

The ascorbate-glutathione cycle (AsA-GSH) and the AhGST23 gene mediate drought and salt stress tolerance in peanut (*Arachis hypogaea* L.)

Zengqiang Li

Henan Institute of Science and Technology

Haifang Dai

Henan Institute of Science and Technology

Muhammad Hafeez Ullah Khan

Henan Institute of Science and Technology

Xiangjun Kong

Henan Institute of Science and Technology

Mengyao Shao

Henan Institute of Science and Technology

Peipei Jia

Henan Institute of Science and Technology

Lijie Li

Henan Institute of Science and Technology

Zhenkun Cui

Henan Institute of Science and Technology

Xin Zhang

Henan Institute of Science and Technology

Mehboob-ur Rahman

National Institute for Biotechnology and Genetic Engineering

Khudayberdi Nazarov

Tashkent State Agrarian University

Zhiyong Zhang

z_zy123@126.com

Henan Institute of Science and Technology

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Abstract

Background

Abiotic stresses such as drought and salinity significantly constrain peanut productivity, nevertheless, their underlying molecular response mechanisms remain unclear.

Results

This study identifies the ascorbate-glutathione cycle (AsA-GSH) and *AhGST23* gene as crucial components in mitigating drought and salt stress in peanut. Under drought (15% PEG6000 for 6 days) and salt (200 mM NaCl for 6 days) stress, peanut seedlings exhibited a marked reduction in biomass, net photosynthetic rate, transpiration rate, and chlorophyll fluorescence parameters. Concurrently, levels of H_2O_2 , O_2^- , and malondialdehyde (MDA), as well as the activities of antioxidant enzymes (APX, GR, SOD, POD, and CAT), were significantly elevated in both leaves and roots. RNA-seq analysis identified 3,780 and 5,019 shared differentially expressed genes (DEGs) in leaves and roots, respectively, which were enriched in pathways including plant hormone signal transduction, starch and sucrose metabolism, glutathione metabolism, and MAPK signaling. The key gene families involved in the AsA-GSH cycle (*AhGRs*, *AhGSTs*, *AhGPXs*, and *AhAPXs*) were highlighted as central players in the antioxidant system. Silencing *AhGST23* disrupted the AsA-GSH cycle in peanut. This disruption was manifested by reduced contents of AsA, DHA, GSH, and GSSG, as well as decreased activities of APX, GST, DHAR, and MDHAR. These disruptions impaired ROS scavenging capacity and heightened sensitivity to drought and salt stress.

Conclusions

In summary, the AsA-GSH cycle, with *AhGST23* as a key regulatory element, plays an essential role in conferring drought and salt stress tolerance in peanut. These findings offer novel insights into antioxidant defense mechanisms and provide valuable genetic resources for enhancing peanut stress resilience.

Introduction

Drought and salt stress significantly constrain crop productivity by impairing plant growth and development, thereby posing serious threats to global agricultural production [1, 2]. These stresses disrupt the uptake of essential macronutrients such as K^+ , Ca^{2+} , and Mg^{2+} , while salt stress specifically leads to cytotoxic Na^+ hyperaccumulation, aggravating ionic imbalance and disruption of osmotic homeostasis. The consequent decline in root-zone water potential, coupled with diminished hydraulic conductivity, exacerbates cellular dehydration and ultimately induces osmotic stress [3–5]. Critically, both stressors trigger a burst of reactive oxygen species (ROS), resulting in oxidative damage to biomolecules (nucleic acids, proteins) and organelles (plasma membranes, chloroplasts, mitochondria). Accumulation of these harmful compounds affects various physiological traits including net photosynthetic rate, respiration, and energy metabolism, thus culminating in arrested growth, reproductive failure, and reduced yield [6–8].

To mitigate these injurious effects of excessive drought and salt, plants enforce multifaceted adaptive strategies: (1) upregulating the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and the contents of antioxidants (glutathione, ascorbate) to restore redox equilibrium; (2) synthesizing osmoprotectants, such as proline and soluble sugar; (3) fine-tuning the ion transporters (SOS1, NHX) for maintaining the K^+/Na^+ and Ca^{2+}/Mg^{2+} homeostasis; (4) activating ABA and MAPK cascades for stress signaling; and (5) reprogramming stress-responsive transcriptome via NAC, MYB, and WRKY transcription factors [9–11]. Within this molecular framework, the role of ascorbate-glutathione (AsA-GSH) cycle is pivotal in responding to oxidative stress by

directly scavenging ROS [11–13]. For example, nitric oxide (NO)-mediated enhancement of the AsA-GSH cycle has been shown to enhance tolerance to salinity in wheat by stabilizing redox homeostasis [14].

Notably, plants have evolved divergent adaptive mechanisms in response to drought and salt stress. For instance, salinity imposes ionic toxicity and osmotic stress which are countered by activating SOS pathway for Na⁺ extrusion and vacuole sequestration in salt-tolerant species [15]. Conversely, drought tolerant species prefer to conserve water through ABA-mediated stomatal closure and remodel root architecture to absorb more water from deeper soil layers. Roots also display stress-specific plasticity: drought often promotes the formation of deeper roots, whereas salinity triggers avoidance strategies [16]. Therefore, it is imperative to elucidate the shared core mechanisms and unique aspects of crop responses to drought and salt stress.

Peanut (*Arachis hypogaea* L.) is a major global oil and economic crop. Its seeds are used for both oil extraction and direct consumption, while the residual plant biomass can be utilized as animal feed [17]. Additionally, peanuts restore soil fertility by establishing symbiotic relationships with rhizobia for biological nitrogen fixation, which significantly reduces their reliance on chemical fertilizers [18]. Consequently, peanut cultivation is characterized by high yield, low input costs, and environmental friendliness [19, 20]. Despite its importance, the molecular mechanisms that mediate peanut responses to drought and salt stress have not been comprehensively explored [21].

The present work focuses on the drought (15% PEG6000) and salt (200 mM NaCl) responses in Yuhua 37, a high-yielding Chinese peanut cultivar. The stress treatments were calibrated using preliminary dose-response assays to achieve physiologically comparable intensities. We comprehensively assessed drought and salt responses by recording various morphological and physiological parameters. Transcriptomic data were also collected to corroborate the molecular and physiological responses. Finally, the role of a key gene was validated using virus-induced gene silencing (VIGS)-mediated functional analysis. The results demonstrate that the AsA-GSH cycle plays a pivotal role in peanut adaptation to both drought and salt stress, with *AhGST23* emerging as key regulatory gene. These molecular insights not only elucidate the mechanistic basis of peanut stress adaptation but also identify the AsA-GSH cycle and *AhGST23* as prime candidates for enhancing drought and salinity tolerance in peanut.

Methods

Plant material cultivation and stress treatments

The widely cultivated peanut cultivar Yuhua 37 (developed by the Henan Academy of Agricultural Sciences) was selected for this study. Seeds produced through controlled selfing were used in the present study. Seeds of uniform size were surface-sterilized with 3% H₂O₂ for 10 min followed by rinsing three times with distilled water, and germinated on moist filter paper under controlled conditions: 10 h light/14 h dark cycle, 27 ± 2°C day/25 ± 2°C night, photosynthetic photon flux density of 350 μmol/(m²·s), and 75% relative humidity. After nine days, a total of 54 uniformly grown seedlings were transplanted into nine hydroponic containers (25 cm×20 cm×15 cm), with six seedlings in each container. These containers were randomly divided into three treatment groups (control, drought, and salt stress), with three containers (biological replicates) per group. All plants were initially acclimated by keeping in half-strength modified Hoagland solution for three days. Stress treatments were imposed for six days. A stepwise stress regime was implemented with increments at two-day intervals. For drought stress, PEG6000 concentrations were increased from 5% to 10% and 15%; for salt stress, NaCl concentrations were increased from 100 mM to 150 mM and 200 mM, respectively. All treatment solutions were replenished daily and maintained in an aerated state to ensure oxygen supply.

Determination of phenotypic traits, photosynthetic performance, and chlorophyll fluorescence parameters

After six days of treatment, three peanut seedlings were randomly selected from each container. The photosynthetic performance (net photosynthetic rate, stomatal conductance, intercellular CO₂ concentration, and transpiration rate) and

chlorophyll fluorescence parameters of the first fully expanded leaf from the upper part of the seedlings were recorded using LI-6800 portable photosynthesis system (LI-COR Biosciences, USA). Root systems were scanned using an Epson Expression 12000XL scanner, and total root length, root surface area, and root volume were analyzed with the WinRHIZO 2007 root analysis system (Regent Instruments, Canada). Weight of fresh roots, stems, and leaves was recorded using an electronic balance (0.00001 g precision). Seedlings were kept at 105°C for 30 minutes, followed by oven-drying at 80°C to constant weight, and re-weighed to determine dry biomass. For the remaining three seedlings in each treatment, leaves and roots were thoroughly rinsed with deionized water, separated, and stored at -80°C for subsequent analyses, including physiological index determination, transcriptome sequencing, and qRT-PCR validation.

Determination of physiological indexes

The O_2^- and H_2O_2 contents were quantified using biochemical kits (Solarbio Science & Technology Co., Ltd, Beijing, BC1290, BC3595). The MDA contents were determined by applying the thiobarbituric acid method, the activities of SOD and POD were determined using the nitroblue tetrazolium method and guaiacol method, respectively, and the activities of CAT, APX, GR, GST were measured using the UV spectrophotometric method, as described by Liu et al [22].

Transcriptome sequencing and qRT-PCR validation

Transcriptome sequencing was performed using established molecular biology protocols. Total RNA was extracted from peanut root tissues using an RNA extraction kit (CoWin Bioscience, Item No. CW0599S). Quality and quantity of RNA were confirmed before synthesizing cDNA. The mRNA was then purified from the total RNA and sheared into short fragments for sequencing. Subsequently, first-strand cDNA was synthesized from the fragmented mRNA templates followed by second-strand cDNA synthesis to create double-stranded DNA. The cDNA underwent purification to remove reaction components followed by end repair to create blunt ends. A poly-A tail was added to facilitate sequencing, and then adapters were ligated to enable PCR amplification. Finally, the library was amplified through PCR to generate sufficient DNA template for high-throughput sequencing. The constructed libraries were sequenced using an Illumina NovaSeq™ X Plus system (LC-Bio Technology Co., Ltd., Hangzhou, China). Gene expression levels were quantified as Fragments Per Kilobase Million mapped reads (FPKM), and differentially expressed genes (DEGs) were identified with thresholds of |fold change| ≥ 2 and q-value < 0.05. Subsequently, based on these DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed [23].

