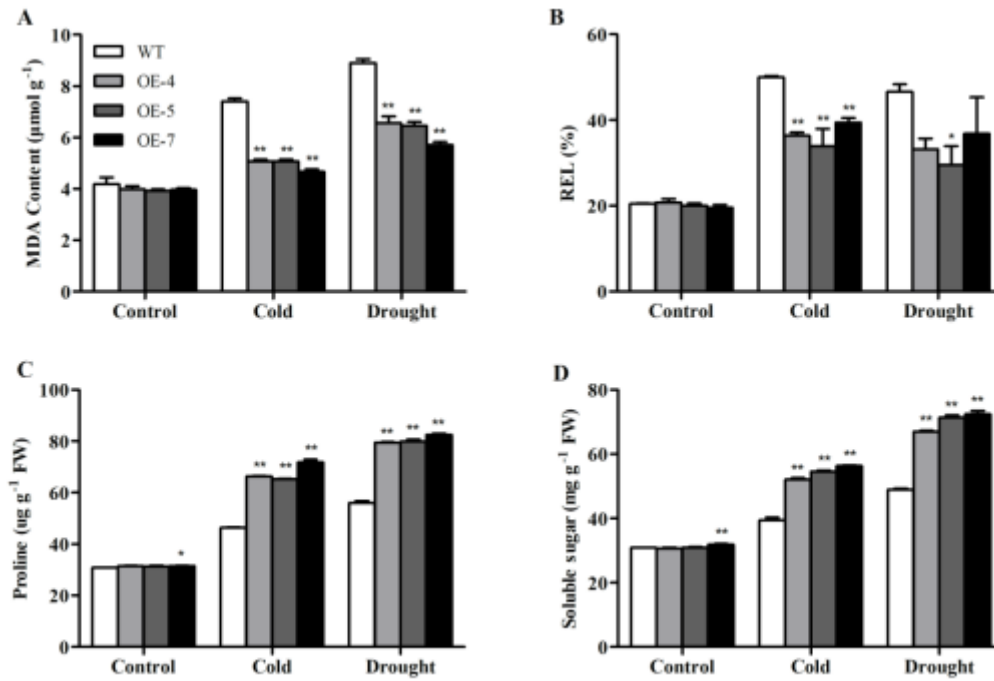
**Phenotype analysis of WT and *SikkCOR413PM1*-expressing seedlings under cold and drought stress.**(A) Analysis of phenotype 20 d of WT and transgenic plants (OE-4, OE-5, and OE-7) under 4°C for 24, 48 and 72 h, respectively; (B) Analysis of phenotype 20 d of WT and transgenic plants (OE-4, OE-5, and OE-7) after 20% PGE6000 treatment 10, 15, and 20 d respectively; (C) Survival rates; (D) Fresh weight ; (E) Dry weight. The data represent the average of three independent biological replicates. Bars represent SDs. \*P < 0.05 and \*\*P < 0.01 indicate significant differences relative to WT cotton plants.



