ROLE AND MECHANISM OF GMHORSTS GENE ON DROUGHT STRESS TOLERANCE OF SOYBEAN

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Abstract. With the frequent occurrence of global drought, some countries and regions are facing reduced soybean production. Therefore, in order to increase soybean yield and ensure global food security, the influence and mechanism of soybean GmHORSTs gene on its drought stress tolerance are analyzed, in order to provide support for improving soybean drought resistance. The experimental results indicated that after drought stress, the fresh weight, dry weight, and water content of pTRV-GmHORSTs were 0.90 g, 0.48 g, and 36.7%, respectively, significantly lower than that of pTRV-00. The content of malondialdehyde and H_2O_2 were both higher than in pTRV-00, with values of 0.41 umlo/g FW and 13.69 umlo/g, respectively. The fresh weight, dry weight, and water content of GmHORSTs over-expressed plants after drought stress were 1.51 g, 0.59 g, and 61.2%, respectively, all higher than those of wild plants, while their H_2O_2 and propylene glycol contents were lower than those of wild plants. Under drought stress, the withering degree of pTRV-GmHORSTs was higher than that of normal plants, while the withering degree of over-expressed plants was significantly lower than that of wild plants. In addition, under drought stress conditions, the stomatal conductance of pTRV-GmHORSTs was 19.7 um^2, which was higher than that of pTRV-00. The stomatal conductance of over-expressed plants was 3.9 um^2, which was lower than that of wild plants. The abscisic acid content of pTRV-GmHORSTs was 45.3 ng/mL, which was lower than that of pTRV-00. The abscisic acid content of over-expressed plants was 84.8 ng/mL and 84.7 ng/mL, both higher than that of wild plants. The above results indicate that the GmHORSTs gene can increase stomatal closure by increasing leaf abscisic acid content, thereby reducing transpiration and improving soybean drought tolerance.
Keywords: *soybean, drought stress, GmHORSTs, VIGS, stomatal conductance, H_2O_2, fatty acid ω - hydroxylase*

Introduction

Drought, as one of the most serious physical hazards in agricultural practice, can lead to a decrease in groundwater or soil moisture and a reduction in river flow, resulting in damage to crops. It is estimated that about 60% of all deaths caused by extreme weather events are due to drought. By 2030, over 700 million people will face displacement due to drought (Galandiuk, 2021; Wang et al., 2021). The main reasons for the frequent occurrence of global droughts include climate change and human activities. According to relevant research predictions, the frequency and severity of droughts in the future will increase year by year, posing a serious threat to food production (Ahmad et al., 2021; Flaute et al., 2024). Therefore, in order to cope with the reduction in grain production caused by drought, it is necessary to optimize the drought stress tolerance of various types of grains. Genes, as the material basis of inheritance, exert a decisive function in the biological traits. Therefore, to improve the drought stress tolerance of crops, many scholars have analyzed it from a genetic perspective to provide support for the cultivation of drought tolerant crops. To improve the drought stress tolerance of Chinese cabbage, Yuan et al. (2022) conducted heterologous over-expression of the BrIQD35 gene in tobacco. The results showed that under drought stress, BrIQD35-over-expressed

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tobacco plants had a milder degree of withering, indicating that BrIQD35- had a positive effect on drought stress (Yuan et al., 2022). To improve the drought resistance, Stührwohldt et al. (2021) analyzed the role of plant sulfofactor peptide precursor genes. The results showed that over-expressed plants improved their tolerance to osmotic stress. Compared with wild-type plants, transgenic plants had higher fresh weight and improved lateral root development. The precursor gene of plant sulfofactor peptide had significant active effects on the drought stress tolerance of plants (Stührwohldt et al., 2021). Guo et al. (2021) transferred the BoWRKY10 gene from cabbage to tobacco to improve its drought resistance. The results showed that over-expressed BoW-RKY10 in tobacco resulted in higher relative water content, proline content, and superoxide dismutase activity, but lower malondialdehyde and hydrogen peroxide content after drought treatment. Moreover, several genes related to the abscisic acid signaling pathway, sucrose and reactive oxygen species scavenging system were obviously up-regulated in transgenic lines. Over-expressed BoW-RKY10 in tobacco exhibited good drought resistance (Guo et al., 2021).

Among numerous crops, soybean is not only an important grain and oil crop, but also an important industrial raw material. Soybeans are native to China and widely cultivated around the world. Their seeds contain abundant plant protein, which can be used not only to produce various foods and extract soybean oil, but also to make poultry and livestock feed. It can be seen that soybeans play an important role in human production and life. However, due to the frequent occurrence of drought and high temperature weather, the yield of soybeans is threatened. Therefore, in order to ensure soybean supply, it is necessary to cultivate soybean plants with excellent drought resistance. Ruby et al. identified and characterized GmPARPs genes in soybean using bioinformatics and molecular analysis. A transgenic soybean GmPARPs-RNAi was proposed. The results showed that the down-regulation of GmPARP1 effectively improved the drought and heat tolerance of soybeans. However, GmPARP2 could only protect soybean plants under drought stress (Ruby et al., 2023). Repke et al. (2022) treated soybeans with a biostimulant derived from seaweed extract to enhance their tolerance to high temperature stress. The application of seaweed extract as a biostimulant could reduce leaf temperature and increase CO_2 assimilation rate, stomatal conductance, transpiration rate, and carboxylation efficiency. The tolerance of soybean plants to heat stress was improved without affecting water use efficiency and chlorophyll content (Repke et al., 2022). Wang et al. investigated the role of soybean TOPP protein phosphatase family genes under drought stress and transformed GmTOPP13 gene into tobacco plants through Agrobacterium mediated method. The results showed that over-expressed GmTOPP13 gene enhanced the drought tolerance of tobacco plants and regulated stress responsive genes including CAT, SOD, ERD10B, and TIP under drought stress (Wang et al., 2021).

In summary, genetic technology is an effective method for improving crop drought stress tolerance. By analyzing the crop itself and other crop related genes, it can provide strong support for improving crop drought resistance. However, due to the current research on soybean drought resistance related genes mostly focusing on phospholipases, there is a lack of research on the fatty acid ω-hydroxylase gene. The GmHORSTs gene is a soybean fatty acid ω - hydroxylase gene, which belongs to the P450 enzyme family. It participates in regulating the synthesis and metabolism of fatty acids together with other fatty acid ω - hydroxylase genes. Fatty acid metabolism is of great significance for the survival and adaptation of plants under drought conditions.