Total RNA was reverse-transcribed into cDNA for qRT-PCR following the manufacturer's instructions for the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, Cat. No. Q311-03). Ten DEGs were randomly selected for qRT-PCR validation to confirm the accuracy of transcriptome data [23].

Virus-induced gene silencing of

Virus-induced gene silencing of *AhGST23*

Peanut *AhGST23*-silenced plants were generated using virus-induced gene silencing (VIGS). Briefly, a pTRV2-*AhGST23* silencing vector was constructed and transformed into *Agrobacterium tumefaciens* strain EHA105. The *Agrobacterium* suspension was infiltrated into peanut leaves using a syringe. Silencing efficiency was confirmed by qRT-PCR. Detailed protocols followed our previous publication [23]. The contents of AsA, dehydroascorbate (DHA), glutathione (GSH), oxidized glutathione (GSSG), and the activities of dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) were determined using biochemical kits (Solarbio Science & Technology Co., Ltd, Beijing, Item No. BC1235, BC1245, BC1175, BC1185, BC0665 and BC0655), respectively.

Statistical analysis

Results and analysis

Drought and salt stress significantly inhibit peanut growth

Both drought and salt stress negatively affected the morphology of peanut seedlings (Fig. 1). Salt stress caused more severe suppression of shoot growth, as evidenced by a significant reduction in seedling height and aboveground biomass (Table 1). In contrast, drought stress led to sharp reduction in root length, surface area, and volume (Table 1). Results showed that whole-plant dry weight decreased by 16.33% under drought conditions while it was 30.61% under salt stress, significantly higher than that under drought stress. Conversely, the reduction in root length was more severe under drought (54.71%) than under salt stress (36.77%). These results indicate that while both stresses inhibit peanut growth, salt stress primarily constrains shoot development, whereas drought stress more severely disrupts root system architecture.

Table 1
Determination of growth indices.

	Plant height (cm)	Fresh weight (g)			Dry weight (g)			Root length (cm)	Surface area (cm ²)	Root volume (cm ³)
		Whole plant	Aboveground	Root	Whole plant	Aboveground	Root			
Control	9.80 a	7.06 a	4.55 a	2.51 a	0.98 a	0.83 a	0.16 a	899.02 a	141.57 a	1.89 a
Drought	6.93 b	4.52 b	3.15 b	1.37 c	0.82 b	0.71 b	0.11 b	568.48 b	94.78 b	1.26 b
NaCl	5.93 c	4.10 b	2.37 c	1.72 b	0.68 c	0.55 c	0.13 b	407.19 c	78.96 b	1.19 b
Note: Different lowercase letters represent significant differences between groups (<i>P</i> < 0.05), the same applies below.										

Drought and salt stress elevate ROS levels and antioxidant activity in peanut seedlings

Under drought stress, the content of O₂⁻ in peanut seedlings increased by 124.44%, while MDA, a key indicator of membrane damage resulting from lipid peroxidation, increased by 63.24% compared to the control (Fig. 2A, 2C). Interestingly, the H₂O₂ content decreased by 21.56% (Fig. 2B), indicating the enhanced enzymatic detoxification of H₂O₂, potentially through CAT or glutathione-dependent pathways. This was supported by increased activities of CAT (112.10%) and GR (121.92%) (Fig. 2F, 2H), suggesting a robust antioxidant response to counteract ROS accumulation. Additionally, SOD and POD activities increased by 14.58% and 27.92%, respectively, further reflecting activation of enzymatic defence systems (Fig. 2D, 2E).

Under salt stress, the oxidative and antioxidant responses followed a different pattern. The H₂O₂ and MDA contents increased sharply by 53.70% and 130.60% (Fig. 2B, 2C), respectively, indicating substantial ROS accumulation and membrane damage. Notably, the O₂⁻ level did not change significantly, possibly due to efficient elimination by upregulated SOD activity (18.75%, Fig. 2D), which converts O₂⁻ into H₂O₂. To mitigate H₂O₂ toxicity, POD activity significantly increased by 70.05% (Fig. 2E), alongside APX by 44.39% (Fig. 2G) and GR by 80.82% (Fig. 2H). However, CAT activity remained unchanged (Fig. 2F).

In roots, drought stress induced marked ROS accumulation and severe oxidative damage. Specifically, O₂⁻, H₂O₂, and MDA contents increased by 309.91%, 153.26%, and 80.91%, respectively, reflecting enhanced ROS generation and lipid peroxidation (Fig. 3A-C). In response, the antioxidant system was strongly activated: SOD, CAT, APX, and GR increased by 44.34%, 237.50%, 39.67%, and 497.87%, respectively (Fig. 3D-H). The pronounced upregulation of CAT (2.4 times) and GR (5.0 times) underscores the importance of both the H₂O₂ scavenging and glutathione metabolism in drought tolerance.

In contrast, salt stress also induced oxidative stress in peanut roots, though to a lesser extent than drought. The contents of O₂⁻, H₂O₂, and MDA increased by 93.79%, 57.86%, and 46.65%, respectively, indicating moderate ROS accumulation (Fig. 3A-C). The SOD, POD, CAT, APX, and GR activities increased by 27.46%, 12.10%, 343.62%, 21.87%, and 68.35%, respectively (Fig. 3D-H). Notably, CAT activity increased 3.4 fold more than the other enzymes, highlighting its crucial role in H₂O₂ detoxification under salt conditions. Collectively, both stress treatments induced pronounced ROS accumulation and upregulated antioxidant enzyme activities in peanut, with drought provoking a stronger oxidative challenge and more pronounced antioxidant activation than salt stress.

Drought and salt stress reduced photosynthetic performance in peanut leaves

Both drought and salt stress significantly decreased the net photosynthetic rate and transpiration rate in peanut seedlings, indicating a marked inhibition of photosynthetic activity (Table 2). In contrast, both drought and salt stress elevated the intercellular CO₂ concentration (Table 2).

Table 2
Measurement results of photosynthetic performance parameters.

	Net photosynthesis rate (μmol CO ₂ /(m ² ·s))	Transpiration rate (mmol/(m ² ·s))	Stomatal conductance (mol/(m ² ·s))	Intercellular CO ₂ concentration (μmol/mol)
Control	6.70 a	0.003 a	395.68 a	288.26 b
Drought	0.59 b	0.001 b	398.48 a	360.91 a
Salt	0.63 b	0.002 b	398.49 a	362.49 a

Both stresses also significantly reduced the potential activity of PSII (Fv/Fo) and the photochemical performance index (PI abs), indicating impaired photosynthetic capacity (Fig. 4). Notably, salt-stressed seedlings exhibited lower PI abs values than those under drought. The coordinated decline in Pn, Tr, and PSII activity, along with elevated Ci, further reflects an overall reduction in photosynthetic capacity and water use efficiency under stress conditions. Overall, both drought and salt stress suppressed photosynthetic performance in peanut seedlings, with salt stress exerting a stronger negative impact on the photochemical efficiency of PSII.

RNA-seq data output and quality assessment

RNA sequencing was conducted on a total of 18 peanut samples, comprising leaves and roots subjected to control, drought, and salt treatments. The output and quality metrics demonstrated high sequencing reliability and consistency across all samples. The number of raw reads per sample ranged from 41.97 to 50.82 million, corresponding to a total base count between 6.38 and 7.62 gigabases (Gb), indicating a sequencing depth sufficient for accurate gene expression analysis. After filtering, the percentage of high-quality reads remained consistently high, ranging from 93.08% to 97.09%. In

addition, Q30 scores were uniformly high (97.30%-98.06%), further confirming the high quality of the sequencing data. The GC contents across all samples ranged from 43.50% to 45.50% as expected for plant transcriptomic data (Table 3).

Table 3
The output statistics of RNA seq data.

Sample	Raw Data		Valid Data		Valid Ratio (%)	Q20 (%)	Q30 (%)	GC content (%)
	Read	Base	Read	Base				
Control_L1	45,811,618	6.87 G	44,051,680	6.61 G	96.16	99.95	97.83	44.5
Control_L2	43,753,050	6.56 G	41,819,538	6.27 G	95.58	99.97	98.05	45.5
Control_L3	42,884,020	6.43 G	41,075,086	6.16 G	95.78	99.97	98.06	44.5
Control_R1	48,148,844	7.22 G	46,485,530	6.97 G	96.55	99.95	97.71	44.0
Control_R2	44,151,058	6.62 G	42,684,540	6.40 G	96.68	99.95	97.64	44.5
Control_R3	48,931,230	7.34 G	47,508,260	7.13 G	97.09	99.97	97.84	44.5
Drought_L1	42,513,658	6.38 G	40,558,748	6.08 G	95.40	99.95	97.92	44.5
Drought_L2	42,811,120	6.42 G	40,569,784	6.09 G	94.76	99.94	97.88	44.5
Drought_L3	44,020,806	6.60 G	41,648,160	6.25 G	94.61	99.94	97.91	43.5
Drought_R1	44,392,754	6.66 G	42,602,560	6.39 G	95.97	99.96	97.67	44.0
Drought_R2	42,915,388	6.44 G	41,128,376	6.17 G	95.84	99.96	97.67	44.0
Drought_R3	43,798,054	6.57 G	41,757,596	6.26 G	95.34	99.96	97.65	44.5
Salt_L1	42,863,772	6.43 G	40,362,816	6.05 G	94.17	99.96	97.69	44.5
Salt_L2	41,971,184	6.30 G	39,067,614	5.86 G	93.08	99.96	97.76	45.0
Salt_L3	43,790,978	6.57 G	41,837,192	6.28 G	95.54	99.94	97.61	43.5
Salt_R1	49,941,354	7.49 G	48,049,324	7.21 G	96.21	99.94	97.30	44.0
Salt_R2	50,824,958	7.62 G	49,034,578	7.36 G	96.48	99.93	97.30	44.0
Salt_R3	45,566,844	6.84 G	43,803,036	6.57 G	96.13	99.95	97.51	44.5

Sample correlation analysis revealed high reproducibility among biological replicates. Most treatment groups showed correlation coefficients greater than 0.98. Slightly lower correlations were observed in the Drought_Leaf (0.91) and Salt_Leaf (0.90) groups, which may be attributed to increased biological variability in leaf tissues under stress conditions. Nevertheless, these values remain within an acceptable range for transcriptomic studies. Overall, the results confirm that biological replicates were consistent and reliable for transcriptome analysis.