**Figure 3**

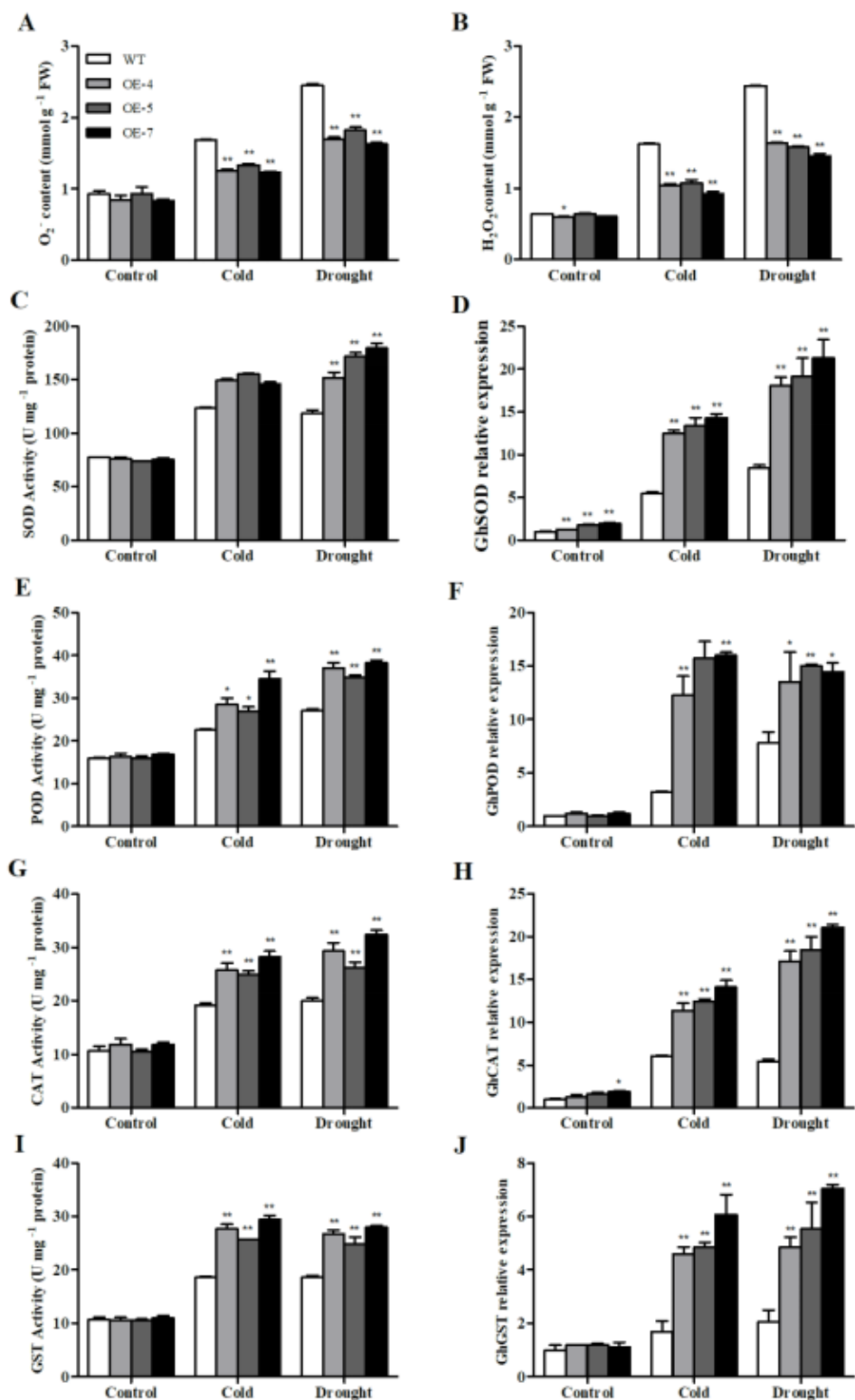
**Phenotype analysis of WT and *SikCOR413PM1*-expressing cotton plants under cold and drought stress.**

(A) Analysis of phenotype 70-d-old WT and transgenic plants OE-4, OE-5, and OE-7 under 4°C for 24 h and 48 h, respectively; (B) Analysis of phenotype 70-d-old WT and transgenic plants OE-4, OE-5, and OE-7 after drought treatment 14 and 21 days, respectively; (C) Fresh weight ; (D) Dry weight . The data represent the average of three independent biological replicates. Bars represent SDs. \*P < 0.05 and \*\*P < 0.01 indicate significant differences relative to WT cotton plants.