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Therefore, GmHORSTs genes may enhance the stability of plant cell membranes and regulate plant hormone levels by affecting the synthesis and metabolism of fatty acids, thereby helping plants cope with water stress. Therefore, to improve the drought tolerance of soybeans and perfect the research on soybean related genes, the research innovatively uses Virus-Induced Gene Silencing (VIGS) to obtain GmHORSTS silenced plants, and analyzes the effect of GmHORSTS genes on drought stress tolerance of soybean and its mechanism, so as to cultivate soybean with excellent drought stress tolerance.

Methods and materials

Experimental materials and equipment

Affected by the greenhouse effect and climate change, the area of arid and semi-arid regions around the world is accelerating, and the intensity of drought frequency has significantly increased. Therefore, cultivating soybeans with drought stress tolerance is of great significance for global food security. Therefore, to explore the effect of GmHORSTS gene on drought stress tolerance of soybean, this study uses VIGS to obtain the soybean with GmHORSTS gene silencing, and analyzes the mechanism of the effect of this gene on drought stress tolerance. The required reagents for the experiment include *NaClO* (Guangdong Wenglong Chemical Reagent Co., Ltd), $CaCl_2$ (Shanghai Umibio Biotechnology Co., Ltd), sucrose (Beijing Biotopped Technology Co., Ltd), Agar powder (Beijing Biotopped Technology Co., Ltd), acetic acid (Shanghai Enzyme Linked Biotechnology Co., Ltd), Anhydrous ethanol (Guangdong Wengjiang Chemical Reagent Co., Ltd), chloral hydrate (Shanghai Yuanye Biotechnology Co., Ltd), PEG6000 (Saiguo Biotechnology Co., Ltd), Hormone abscisic acid (Shanghai Yuanye Biotechnology Co., Ltd), and various types of culture media and Holland nutrient solution. The required culture media include Liquid Co Culture Medium (LCCM), Solid Co Culture Medium (SCCM), Sprout Induced Medium (SIM), Stem Elongation Medium (SEM), and Rooting Medium (RM). The required instruments include a sterilization pot (Shanghai Boxun Medical Biological Instrument Co., Ltd.), a fluorescence quantitative PCR instrument (Thermo Fisher Scientific), a low-temperature freeze centrifuge (Sigma-Aldrich), a constant temperature incubator (Shaoxing Wanli Instrument Co., Ltd.), and a high-precision pipette (Taiwan Weierkang Medical Supplies Co., Ltd.). The configuration of the culture medium is shown in *Table 1*.

Experimental methods

In order to obtain vectors with different GmHORSTs expression conditions, construct genetic recombinant vectors. Construction of GmHORSTs gene expression vector: Firstly, the selected restriction enzyme cleavage sites (BamH1 and EcoR1) are subjected to double enzyme cleavage treatment and recovered. After measuring the concentration, they are stored at -20℃. Next, homologous arm primers (pTRV2-F, pTRV2-R) are designed based on the double enzyme cleavage sites and vector/gene sequences. The GmHORSTs gene is cloned using LA tap enzyme (Chen et al., 2021; Winck et al., 2023). Finally, the target gene is ligated to the enzyme digested vector using homologous recombination, with ligation time and temperature of 20 minutes and 50℃, respectively. The method was repeated 100 times to obtain enough samples.

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Table 1. *Configuration of the culture medium*

Reagent	LCCM / 500 mL	SCCM/1L	SIM-1/1L	SIM-2/1L	SEM/1L	RM/500 mL
B5 powder	0.155 g	0.31 g	3.1 g	3.1 g	/	/
MES	1.95 g	3.9 g	0.59 g	0.59 g	0.6 g	0.3 g
DTT	/	1.54 mL			/	/
6-BA	835 uL	1.67 mL	1.67 mL	1.67 mL	/	/
Sucrose	15 g	30 g	30 g	30 g	30 g	10 g
B5 Vitamin	500 uL	1 mL	1 mL	1 mL	1 mL	500 uL
L-Cys	2 mL	4 mL	/	/	/	/
As	400 uL	800 uL	/	/	/	/
Switte-77	100 uL	/	/	/	/	/
GA3	125 uL	/	/	/	500 uL	/
Agar	/	8 g	/	/	/	4.5 g
Phytagel	/	/	2.75 g	2.75 g	3 g	/
Timentin	/	/	1 mL	1 mL	1 mL	500 uL
Glufosinate	/	/	/	600 uL	500 mL	/
Cefotaxime	/	/	1.5 mL	1.5 mL	1.5 mL	500 uL
MS powder	/	/	/	/	4.33 g	1.11 g
L-Asp	/	/	/	/	1 mL	500 uL
IAA	/	/	/	/	250 uL	/
L-Pyr	/	/	/	/	1 mL	/
ZR	/	/	/	/	1 mL	/
IBA	/	/	/	/	/	500 uL

To obtain soybean plants with stable GmHORSTs gene silencing traits, an induction treatment was required. Silencing of GmHORSTs gene: Firstly, the recombinant vector and empty plasmid of GmHORSTs gene are transferred to Agrobacterium tumefaciens (GV3101) and identified by PCR. Then, they are stored at -80℃ for future use. Then, the processed GV3101 agrobacterium is inoculated into YEP liquid medium (7 ml) containing potassium ions and Rif, and subjected to constant temperature shaking culture for 20 hours and 28℃, respectively. After shaking cultivation, it is transferred to YEP liquid medium (40 ml) and subjected to constant temperature shaking cultivation at the same temperature until the OD600 range is 1.0-1.2 (Li et al., 2022; Souza Júnior et al., 2023). The processed pTRV1 bacterial solution is mixed with equal volumes of pTRV2 bacterial solution and pTRV2-GmHORSTs bacterial solution, and centrifuge to remove YEP. The centrifugation speed is 4000 rpm and the centrifugation time is 15 minutes. Resuspend the centrifuged bacterial solution with *MgCl* (10 mm) until OD600 reaches 1.0, and add As to prepare the infection solution. Leave the infection solution at room temperature in the dark for 2 hours. Next, inject the infection solution into the second pair of compound leaves of soybean. After 12 hours of injection, detect GmHORSTs gene expression and perform drought stress. The method was repeated 100 times to obtain enough samples.