Identification and functional analysis of differentially expressed genes

A total of 66,979 genes and 84,455 transcripts were annotated from the sequencing data (Table S1). Among these, 5,063 DEGs were identified in leaves under drought stress, including 3,305 upregulated and 1,758 downregulated genes (Fig. 5A, Table S2). In contrast, salt stress triggered a more extensive response in leaves, with 13,329 DEGs (8,247 upregulated and 5,082 downregulated). In roots, the expression pattern was reversed: drought stress induced 15,734 DEGs (5,599 upregulated, 10,135 downregulated), whereas salt stress resulted in 8,004 DEGs (3,671 upregulated, 4,333 downregulated).

These results indicate that drought stress has a greater impact on root transcriptomes, whereas salt stress exerts stronger effects on leaves.

Notably, DEG profiles contrasted markedly between leaves and roots under the two stresses (Fig. 5A). Leaves under salt stress showed the highest number of DEGs, consistent with the stronger growth inhibition observed in aboveground tissues. Conversely, roots under drought stress exhibited the highest DEG count, aligning with the more severe physiological damage in root systems. Furthermore, upregulated genes outnumbered downregulated ones in leaves under both stresses, whereas downregulated genes predominated in roots, especially under drought.

Analysis also revealed that 2,103 DEGs in leaves and 2,905 DEGs in roots were commonly expressed under both drought and salt stress conditions (Fig. 5B). A total of 3,780 DEGs under drought and 5,019 under salt stress were commonly regulated in both leaves and roots. The higher number of shared DEGs under salt stress implies a broader systemic transcriptional reprogramming under salinity. Moreover, the total number of stress-responsive genes was greater in roots than in leaves, consistent with the direct exposure of roots to PEG6000 and NaCl solutions, positioning them as the primary site of stress perception and response. Importantly, 636 DEGs were consistently differentially expressed across all treatment comparisons, across both stress types and tissues, highlighting their role in countering both stresses in peanut.

GO and KEGG enrichment analysis of DEGs

To elucidate the biological function of DEGs involved in peanut responses to drought and salt stress, GO and KEGG pathway enrichment analyses were performed across all treatment groups (Table S3). The top ten significantly enriched terms in each category, biological process (BP), cellular component (CC), and molecular function (MF), were shown in Fig. 6A. For example, in the BP category, DEGs were primarily enriched in transcription regulation, oxidation-reduction processes, defense response, and response to water deprivation. Within the CC category, DEGs were strongly associated with the nucleus, plasma membrane, cytoplasm, and chloroplasts. In the MF category, significantly enriched terms included protein binding, ATP binding, metal ion binding, and protein serine/threonine kinase activity.

KEGG pathway analysis revealed that DEGs were significantly enriched in several stress-related pathways (Table S4, Fig. 6B). These included plant hormone signal transduction, plant-pathogen interaction, starch and sucrose metabolism, phenylpropanoid biosynthesis, MAPK signaling pathway, and glutathione metabolism. In summary, these enrichment analyses reveal a highly coordinated molecular response in peanuts under drought and salt stress, involving transcriptional control, signal transduction, metabolic adjustment, and antioxidant defense.

Phylogenetic and expression analysis of ROS and antioxidant enzyme-related gene families

Based on physiological and transcriptomic data, we conducted phylogenetic and expression analyses of key gene families involved in antioxidant metabolism. The study specifically focused on genes associated with the AsA-GSH cycle, which had been significantly enriched in our KEGG analysis. Results revealed that gene families such as *AhGRs*, *AhGSTs*, *AhGPXs*, and *AhAPXs* were broadly and significantly induced under both drought and salt stress. Notably, a large proportion of the DEGs from these families were strongly upregulated, particularly members of the *AhGST* family (Fig. 7A).

In addition, most *AhRbohs* genes were upregulated under stress conditions, indicating their active involvement in ROS production (Fig. 7B). Collectively, these findings elucidated the important role of *AhRbohs* in ROS generation, while the AsA-GSH cycle facilitates efficient scavenging of excess ROS, thereby preventing oxidative damage and maintaining cellular redox balance.

qRT-PCR validation of transcriptome sequencing results

To validate the transcriptome sequencing results, qRT-PCR was performed on 10 selected DEGs involved in the AsA-GSH cycle and ROS regulation, including six *AhGSTs*, two *AhRbohs*, one *AhGR*, and one *AhGPX*. The expression patterns of all ten genes, as validated by qRT-PCR, aligned with the RNA-seq data. Specifically, genes that were up- or down-regulated in RNA-seq showed corresponding directional changes in qRT-PCR (Fig. 8), which validated the transcriptomic results. Although the overall expression trends were consistent, minor discrepancies in fold-change values were observed between the two methods. These differences can be attributed to the distinct computational approaches used for gene expression quantification: RNA-seq data were analyzed using the FPKM method, which normalizes for transcript length and sequencing depth, whereas qRT-PCR data were processed using the $2^{-\Delta\Delta CT}$ method, which relies on cycle thresholds and reference gene normalization. These methodological differences typically affected the magnitude of fold changes but did not alter the overall expression patterns. In summary, the strong concordance in gene expression trends between qRT-PCR and RNA-seq supports the accuracy and reproducibility of the transcriptome sequencing data.

Disruption of the AsA-GSH cycle in *AhGST23*-silenced peanuts reduces salt tolerance

To further investigate the functional role of *AhGST23* in conferring drought and salt stress tolerance, particularly through its involvement in regulating AsA-GSH cycle, a VIGS approach was employed. The *AhGST23* gene (Gene ID: G3R5KE), a representative member of the Glutathione S-transferase family, was selected based on its significant upregulation under both stresses. A gene silencing vector (pTRV2-*AhGST23*) was constructed and used to generate 17 *AhGST23*-silenced peanut plants through leaf infiltration. The pTRV2-*AhGST23* treated plants and control (infiltrated with the empty pTRV2 vector) were subjected to drought and salt stress treatments.

Under drought and salt stress, *AhGST23*-silenced plants exhibited earlier and more severe wilting, along with a significant increase in water loss (Fig. 9A, B), and a marked reduction in fresh weight by 10.98% and 9.16%, respectively (Fig. 9C). Also, under control conditions, the contents of AsA, DHA, GSH and GSSG in peanut *AhGST23*-silenced plants were decreased by 22.30%, 35.74%, 40.86% and 29.79%, respectively, compared to the control plants (Fig. 10A-D). Under drought and salt stress, the contents of AsA, DHA, GSH and GSSG in both control and *AhGST23*-silenced plants showed a significant increasing trend, but the elevation was much lower in silenced plants.

The activities of APX, GST, DHAR, and MDHAR followed the similar trends as observed for antioxidant contents. Compared to the empty-vector control plants, the *AhGST23*-silenced plants showed significantly reduced activities of APX (by 42.20%), GST (50.17%), DHAR (35.48%), and MDHAR (41.67%) under non-stress conditions (Fig. 10E-H). Under drought and salt stress, while these enzyme activities increased in both control and silenced plants, the extent of increase was markedly lower in the silenced plants. Furthermore, under stress conditions, the activities of DHAR and MDHAR in *AhGST23*-silenced plants did not differ significantly from their levels under non-stress conditions (Fig. 10).

In summary, our results showed that silencing *AhGST23* expression not only reduced the GST activity and GSH content in peanut plants, but also significantly diminished the activity of key antioxidants and enzymes involved in the AsA-GSH cycle. The disruption of the AsA-GSH cycle system impaired the plants' ability to effectively scavenge excess ROS, ultimately leading to a substantial decline in salt stress tolerance.

Discussion

The abiotic stressors of drought and salinity disrupt cellular homeostasis in plants primarily through the overaccumulation of ROS. While ROS act as signaling molecules at moderate levels, they cause severe oxidative damage when present in excess [6, 7]. Therefore, plant survival under such stresses critically depends on a fine-tuned balance between ROS production and scavenging. In the present study, we identified two key components involved in the antioxidant defense of peanuts under drought and salt stresses: AsA-GSH cycle and NADPH oxidase-mediated ROS regulation, particularly examining the roles of *AhGST23* and *AhRbohs* gene families.

The AsA-GSH cycle and the *AhGST23* gene in peanut stress tolerance

The AsA-GSH cycle plays a vital role in plant defence against abiotic stresses by effectively scavenging ROS, thus maintaining redox homeostasis within cells. Glutathione (GSH) metabolism begins with the synthesis of γ -glutamylcysteine from L-glutamate and L-cysteine, catalyzed by glutamate-cysteine ligase (GCLC), followed by the formation of GSH through glutathione synthase (GS) [24]. Under oxidative stress, GSH serves as a substrate for GPX to reduce H_2O_2 to water, concurrently producing oxidized glutathione (GSSG) [25]. The GST further enhances detoxification by conjugating GSH with electrophilic compounds, thereby mitigating ion toxicity and neutralizing xenobiotics [26, 27]. To replenish the GSH pool, GR converts GSSG back to GSH using NADPH, thereby sustaining a high GSH/GSSG ratio, which is critical for the sustained activity of GPX and GST [12, 26]. Moreover, GSH acts synergistically with ASA in the ASA-GSH cycle. In this cycle, ASA is oxidized by APX during the reduction of H_2O_2 to water. The oxidized forms of ASA, monodehydroascorbate (MDHA) and dehydroascorbate (DHA), are regenerated by MDHA reductase (MDHAR) and DHA reductase (DHAR), respectively, ensuring continuous antioxidant capacity [28]. This pathway constitutes a central mechanism for ROS scavenging in stressed plants [13, 29].