**Figure 4**

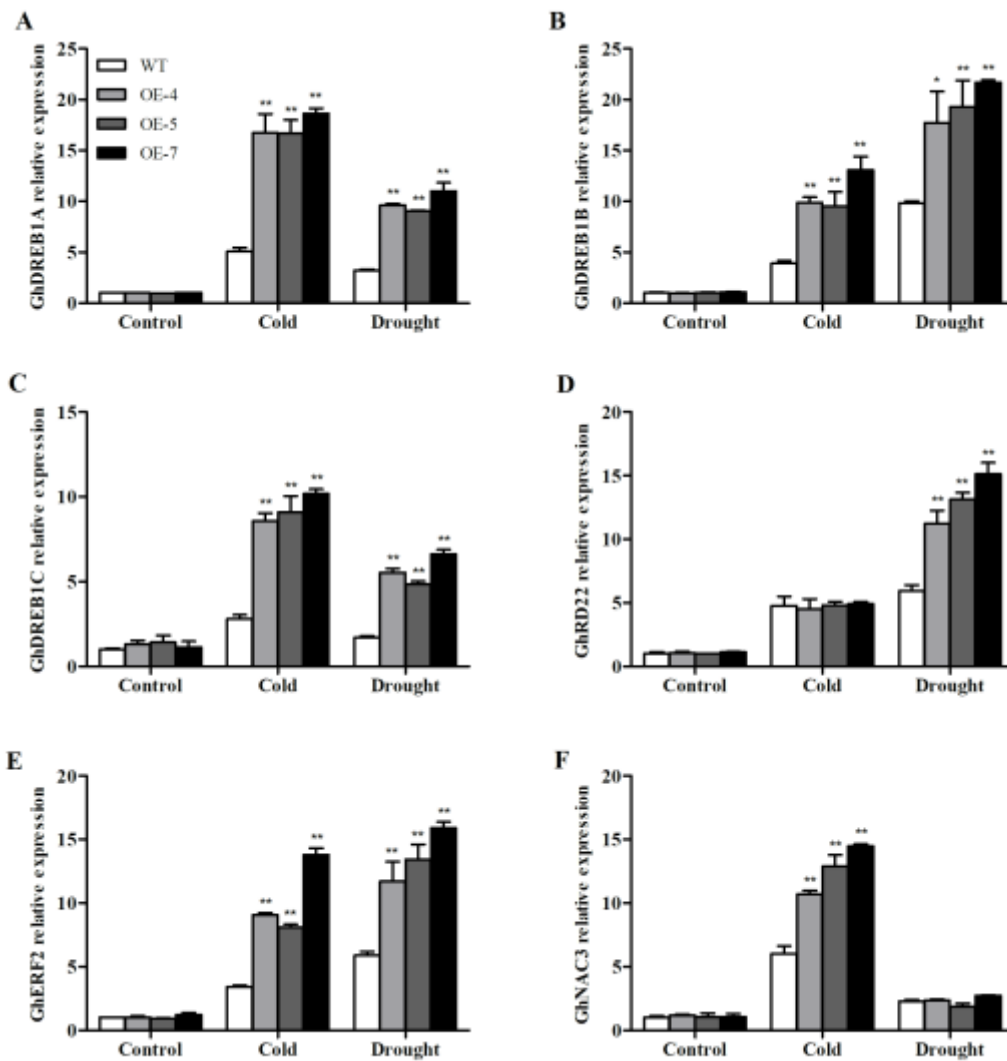
**Plant physiological analysis in WT and *SikCOR413PM1*-expressing transgenic plants after cold and drought stress treatments.**(A) Malondialdehyde content; (B) Relative electrolyte leakage; (C) Proline content; (D) Soluble sugar content. Data for the WT are means of three replicates, whereas data for *SikCOR413PM1* transgenic plants are means of three different lines. Bars represent SDs. \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant differences relative to WT cotton plants.



**Figure 5**

**Analysis of ROS and antioxidant enzyme activity and expression of related genes in WT and transgenic *SikCOR413PM1* plants under cold and drought stress.** (A)  $O_2^-$  contents; (B)  $H_2O_2$  contents; (C) SOD activity; (D) Expression level of *GhSOD*; (E) POD activity; (F) Expression level of *GhPOD*; (G) CAT activity; (H) Expression level of *GhCAT*; (I) GST activity; (J) Expression level of *GhGST*. Data for the WT are means