To achieve stable inheritance of GmHORSTs gene silencing traits, genetic transformation of soybean is required. Stable genetic transformation method for soybeans: Select 50 soybean single grains with similar shapes and sizes and spread them flat in a culture dish. Place the culture dish and two bottles of *NaClO* (96 mL) on the upper layer of a double-layer plastic basin, and place a container containing sufficient $CaCl_2$ on the lower layer. Then, seal the plastic basin with Vaseline and cling film. Then,

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inject 5 ml of concentrated hydrochloric acid into each of the two bottles of *NaClO*, and seal the injection holes to thoroughly disinfect the soybeans for 20 hours. After disinfection, remove the soybeans and blow off the chlorine gas for 40 minutes. Then immerse the soybeans in sterile water and avoid light for 24 hours. Take 100 ul of Agrobacterium tumefaciens GV3101 and evenly coat it on YEP solid medium containing potassium ions, As, and Rif. Perform constant temperature inversion cultivation for 20 hours and 28℃, respectively. Remove the seed coat of soaked soybeans and divide it longitudinally with a sterile knife. Take the hypocotyl and make 4 horizontal and 3 vertical cuts at a distance of 3 mm from the cotyledons (Repke et al., 2022; Basit et al., 2023). Then collect Agrobacterium and resuspend it in LCCM, and prepare the resuspended bacterial solution into an infection solution with a resuspended range of 0.6-0.8 (OD600). Next, place the processed soybeans in the infection solution and perform constant temperature shaking bed infection for 20 hours at a temperature of 28℃. After infection, take out the soybeans, use filter paper to absorb the liquid on their surface, and incubate them in SCCM for 72 hours in the dark. After cultivation, remove the cotyledons of soybeans, cut off 2/3 of the hypocotyls, and then cut off 1/3 of the remaining hypocotyls. Then, insert the embryonic axis at a 45° downwards into SIM-1 and culture it upside down for one week. Remove the large buds from the soybean clusters and insert them at a 45° into SIM-2, culture for 2 weeks, and screen twice. After cultivation, remove the explant and remove its cotyledons. Place the clustered buds in SEM for cultivation until the buds sprout to 7 cm. Remove the remaining part of the stem and insert it into RM. After rooting, add a small amount of pure water to the culture dish and refine the seedlings for 48 hours. Next, transfer the soybean seedlings to potted plants and perform sealed cultivation. After the seedlings grow, use immunochromatographic test strips to detect the expression of GmHORSTs genes.

To obtain soybean plants affected by drought stress, soybean seedlings were treated to drought stress. Drought stress treatment method for soybean seedlings: Mix peat soil and sandy soil in a volume ratio of 1:1, select plump soybean seeds and sow them, and water during the period to keep the soil moist. After 7 days, when the root length of soybean seedlings is about 10 cm, they are transferred to Hogland nutrient solution for hydroponic cultivation. After 10 days, the first triple compound leaf of soybean seedlings has fully unfolded, and drought stress treatment begins at this point. The drought stress treatment method is to add PEG8000 with a final concentration of 20% to the Hogland solution, and after complete dissolution, transfer soybean seedlings into it. Before treatment (0 h as a control) and after treatment 1, 2, 5, 10, Cut 0.1 g of soybean seedling leaves for 24 hours, immediately place them in liquid nitrogen, and transfer them to a -80 ℃ ultra-low temperature freezer. It is worth noting that the soybeans used in the experiment were divided into three groups: normal plants, GmHORSTs gene silenced plants, and wild plants, with 50 samples in each group. Control ls were corresponding normally growing plants, similarly 50 in each group.

To measure the GmHORSTs gene expression of different plants, the expression level was examined. GmHORSTs gene expression detection method: Design quantitative primers (F-GmActin，R-GmActin，F-GmHORSTs，R-GmHORSTs) for GmHORSTs genes using NCBI. Then, extract RNA from soybean leaves using the Trizol method and synthesize cDNA from soybean leaves. Finally, perform real-time fluorescence quantitative PCR with a reaction temperature and time cycle of 94℃/30 s-94℃/5 s-60℃/15 s-72℃/10 s, for a total of 40 cycles.

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In order to reflect the growth status of different soybean plants, their physiological and biochemical indexes were measured. Physiological and biochemical index measurement: Firstly, measure the malondialdehyde content, fresh weight, and dry weight of soybean leaves, and measure their relative water content and H_2O_2 content. Malondialdehyde content measurement: Weigh 0.1 g of the sample and place it in a mortar, then add 1 ml of TCA solution (10%) and a small amount of quartz sand for grinding. Centrifuge the ground sample under the condition of 12000 g/15 min. Add an equal amount of TBA solution (0.5%) to the supernatant and heat it in a boiling water bath for 30 minutes. Remove the supernatant, cool it, and centrifuge at 12000 g for 10 minutes. Extract 300 uL of supernatant and measure its OD450, OD532, and OD600 absorbance to calculate the content of malondialdehyde (Sheteiwy et al., 2021; Niazian et al., 2022). Relative water content measurement: After measuring the fresh weight of 12 soybean plants, soak them in deionized water for 12 hours and measure their saturated weight. Then take out the plants and wrap them in newspaper for withering at 108℃ and 15 minutes, respectively. Place the green plants in an oven for drying until they reach a constant weight, with a drying temperature of 60℃. Measure the dry weight of the plant and calculate its relative water content. H_2O_2 content measurement: Use H_2O_2 detection kit to measure the content of H_2O_2 supernatant. Weigh 0.05 g of the sample and add 450 uL of *NaCl* solution (0.9%) to it, then grind it at 4℃. Then centrifuge the ground sample at a speed of 10000rpm and for 10 minutes. Use an enzyme-linked immunosorbent assay (ELISA) reader to detect the absorbance of the supernatant at a wavelength of 405 nm.

To stain the amount of reactive oxygen species in the leaves of different plants. Leaf staining method: Diaminobenzidine (DAB) staining and Nitro Blue Tetrazolium (NBT) staining are carried out on the leaves. DAB staining: Soak the leaves in DBA staining solution for 14 hours, then heat and boil for 2 minutes. Then, remove the leaves and place them in a centrifuge tube, and add 40 mL of ethanol (90%) for decolorization (Rahman et al., 2021). NBT staining: After immersing the leaves in NBT staining solution for 14 hours, remove the leaves and place them in a centrifuge tube. Add 40 ml ethanol (90%) water bath for decolorization at a temperature of 80℃. When decolorizing, replace ethanol every 10 minutes to ensure the degree of decolorization (El-Sappah et al., 2023).

To reflect the effect of abscisic acid on drought resistance, the abscisic acid content in different plants was measured. Abscisic Acid (ABA) content detection method: ABA in soybean plants is detected using an ABA ELISA detection kit. The soybean plant sample is mixed with phosphate buffered saline solution in a ratio of 1:9, and then the solution is centrifuged at 10000 g/20 min. The supernatant is subjected to OD450 detection.