In this study, gene expression profiling revealed a significant upregulation of AsA-GSH cycle related gene families, *AhGRs*, *AhGSTs*, *AhGPXs*, and *AhAPXs*, under both drought and salt stress conditions (Fig. 7). These results align with previous reports on the role of glutathione metabolism and the AsA-GSH cycle in conferring drought and salinity tolerance in maize and rice [25, 29, 30]. Among these, the *AhGSTs* family was most markedly upregulated, with *AhGST23* showing the greatest increase. Under drought and salt stress, its expression increased by 2.33- and 28.12-fold in leaves, and by 22.98- and 13.17-fold in roots, respectively (Fig. 7), suggesting a central role within the family. This was further confirmed through VIGS experiments: silencing *AhGST23* significantly reduced the contents of AsA, DHA, GSH, and GSSG, as well as the activities of APX, GST, DHAR, and MDHAR. These disruptions led to dysfunction of the AsA-GSH cycle, which impaired the plants' ability to scavenge ROS. Consequently, severe oxidative stress accumulated, rendering the plants more vulnerable to drought and salt stress. These findings demonstrate that the *AhGST23* plays a pivotal role in peanuts responses to drought and salt stress by participating in the AsA-GSH cycle. The successful identification and functional characterization of the *AhGST23* gene provide a theoretical foundation for the future use of this gene in developing salt-tolerant peanut germplasm.

AhRbohs-mediated ROS production impacts peanut stress responses

The ROS production in plants is primarily mediated by NADPH oxidases (also known as respiratory burst oxidase homologs, Rbohs), along with enzymes such as peroxidases, amine oxidases, and aldehyde oxidases [31, 32]. Rbohs catalyze the transfer of electrons from NADPH to oxygen, generating O_2^- , which is then converted into H_2O_2 [33, 34]. While low to moderate ROS levels serve as signalling molecules to trigger defence and acclimation responses, their excessive accumulation leads to oxidative stress and cellular damage [31, 35]. Previous studies have highlighted the dual role of *Rbohs* in plant stress tolerance. For instance, overexpression of *CmoRbohD1* and *CmoRbohD2* in cucumber enhanced salt tolerance by activating ROS signalling pathways [36], while knockout of *OsRbohA* in rice reduced ROS overaccumulation and improved salt stress tolerance by maintaining K^+/Na^+ balance [37]. In peanuts, *AhRbohs* have been implicated in ROS burst under arsenic stress [38], and prior research has shown that silencing *AhRbohB6* and *AhRbohC18* significantly enhanced salt tolerance by reducing ROS levels [23].

In this study, RNA-seq analysis revealed that expression of *AhRbohs* was significantly higher than that of the other genes belonging to ROS-related families under drought and salt stress, with most DEGs being significantly upregulated (Fig. 7). However, excessive ROS production can be detrimental, suggesting that selective downregulation or genetic silencing of key *AhRboh* genes could be an effective breeding strategy to minimize the oxidative damage and enhance peanut resilience. These insights present *AhRbohs* as both crucial contributors to stress signalling and potential targets for molecular breeding to fine-tune ROS homeostasis.

Differential responses of peanut to drought and salt stress

This study compared the effects of equivalent-intensity PEG-induced drought and NaCl-induced salt stress on peanut plants. Phenotypically, salt stress caused more severe overall growth inhibition than drought stress. Notably, salt stress primarily impaired shoot development, whereas drought stress caused a greater reduction in root growth (Fig. 1, Table 1). RNA-seq results supported these phenotypic observations: salt-stressed leaves exhibited 8,266 more DEGs than drought-stressed leaves, while drought-stressed roots showed 2,405 more DEGs than salt-stressed roots (Fig. 5). This tissue-specific enrichment of DEGs suggests that organs subjected to greater physiological damage tend to activate broader transcriptional responses to mitigate stress.

Functional enrichment analysis further revealed distinct stress- and tissue-specific metabolic adaptations. In leaves, DEGs under salt stress were significantly enriched in pathways such as glutathione metabolism, MAPK signalling, flavonoid biosynthesis, and isoflavonoid biosynthesis, which are closely associated with antioxidant defence and cellular signaling. In roots, DEGs under drought stress showed significant enrichment in flavonoid and isoflavonoid biosynthesis, indicating a prominent role of secondary metabolites in root-specific drought adaptation. These findings highlight the central role of glutathione metabolism and the AsA-GSH cycle in leaf responses to salt stress, particularly through GST-mediated detoxification, and emphasize the contribution of flavonoid pathways in roots under drought.

Interestingly, the most affected tissues under each stress condition (leaves under salt stress and roots under drought) also displayed the strongest enrichment of adaptive metabolic pathways. This pattern suggests that stress-responsive gene networks are selectively activated in a tissue-specific manner to respond to environmental challenges. Future research should focus on dissecting these key metabolic pathways and their core regulatory genes to understand their precise molecular mechanisms in drought and salt tolerance. Employing molecular tools to trigger early expression of these crucial genes could enhance peanut resilience at the onset of stress exposure, thereby maximizing resistance. These insights provide the foundation for developing genetically improved peanut varieties with superior drought and salt tolerance.

Conclusions

In summary, this study elucidates the molecular and physiological mechanisms mediating peanut responses to drought and salt stress. Although both stresses significantly suppressed plant growth, salt stress more strongly inhibited shoot development, whereas drought stress caused greater impairment to root growth, reflecting distinct adaptive strategies under different abiotic conditions. Drought and salt stresses rapidly induced the expression of *AhRbohs* genes, leading to ROS overaccumulation. Concurrently, key family genes involved in the AsA-GSH cycle, including *AhGSTs* (especially *AhGST23*), *AhGRs*, *AhGPXs*, and *AhAPXs*, were markedly upregulated, thereby facilitating ROS detoxification and alleviating oxidative damage (Fig. 11). Silencing of *AhGST23* led to a significant decline in GST activity and content, along with reduced levels of key antioxidants and enzymes involved in the AsA-GSH cycle, ultimately disrupting redox homeostasis and increasing plant sensitivity to both drought and salt stress.

Collectively, this study provides a molecular framework for understanding drought and salt stress responses in peanut and highlights candidate genes and pathways for genetic improvement. Targeting early activation of these defense mechanisms could facilitate the development of stress-resilient peanut varieties, helping to maintain stable yields under changing climatic conditions.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Zengqiang Li conducted experiments, analyzed data, and wrote the original draft. Zhiyong Zhang conceptualized the project, and reviewed the manuscript. Muhammad Hafeez Ullah Khan, Mehboob-ur Rahman, Khudayberdi Nazarov, Xiangjun Kong and Xin Zhang participated in manuscript revision. Peipei Jia and Zhenkun Cui enrolled in this research. Haifang Dai, Mengyao Shao and Lijie Li conducted data analysis.

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Availability of Data and Materials

The datasets generated during the current study are available in the National Center for Biotechnology Information: Submission ID SUB15859906.

All data, with the exception of the mRNA-Seq data, are available in the article and its supplementary information.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests

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Figures

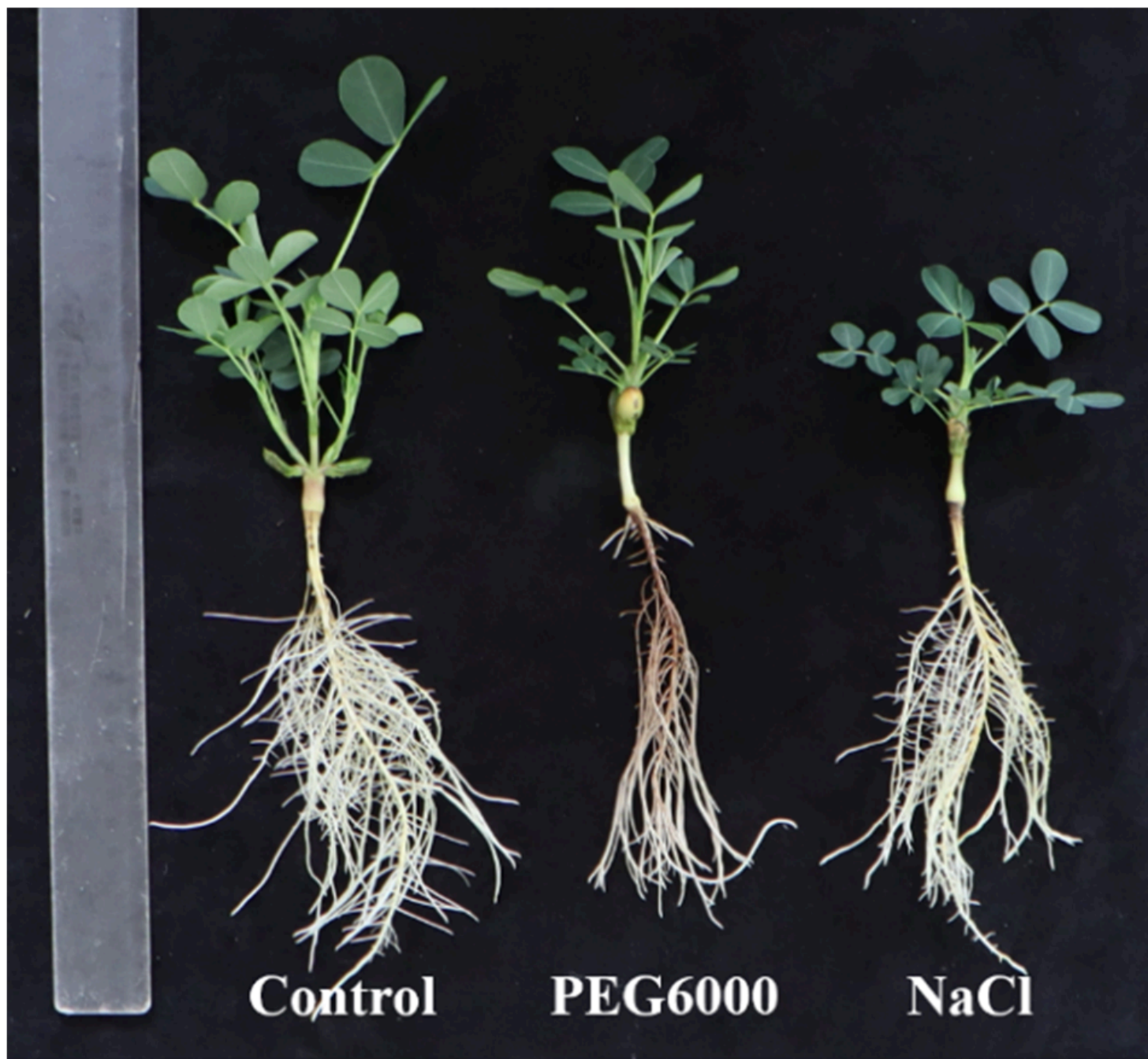


Figure 1

Observation on the growth of peanut seedlings under drought and salt stress.