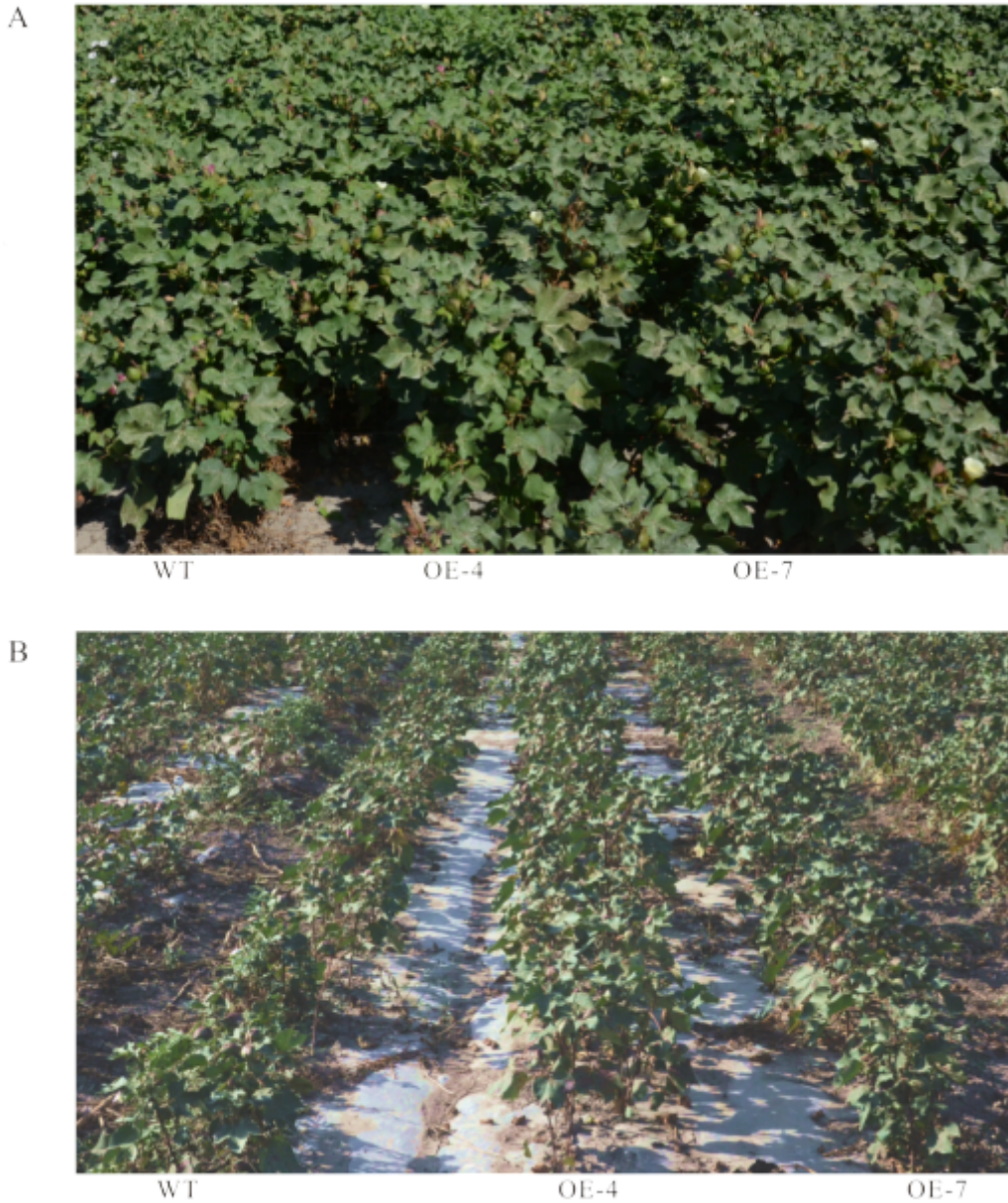
of three replicates, whereas data for *SikCOR413PM1* transgenic plants are means of three different lines. Bars represent SDs. \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant differences relative to WT cotton plants.



**Figure 6**

**Relative expression of stress-related genes in WT and transgenic lines under cold and drought stress.**

(A) Expression level of *GhDREB1A*; (B) *GhDREB1B*; (C) *GhDREB1C*; (D) *GhRD22*; (E) *GhERF2*; (F) *GhNAC3*. Data are means  $\pm$  SD of three replicates. Asterisk(s) indicate a significant difference between the WT and transgenic plants: \* represents  $P < 0.05$ , and \*\* represents  $P < 0.01$ .



**Figure 7**

**Phenotypic analysis of WT and transgenic cotton lines under drought stress in the field.**(A) Plants grown under normal conditions with regular irrigation, photos taken on day 70. (B) Plants grown under drought stress, photographs taken on day 70.

## Supplementary Files

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