In order to reflect the change, the stomatal conductivity of soybean plants was measured. Pore conductance detection method: firstly, irrigate the two groups of soybeans in vermiculite with Holland nutrient solution until the second pair of compound leaves of the soybeans are unfolded. Then, process two groups of soybean seedlings with PEG6000 stress solution (10%) for 4 hours. One group does not receive additional treatment, while the other group is sprayed with ABA solution (6 uM) on its leaves. Cut off soybean leaves and treat them in a mixture of 6 ethanol/1 acetic acid solution for 24 hours. Place the processed leaves in ethanol (70%) for dehydration treatment, and dehydrate them three times using anhydrous ethanol for 30 minutes each time. Place the dehydrated leaves in a solution of hydrated chloral/glycerol/water ratio

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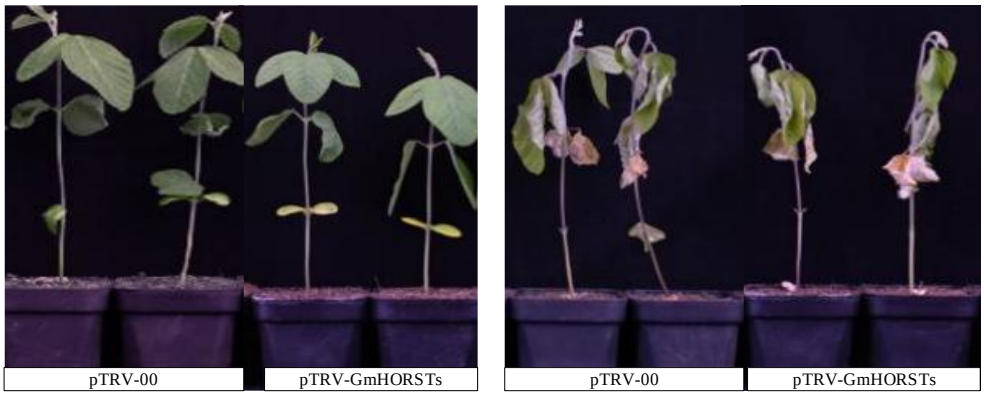
of 8:2:1 for 10 hours. Then, observe the stomata of the leaves using a confocal laser microscope and measure their stomatal conductance.

Statistical methods: Data processing and plotting were performed using Microsoft Excel 2016, and SPSS 21 was used The significance test between treatments was conducted using Duncan's method, a statistical software (P<0.05).

Results

Analysis of drought stress tolerance of soybean by GmHORSTs gene

To explore the effect of GmHORSTs gene on drought stress tolerance of soybean, pot experiments are carried out. The physiological and biochemical indexes of soybean plants are measured and leaf staining analysis is carried out. The drought stress phenotypes of various soybean plants are displayed in *Figure 1*.



(a) Normal irrigation (b) Drought stress

Figure 1. *Drought stress phenotypes of different soybean plants*

According to *Figure 1(a)*, under normal irrigation conditions, pTRV-00 and pTRV-GmHORSTs both grew well. From *Figure 1(b)*, the withering degree of pTRV-GmHORSTs was more severe compared with pTRV-00. The GmHORSTs gene plays a vital function in the drought stress tolerance of soybean. In order to further explore the reasons for the phenotypic changes of soybean under drought stress, physiological and biochemical indicators are measured. The physiological and biochemical indicators of soybeans are shown in *Table 2*.

According to *Table 1*, under normal irrigation conditions, the fresh weight, dry weight, and water content of pTRV-00 and pTRV-GmHORSTs were the same. However, after drought stress, the fresh weight, dry weight, and water content of pTRV-GmHORSTs were 0.90 g, 0.48 g, and 36.7%, respectively, significantly lower than pTRV-00. Under normal irrigation conditions, the malondialdehyde and H_2O_2 were basically the same. However, after drought stress, the malondialdehyde and H_2O_2 levels of pTRV-GmHORSTs were 0.41 umlo/g FW and 13.69 umlo/g, respectively, both higher than pTRV-00. The above results indicate that the silencing of pTRV-GmHORSTs gene led to the change of drought phenotype in soybean. The DAB staining results of soybean leaves are shown in *Figure 2*.

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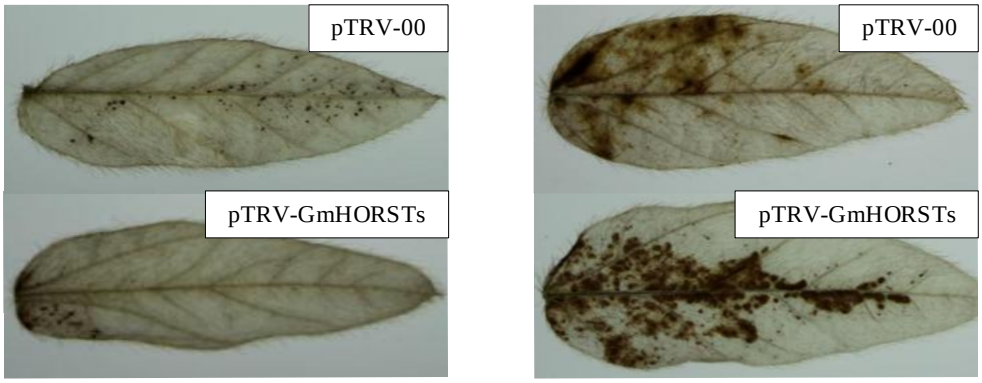
Table 2. *Physiological and biochemical indicators of soybeans*

Index		pTRV-00	pTRV-GmHORSTs
Average fresh weight/g	Normal irrigation	2.52	2.52
	Drought stress	1.61*	0.90*
Average dry weight/g	Normal irrigation	0.60	0.60
	Drought stress	0.54	0.48
Average water content/%	Normal irrigation	90.2	90.2
	Drought stress	59.8*	36.7*
Average content of malondialdehyde (umlo/g FW)	Normal irrigation	0.22	0.22
	Drought stress	0.29	0.41
Average content of H_2O_2 (umlo/g)	Normal irrigation	5.74	5.82
	Drought stress	9.78*	13.69*

Note: * indicates P <0.05



(a) Normal irrigation (b) Drought stress

Figure 2. *DAB staining results of soybean leaves*

According to *Figure 2(a)*, under normal irrigation conditions, the damaged area of pTRV-00 and pTRV-GmHORSTs leaves was basically the same. According to *Figure 2(b)*, under drought stress conditions, the leaf damage area of pTRV-GmHORSTs was significantly larger compared with normal leaves. From this, the H_2O_2 content in the leaves of pTRV-GmHORSTs is higher. The NBT staining results of soybean leaves are shown in *Figure 3*.

As shown in *Figure 3(a)*, the NBT staining results indicated that the leaf damage areas of pTRV-00 and pTRV-GmHORSTs under normal irrigation were roughly equivalent. The accumulation of reactive oxygen species in leaves under normal conditions was basically the same. From *Figure 3(b)*, under drought stress conditions, the leaf damage area of pTRV-GmHORSTs was significantly larger than that of pTRV-00, which was consistent with the staining results of DAB. The above results once again confirm the important role of GmHORSTs genes in soybean drought stress tolerance. To further investigate the specific role of GmHORSTs gene in drought stress tolerance of soybean, a stable genetic transformation experiment is conducted to obtain soybean plants over-expressed GmHORSTs gene. The gene expression detection results of GmHORSTs over-expressed plants are shown in *Figure 4*.