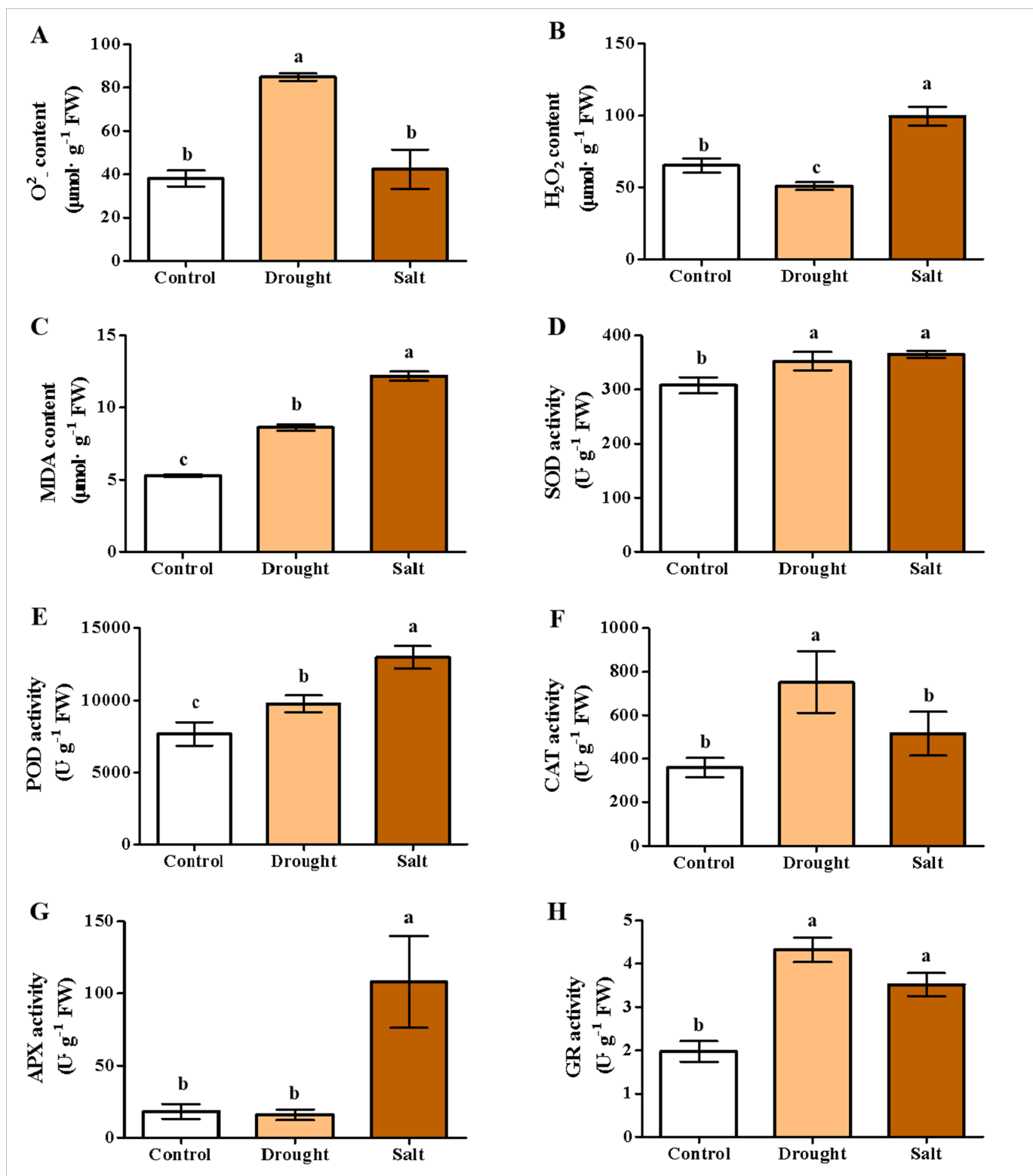


Figure 2

Determination results of ROS and antioxidant enzyme related indicators in peanut leaves. **A** O_2^- content; **B** H_2O_2 content; **C** MDA content; **D** SOD activity; **E** POD activity; **F** CAT activity; **G** APX activity; **H** GR activity.

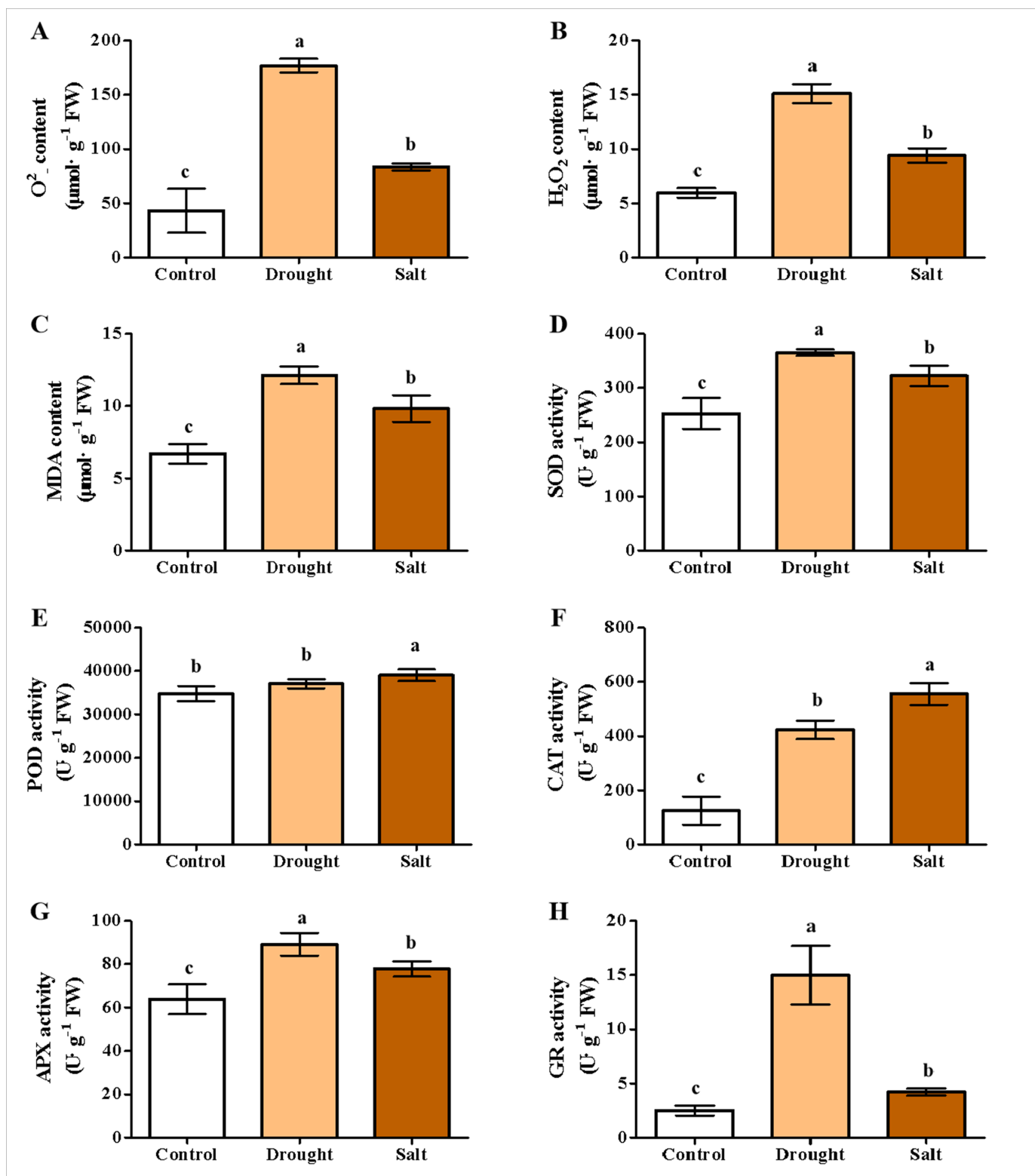


Figure 3

Determination results of ROS and antioxidant enzyme related indicators in peanut roots. **A** O_2^- content; **B** H_2O_2 content; **C** MDA content; **D** SOD activity; **E** POD activity; **F** CAT activity; **G** APX activity; **H** GR activity.

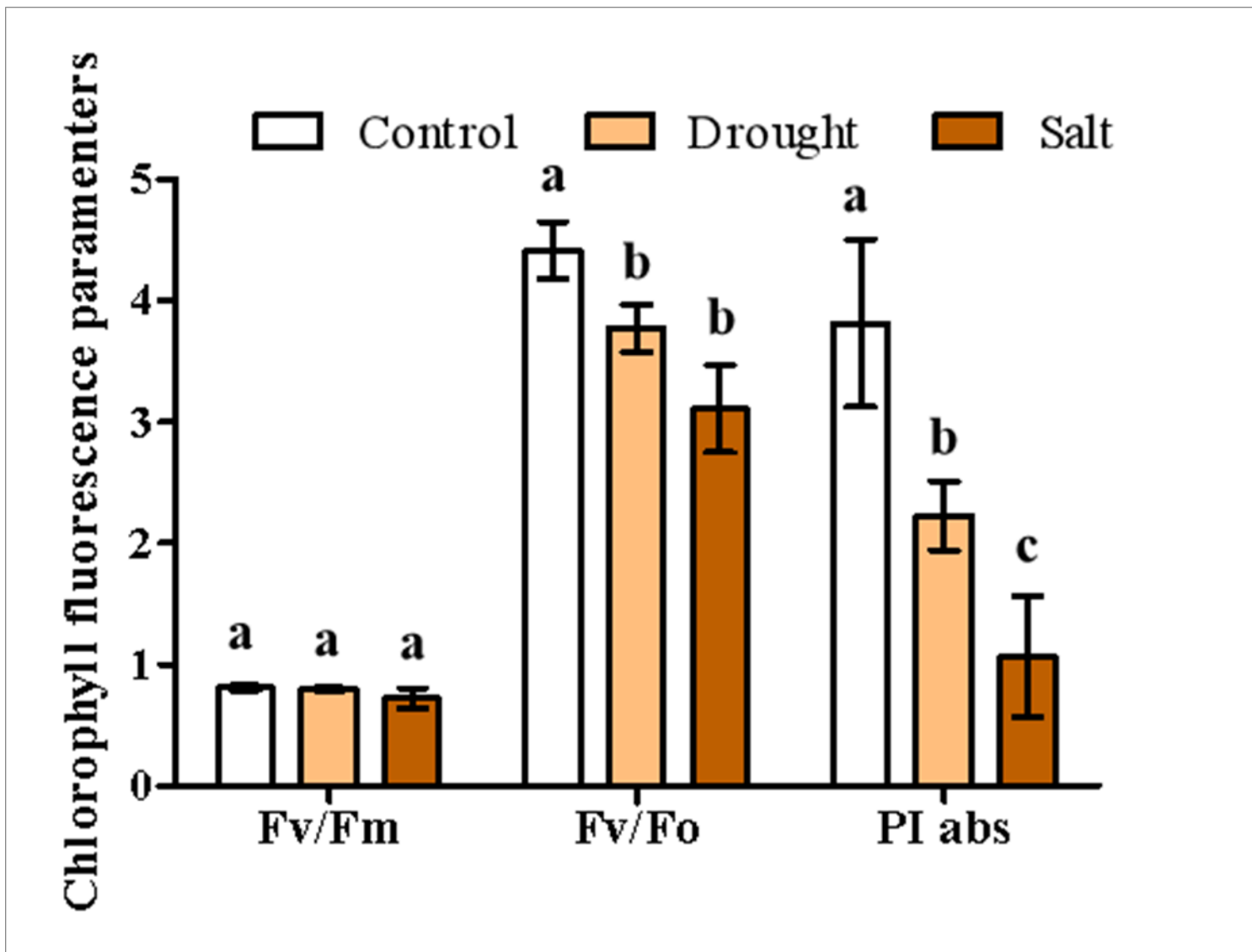


Figure 4

Measurement results of chlorophyll fluorescence parameters.

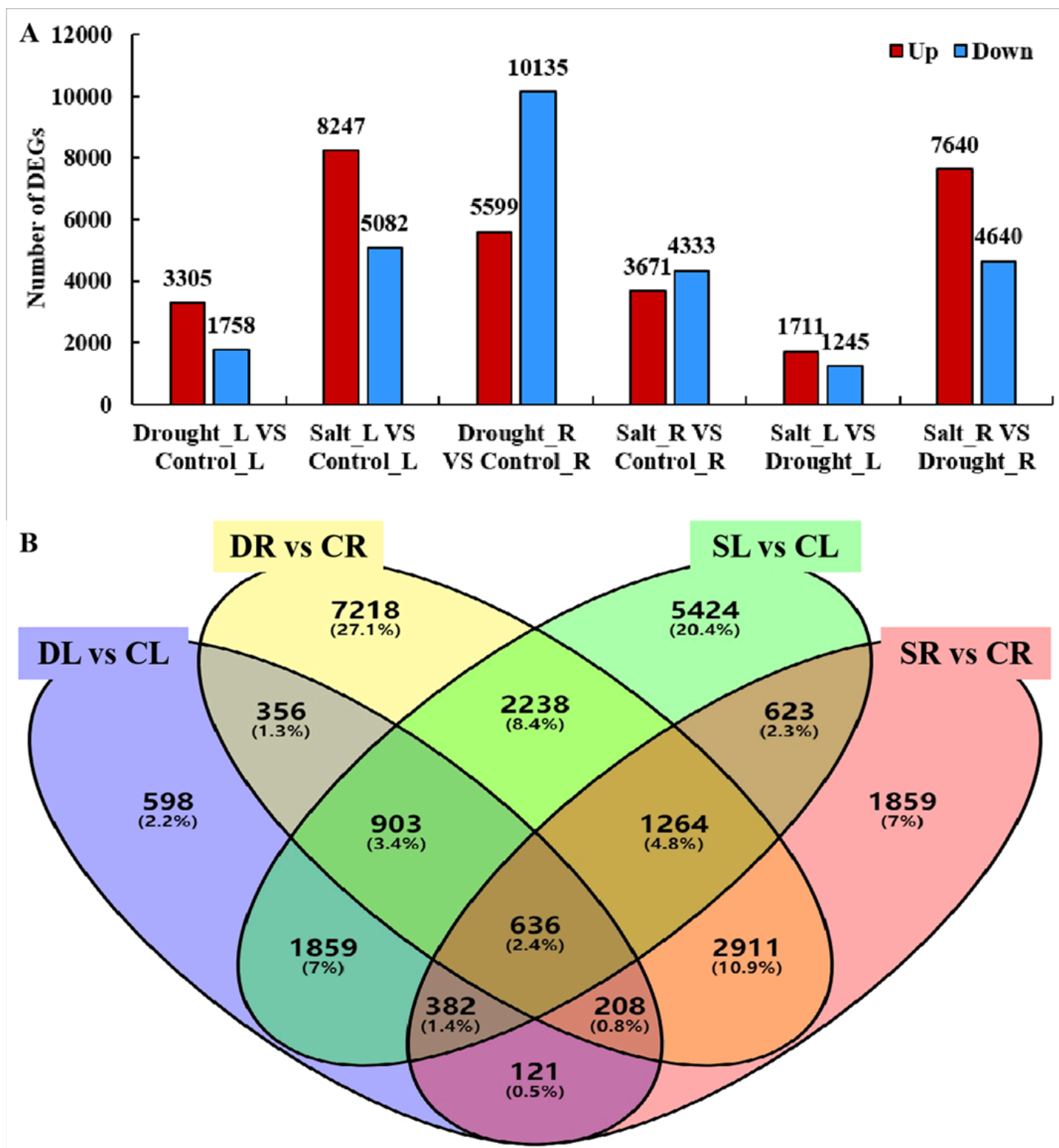


Figure 5

Statistical analysis (A) and Venn analysis (B) of DEGs in comparison group. Drought_L, Salt_L, Control_L, Drought_R, Salt_R and Control_R stand for Drought_Leaf, Salt_Leaf, Control_Leaf, Drought_Root, Salt_Root and Control_Root, respectively. DL, SL, CL, DR, SR, and CR stand for Drought_Leaf, Salt_Leaf, Control_Leaf, Drought_Root, Salt_Root, and Control_Root, respectively, same as below.

A						
Go terms	DL vs CL	DR vs CR	SL vs CL	SR vs CR	SL vs DL	SR vs DR
regulation of transcription	359	896	736	551	149	709
transcription, DNA-templated	300	757	620	467	130	610
obsolete oxidation-reduction process	173	661	532	335	154	593
protein phosphorylation	122	563	460	344	84	460
defense response	163	531	445	325	105	452
response to salt stress	114	295	261	155	53	266
response to abscisic acid	112	213	236	185	57	211
signal transduction	66	274	202	129	35	220
response to water deprivation	112	198	202	144	39	184
nucleus	1343	3571	3268	1929	601	2727
plasma membrane	660	2321	1924	1196	450	1911
cytoplasm	686	2005	1924	998	459	1614
integral component of membrane	555	1996	1624	990	359	1530
chloroplast	503	1661	1600	822	388	1253
cytosol	414	1238	1268	538	183	929
mitochondrion	326	1067	981	466	227	785
extracellular region	266	971	662	513	172	833
membrane	230	917	725	448	174	740
plasmodesma	175	580	504	299	110	479
protein binding	549	1579	1430	875	276	1200
ATP binding	313	1053	871	554	178	781
DNA binding	282	813	632	509	121	616
metal ion binding	241	682	670	367	148	541
protein serine/threonine kinase activity	136	601	457	370	104	501
kinase activity	124	522	395	256	90	396
zinc ion binding	141	338	351	174	61	256
RNA binding	164	306	386	129	35	191
oxidoreductase activity	55	231	170	98	67	203
catalytic activity	60	147	149	59	33	122
B						
Pathway name	DL vs CL	DR vs CR	SL vs CL	SR vs CR	SL vs DL	SR vs DR
Ribosome	267	319	449	95	20	93
Plant hormone signal transduction	167	280	260	179	88	269
Plant-pathogen interaction	95	270	256	177	74	235
Starch and sucrose metabolism	75	321	194	138	72	267
Phenylpropanoid biosynthesis	56	274	148	142	49	244
MAPK signaling pathway - plant	100	185	186	126	37	198
Protein processing in endoplasmic reticulum	72	186	213	74	54	136
Endocytosis	62	164	179	79	39	128
Spliceosome	91	144	220	80	22	87
Nucleocytoplasmic transport	98	133	224	56	18	94
Amino sugar and nucleotide sugar metabolism	59	180	122	66	37	140
Purine metabolism	46	182	154	67	24	128
Pentose and glucuronate interconversions	56	180	104	97	21	137
Glycolysis / Gluconeogenesis	34	160	116	67	34	150
Glutathione metabolism	30	111	127	74	48	80
Cysteine and methionine metabolism	46	135	82	69	26	109
Flavonoid biosynthesis	21	136	77	55	49	118
Galactose metabolism	29	137	74	64	17	126
Pyrimidine metabolism	30	132	111	62	15	87
Pyruvate metabolism	29	122	71	34	29	116

Figure 6

GO (A) and KEGG (B) enrichment analysis statistics of DEGs. The numbers in the chart represent the number of enriched DEGs.