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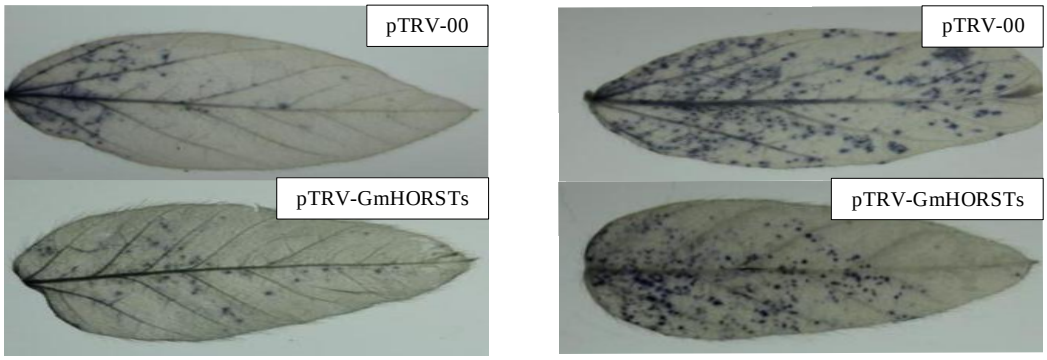
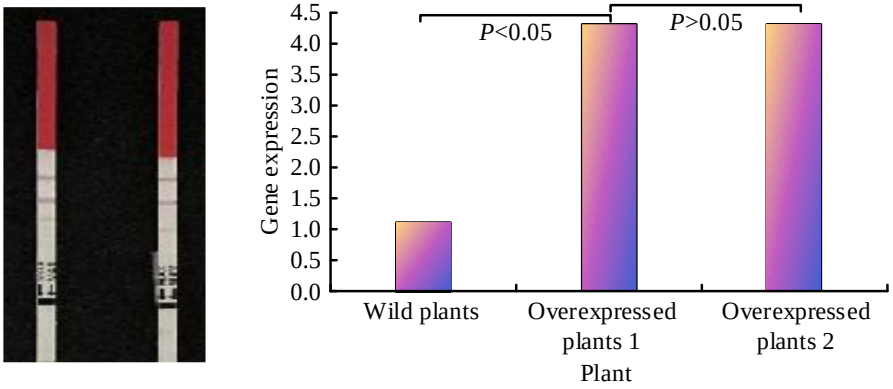


(a) Normal irrigation (b) Drought stress

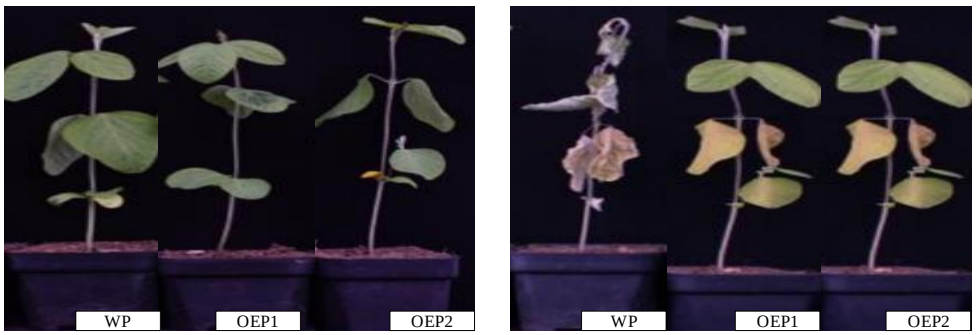
Figure 3. *NBT staining results of soybean leaves*



(a) Immunoassay test strip detection results (b) Gene expression

Figure 4. *Gene expression detection results*

As shown in *Figure 4(a)*, the plants obtained from soybean stable genetic transformation experiments all exhibited over-expressed GmHORSTs genes. According to *Figure 4(b)*, in comparison with the wild plants, the GmHORSTs gene was only 1.1, while the GmHORSTs gene in both groups of over-expressed plants was 4.3, which was significantly different from the wild plants. The pot experiment results of over-expressed plants are shown in *Figure 5*.



(a) Normal irrigation (b) Drought stress

Figure 5. *Potted experiment results of over-expressed plants. Note: WT stands for wild plants, OEP stands for over-expressed plants*

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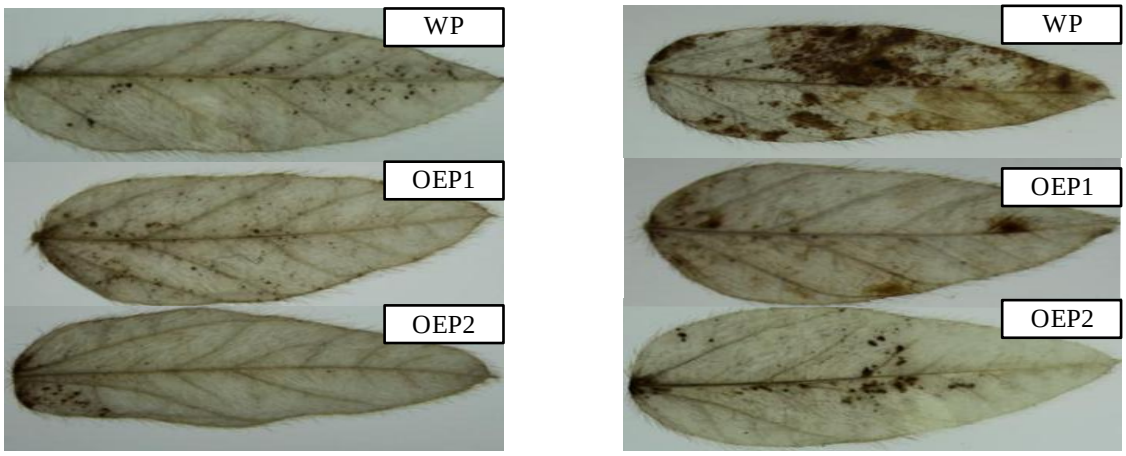
According to *Figure 5(a)*, under normal irrigation conditions, both the wild plant and the two over-expressing plants grew well. From *Figure 5(b)*, compared with wild plants, over-expressed plants had a lighter degree of withering under drought stress, and only showed slight leaf yellowing. The above results indicate that over-expressed GmHORSTs gene can effectively enhance the drought stress tolerance of soybean. The physiological and biochemical indicators of over-expressed plants are shown in *Table 3*.