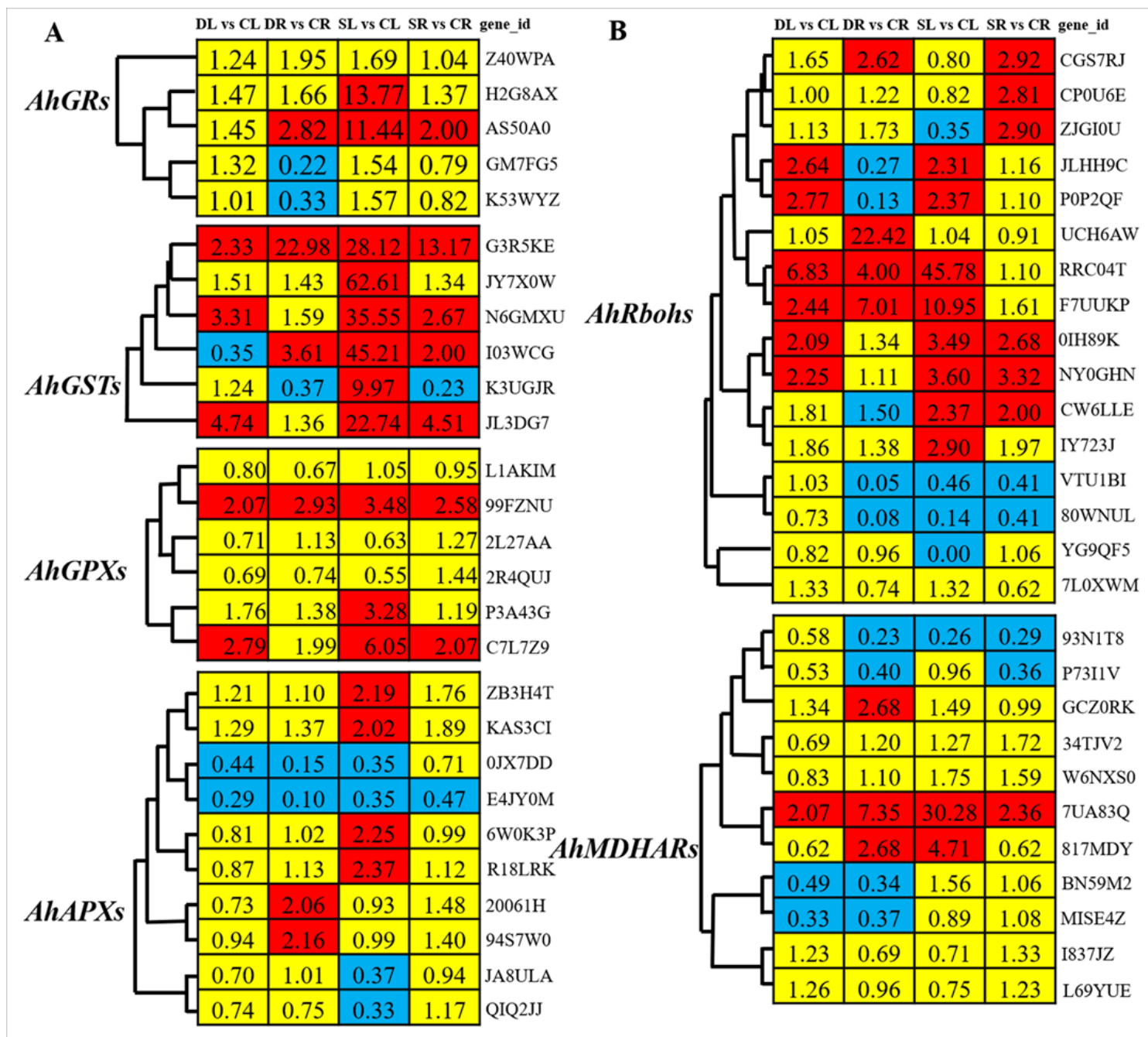


Figure 7

Phylogenetic and expression multiples analysis of AsA-GSH cycle (A) and *AhRbohs* gene families (B). The numbers in the figure represent the multiples of expression level among the comparison groups, the red, blue, and yellow represent significant upregulation, significant downregulation, and no significant difference, respectively.

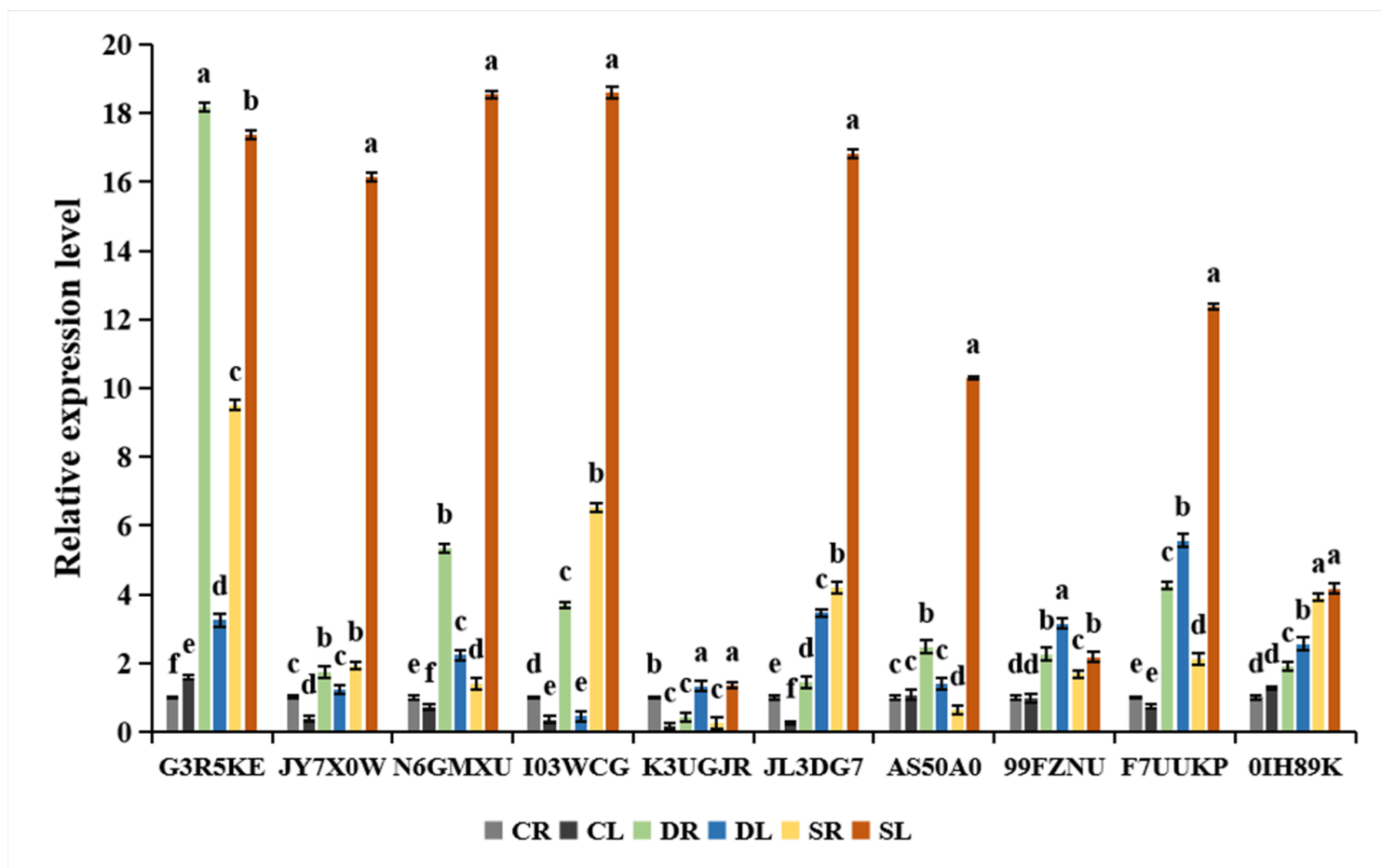


Figure 8

qRT-PCR analysis of some DEGs.

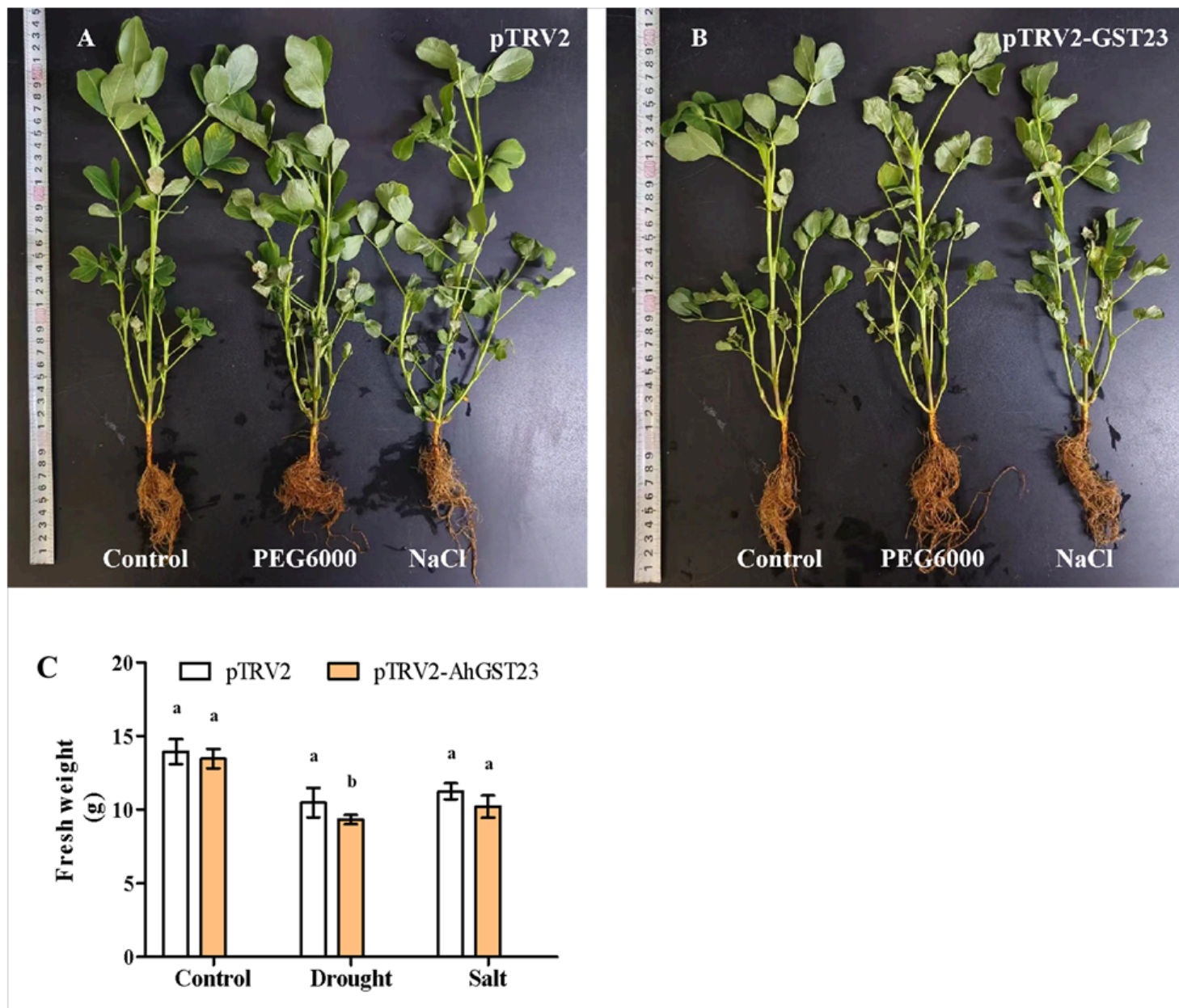


Figure 9

Phenotypic observation (A and B) and biomass analysis (C) of peanut *AhGST23*-silenced plant under drought and salt stress.