Table 3. *Physiological and biochemical indicators of over-expressed plants*

Index		Wild plants	Over-expressed plants 1	Over-expressed plants 2
Average fresh weight/g	Normal irrigation	2.55	2.52	2.52
	Drought stress	0.84*	1.59*	1.59*
Average dry weight/g	Normal irrigation	0.85	0.85	0.85
	Drought stress	0.48	0.59	0.59
Average water content/%	Normal irrigation	91.6	91.5	91.5
	Drought stress	30.3*	61.2*	61.2*
Average content of H_2O_2 (umlo/g)	Normal irrigation	5.81	5.85	5.82
	Drought stress	8.64*	6.71*	6.69*
Average content of malondialdehyde (umlo/g FW)	Normal irrigation	0.07	0.08	0.08
	Drought stress	0.15	0.11	0.11

Note: * indicates P <0.05

According to *Table 3*, under normal irrigation conditions, the physiological and biochemical indicators of wild plants and over-expressed plants were basically the same. The fresh weight, dry weight, and water content of over-expressed plants after drought stress were 1.59 g, 0.59 g, and 61.2%, respectively, all higher than those of wild plants, while their H_2O_2 and propylene glycol contents were lower than those of wild plants. The above results indicate that the GmHORSTs gene has a positive effect on drought stress tolerance in soybean. The DAB staining results of over-expressed plants are shown in *Figure 6*.



(a) Normal irrigation (b) Drought stress

Figure 6. *DAB staining results of over-expressed plants*

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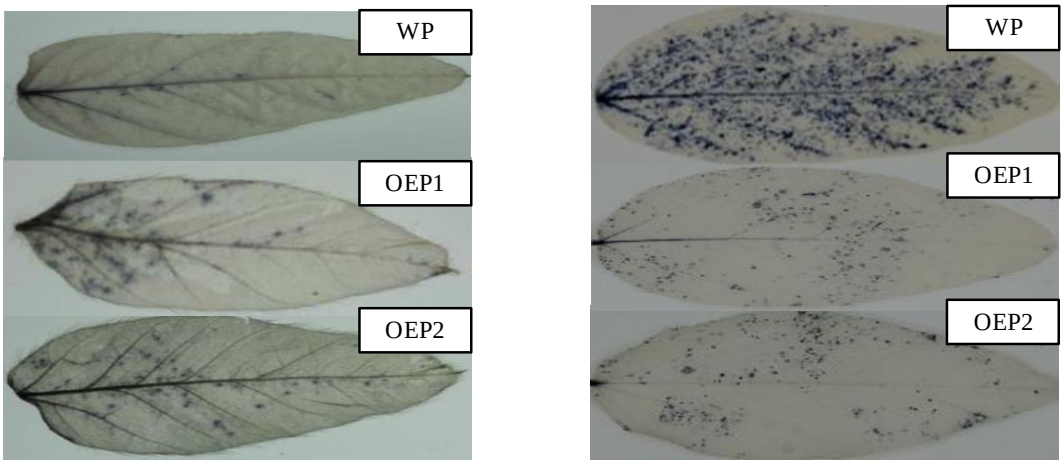
From *Figure 6(a)*, the DAB staining of wild soybean plants and over-expressed plants under normal irrigation was basically the same, indicating that no significant difference existed in H_2O_2 content among the three. From *Figure 6(b)*, compared with wild plants, over-expressed plants had less leaf damage under drought stress. The above results indicate that over-expressed plants accumulate less H_2O_2 under drought stress. The NBT staining results of over-expressed plants are shown in *Figure 7*.



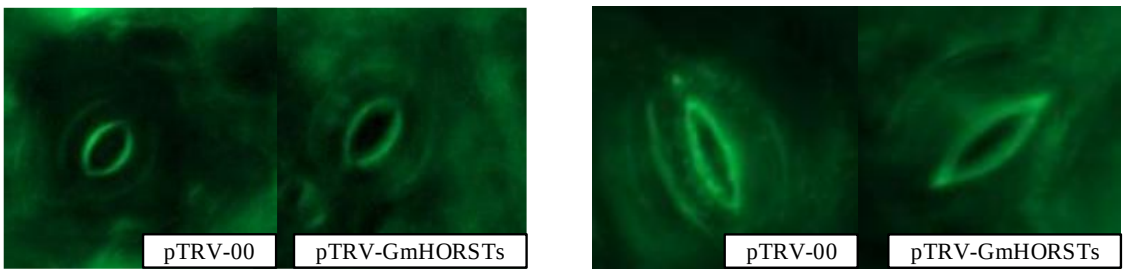
(a) Normal irrigation (b) Drought stress

Figure 7. *NBT staining results of over-expressed plants*

From *Figure 7(a)*, the NBT staining of wild soybean plants under normal irrigation was basically consistent with that of over-expressed plants. According to *Figure 7(b)*, under drought stress conditions, the leaf damage area in wild plants was significantly larger than that in over-expressed plants, which was consistent with the staining results of DAB. Plants over-expressed GmHORSTs have less accumulation of reactive oxygen species under drought stress conditions.

Mechanism of GmHORSTS gene on drought stress tolerance in soybean

To explore the mechanism of GmHORSTS gene on drought stress tolerance of soybean, PEG6000 is used to simulate drought stress, and its ABA and stomatal conductance are detected. The stomatal changes of soybean silenced by GmHORSTs gene are shown in *Figure 8*.



(a) Normal moisture content (b) PEG6000 coercion

Figure 8. *Changes in stomata of soybean silenced by GmHORSTs gene*

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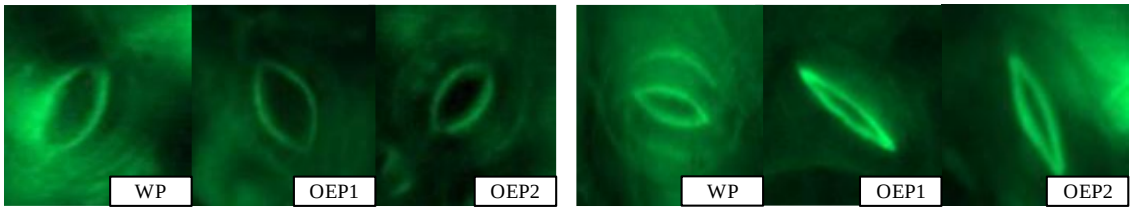
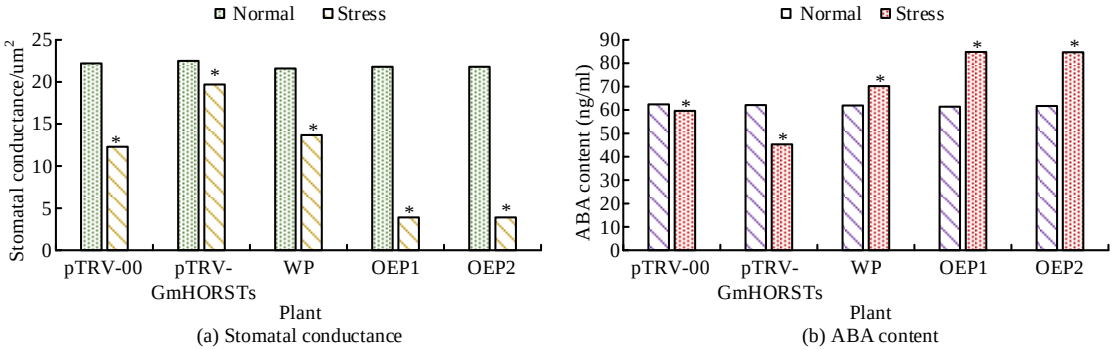
According to *Figure 8(a)*, under normal water conditions, the stomatal changes in the leaves of pTRV-00 and pTRV-GmHORSTs were basically the same. As shown in *Figure 8(b)*, under osmotic stress of PEG6000, the stomata of pTRV-GmHORSTs were larger than those of pTRV-00. The above results indicate that the GmHORSTs gene has an obviously impact on the stomata of plants. The stomatal changes of over-expressed plants are shown in *Figure 9*.



(a) Normal moisture content (b) PEG6000 coercion

Figure 9. *Changes in stomata of over-expressed plants*

According to *Figure 9(a)*, under normal water conditions, the stomatal changes of wild plants and over-expressed plants were basically the same. According to *Figure 9(b)*, under the osmotic stress condition of PEG6000, the stomata of over-expressed plants were smaller than those of wild plants. From this, the over-expressed GmHORSTs genes can cause plants to shrink stomata and retain more water under drought stress conditions. The stomatal conductance and ABA content of different plants are shown in *Figure 10*.



(a) Stomatal conductance (b) ABA content

Figure 10. *Stomatal conductance and ABA content of different plants. Note: * indicates P <0.05*

According to *Figure 10(a)*, under normal conditions, the stomatal conductance of pTRV-00 and pTRV-GmHORSTs was basically the same. Under drought stress conditions, the stomatal conductance of the two are 12.3 um^2 and 19.7 um^2, respectively. Under normal conditions, the stomatal conductance of wild plants and over-expressed plants was basically the same, while under osmotic stress conditions, the stomatal conductance of the three are 13.7 um^2, 3.9 um^2, and 3.9 um^2, respectively. According to *Figure 10(b)*, under normal conditions, the ABA content of pTRV-00, pTRV-GmHORSTs, wild plants and over-expressed plants was basically the same. Under stress conditions, the ABA content was 59.6 ng/mL, 45.3 ng/mL, 70.2 mg/mL, 84.8

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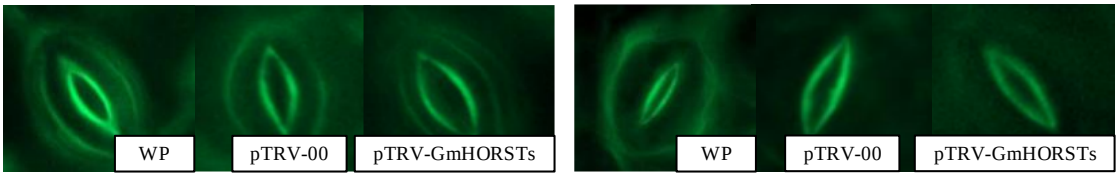
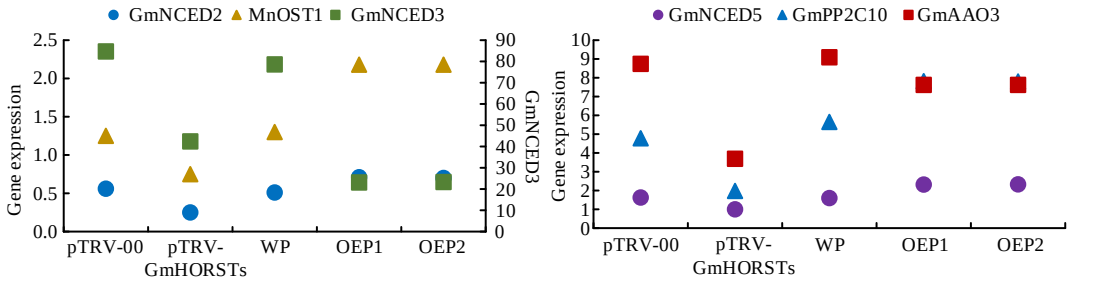
ng/ML, and 84.7 ng/mL, respectively. The above results indicate that the GmHORSTs gene can promote the closure of stomata in plants under drought conditions and increase the content of ABA. The changes in stomata after exogenous application of ABA are shown in *Figure 11*.



(a) PEG6000 coercion (b) PEG6000 stress and application of exogenous ABA

Figure 11. *Changes in stomata after exogenous application of ABA*

According to *Figure 11(a)*, after applying PEG6000 osmotic stress, the stomata of wild plants, pTRV-00, and pTRV-GmHORSTs were all closed, but the stomatal closure degree of pTRV-GmHORSTs was lower than that of wild plants and pTRV-00. From *Figure 11(b)*, after applying PEG6000 osmotic stress and exogenous ABA, the stomata of wild plants, pTRV-00, and pTRV-GmHORSTs were smaller than those under PEG6000 osmotic stress alone, but the stomatal closure degree of pTRV-GmHORSTs was still lower than that of wild plants and pTRV-00. The above results indicate that the GmHORSTs gene is involved in ABA regulated stomatal closure. The related genes under drought stress is shown in *Figure 12*.



(a) Expression levels of GmNCED2, GmNCED3, and MnOST1 (b) Expression levels of GmNCED5, GmPP2C10, and GmAAO3

Figure 12. *Expression of related genes under drought stress*

According to *Figure 12(a)*, under drought stress, the GmNCED2 gene in pTRV-00 and pTRV-GmHORSTs plants were 0.56 and 0.25, respectively. The GmNCED2 gene in wild plants and two over-expressed plants were 0.51, 0.71, and 0.70, respectively. The expression levels of GmNCED3 gene in pTRV-00, pTRV-GmHORSTs, wild plants and two over-expressed plants were 84.7, 42.4, 78.6, 23.1, and 23.3, respectively. The expression levels of MnOST1 gene were 1.25, 0.75, 1.30, 2.18, and 2.18, respectively. Compared with GmHORSTs silenced plants, over-expressed plants showed an increase in the GmNCED2 and MnOST1 genes, while the GmNCED3 was significantly reduced. According to *Figure 12(b)*, under drought stress, the GmNCED5 gene in wild plants and two over-expressed plants were 1.63, 1.00, 1.60, 2.32, and 2.33, respectively. The expression levels of GmPP2C10 were 4.78, 1.98, 5.65, 7.82, and 7.80, respectively. The

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expression levels of GmAAO3 were 8.74, 3.69, 9.09, 7.62, and 7.62, respectively. The expression levels of GmNCED5, GmPP2C1, and GmAAO3 in over-expressed plants were significantly increased. GmHORSTs genes have a significant impact on the ABA synthesis related genes, thereby improving the response speed of stomata to ABA and enhancing soybean drought stress tolerance.