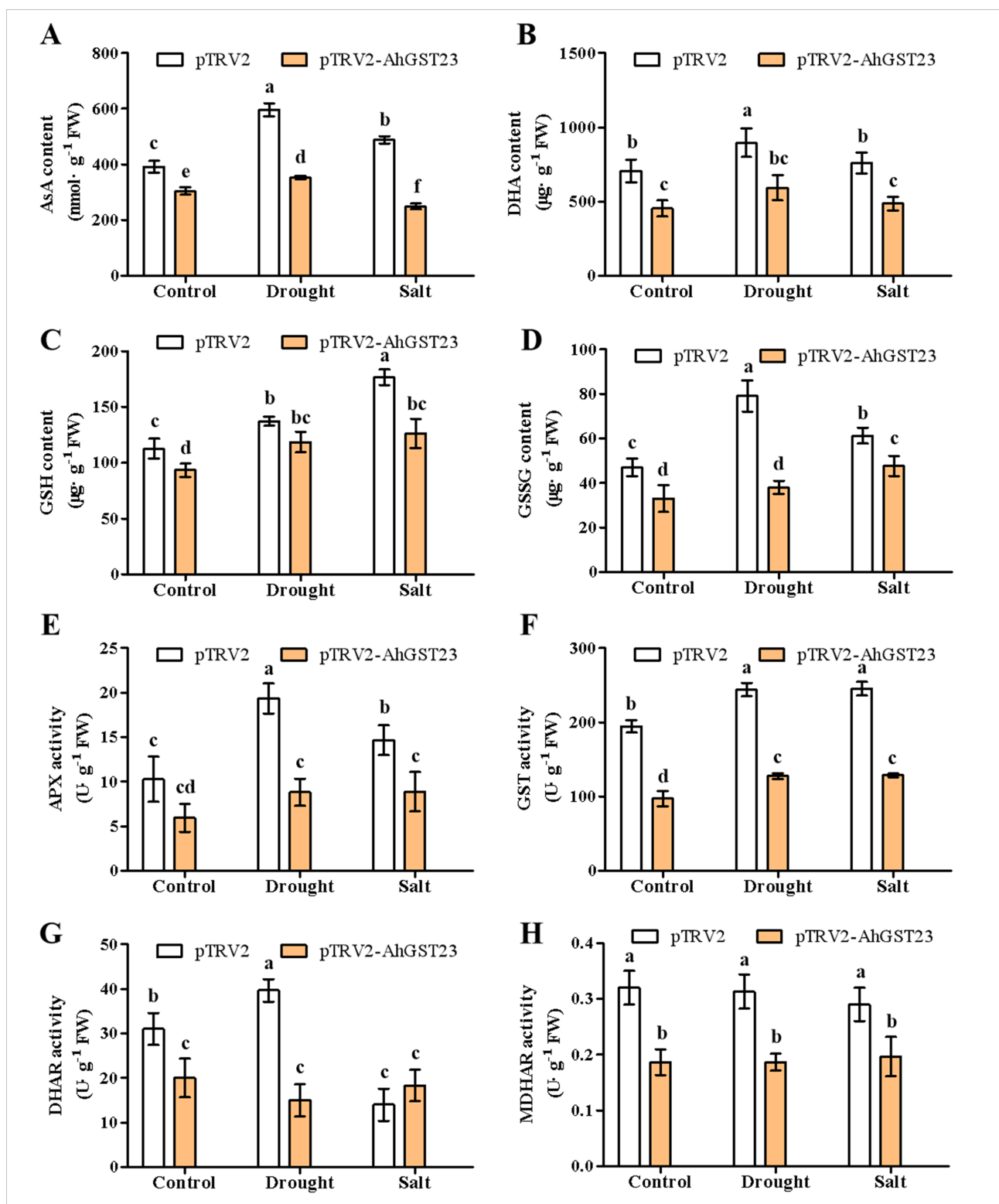


Figure 10

The contents of AsA (A), DHA (B), GSH (C), GSSG (D) and the activities of APX (E), GST (F), DHAR (G), MDHAR (H) in peanut *AhGST23* silenced plants under drought and salt stress.

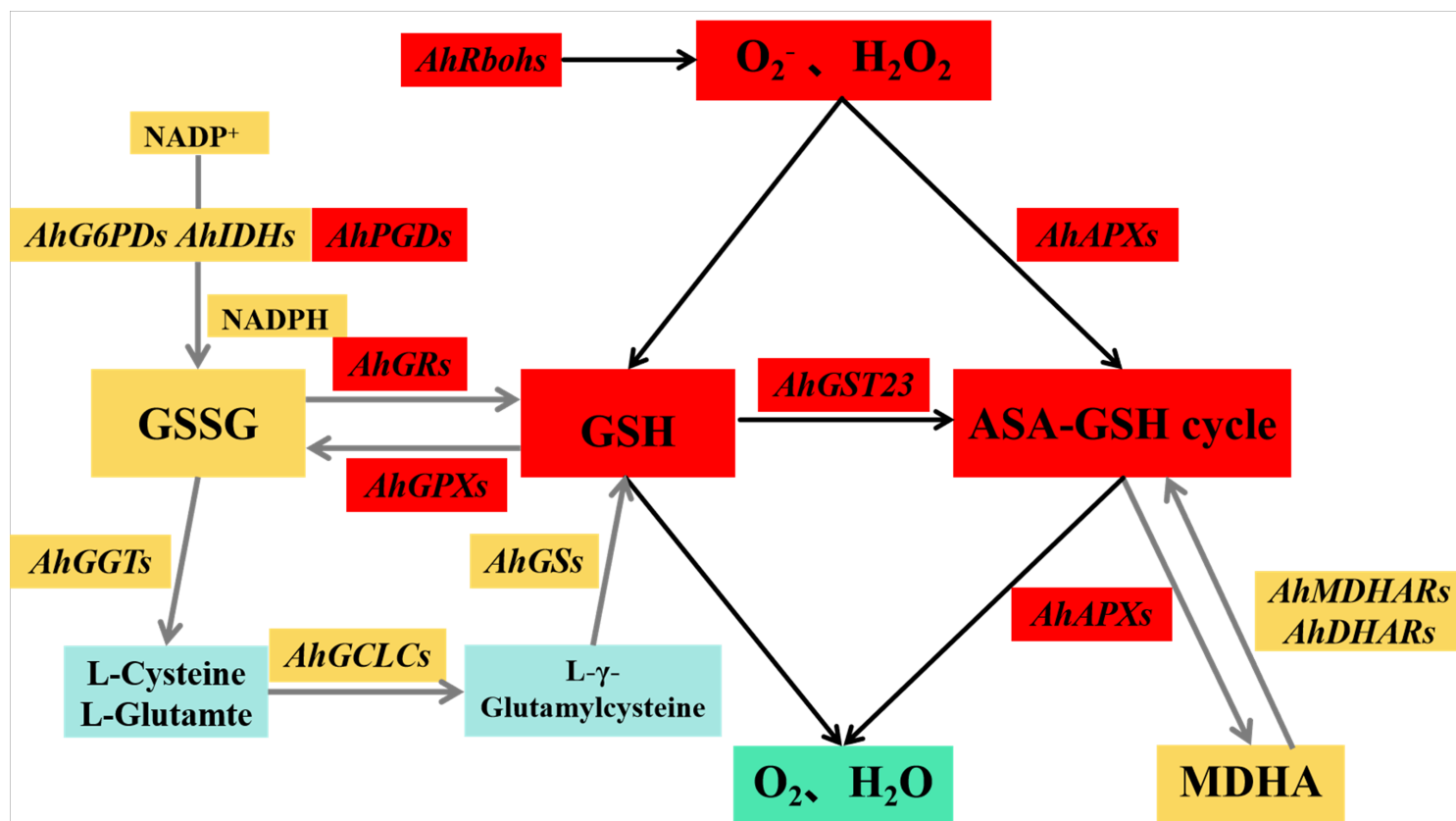


Figure 11

The schematic diagram of the role of AsA-GSH cycle in peanut response to drought and salt stress. Under drought and salt stress, peanut plants exhibit upregulated expression of *AhRboh*s family genes, which mediate ROS production and result in the accumulation of H_2O_2 and O_2^- . Meanwhile, multiple gene families involved in maintaining the dynamic balance of the GSH/GSSG ratio and the ASA-GSH cycle, including *AhPGDs*, *AhGRs*, *AhGPXs*, *AhGSs*, *AhGSTs*, and *AhAPXs*, exhibit upregulated expression. These changes enhance the capacity of ASA-GSH cycle to scavenge H_2O_2 and O_2^- , thereby alleviating the damage caused by drought and salt stress in peanuts. The red, blue, and yellow represent significant upregulation, significant downregulation, and non-significant changes, respectively.

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