Discussion

In order to analyze the effect of GmHORSTS gene on drought stress tolerance of soybean and its mechanism, soybean plants with GmHORSTS gene silencing are obtained by VIGS, and their physiological and biochemical indicators are detected. The experimental results showed that under drought stress conditions, compared with pTRV-00, pTRV-GmHORSTs withered more severely. After drought stress, the fresh weight, dry weight, and water content of pTRV-GmHORSTs were 0.90 g, 0.48 g, and 36.7%, respectively, significantly lower than pTRV-00. The content of malondialdehyde and H_2O_2 were both higher than pTRV-00, with values of 0.41 umlo/g FW and 13.69 umlo/g, respectively. Meanwhile, DAB and NBT staining results demonstrated that under drought stress conditions, the leaf damage area of pTRV-GmHORSTs was significantly larger than that of pTRV-00. From this, the H_2O_2 content in the leaves of pTRV-GmHORSTs was higher. The above results indicated that in plants with GmHORSTs gene silencing, the accumulated dry matter in their leaves was less, but the accumulation of reactive oxygen species was higher. For GmHORSTs over-expressed plants, compared with wild plants, their withering degree under drought stress was lighter, and the plants only showed slight leaf yellowing. After drought stress, their fresh weight, dry weight, and water content were 1.51 g, 0.59 g, and 61.2%, respectively, all higher than wild plants, while their H_2O_2 and propylene glycol content was lower than that of wild plants. Meanwhile, DAB and NBT staining results demonstrated that the accumulation of reactive oxygen species in over-expressed plants was below that in wild plants. This demonstrated that the GmHORSTs gene had active effects on the drought stress tolerance of soybean. The difference in leaf reactive oxygen species content among plants with different drought stress tolerance is due to the dynamic balance between the production and clearance of reactive oxygen species in plant cells under normal environmental conditions. When plants experience drought stress, this balance is disrupted, leading to disturbances in the production and metabolism of reactive oxygen species within the plant. Reactive oxygen species mediated oxidative stress can cause various harmful cellular effects such as biofilm peroxidation, cell nucleus damage, hindered photosynthesis, and abnormal respiration, thereby reducing plant drought stress tolerance (Chen et al., 2021; Asha et al., 2021; Li et al., 2024). Vijayaraghavareddy et al. (2022) investigated the effects of reactive oxygen species production and clearance on the sensitivity of rice and wheat to drought stress. The results showed that an increase in non-photochemical quenching levels led to a decrease in the accumulation of activity in wheat, indicating that wheat had better drought stress tolerance than rice (Vijayaraghavareddy et al., 2022). Clearing reactive oxygen species had a significant impact on improving drought tolerance.

To further explore the mechanism of GmHORSTS gene on drought stress tolerance of soybean, its stomatal conductance and ABA content were detected. The results showed that under normal conditions, the stomatal conductance of different plants was basically equal. Under drought stress conditions, the stomatal conductance of pTRV-

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GmHORSTs was 19.7 um^2, which was higher than that of pTRV-00. The stomatal conductance of over-expressed plants is 3.9 um^2, which was lower than that of wild plants. Meanwhile, the ABA content of pTRV-GmHORSTs was 45.3 ng/mL, lower than that of pTRV-00. The ABA content of over-expressed plants was 84.8 ng/ml and 84.7 ng/mL, both higher than that of wild plants. In addition, after the application of PEG6000 osmotic stress and exogenous ABA, the stomata of wild plants, pTRV-00, and pTRV-GmHORSTs were smaller than those under PEG6000 osmotic stress alone, but the stomatal closure degree of pTRV-GmHORSTs was still lower than that of wild plants and pTRV-00. The GmHORSTs gene had a significant impact on ABA regulation of stomatal conductance. This is because under drought conditions, the ABA content in plant leaves increases, which promotes the efflux of potassium ions, chloride ions, and malic acid ions, thereby causing stomatal closure and reducing transpiration rate (Woraathasin et al., 2021; Khosravi-Nejad et al., 2022; Gao et al., 2023). Baek et al. (2023) analyzed the role of the pepper homologous box abscisic acid signal related transcription factor CaHAT1 in drought response. The results showed that plants silenced by CaHAT1 exhibited a drought sensitive phenotype. Compared with control plants, ABA mediated stomatal closure was reduced and stress response gene expression was decreased. The drought resistance of CaHAT1 transgenic arabidopsis significantly increased (Baek et al., 2023). Abscisic acid can improve the drought stress tolerance of plants by promoting stomatal closure. In addition, the study also analyzed the expression of ABA synthesis related genes. Compared with gene silenced plants, over-expressed plants had significantly increased expression levels of GmNCED2, MnOST1, GmNCED5, GmPP2C1, and GmAAO3. The increase in the expression levels of GmNCED2, NCED5, and GmAAO3 significantly enhanced the content of ABA, while the increase in the expression levels of MnOST1 and GmAAO3 promoted ABA mediated defense cell signaling transduction (Kumar et al., 2021; Molinari et al., 2022).

In summary, GmHORSTs exerts a crucial positive regulatory role in the drought stress tolerance of soybeans. They can control the closure of leaf stomata by regulating the content of ABA in plant leaves, thereby reducing transpiration and reducing water loss in plants under drought conditions.

Conclusion

In order to cope with the impact of climate change, increase soybean production, and ensure global food security, VIGS was used to obtain soybean plants with GmHORSTs gene silencing. The role of soybean GmHORSTs gene in drought stress tolerance was analyzed. The experimental results showed that the GmHORSTs gene could significantly reduce the content of H_2O_2 and propylene glycol in soybean leaves. Moreover, it can also effectively regulate the ABA content in the leaves, promote the closure of leaf stomata by increasing ABA content, and improve soybean's drought stress tolerance. However, the study only analyzes the stomatal conductance of plants with the GmHORSTs gene and does not consider its effects on roots and other related indicators. There are certain limitations to the research. Therefore, in the future, a more comprehensive analysis of the drought stress tolerance of the GmHORSTs gene will be conducted, and downstream target genes will be identified to fully explore the mechanism of action of the GmHORSTs gene.

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