

A tau class glutathione S-transferase in alfalfa, MsGSTU17, regulates salt tolerance in transgenic *Arabidopsis thaliana*

Shuhan Yu¹, Xu Zhuang², Shiyao Chen¹, Yumiao Xie¹ and Lu Zhang^{1*}

¹ College of Landscape and Architecture, Zhejiang A&F University, Hangzhou 311300, China

² Institute of Forage and Grassland Sciences, Heilongjiang Academy of Agricultural Sciences, Heilongjiang, Harbin 150086, China

* Corresponding author, E-mail: zhanglu@zafu.edu.cn

Abstract

High soil salinity negatively affects crop yield, intrinsic quality, and economic profitability. The tau class of glutathione S-transferases (GSTs) is pivotal in plant defense mechanisms against salt stress. In this study, we characterized a salt-responsive tau-class GST gene, designated as *MsGSTU17*, in *Medicago sativa*. The *MsGSTU17* gene was found to be located in the cytoplasm and had high expression levels in both leaves and roots. Compared to the wild-type plants, overexpression of *MsGSTU17* in *Arabidopsis* under salt stress led to increased root length, shoot weight, and survival rate, along with a reduced ion leakage rate. At the same time, the transgenic *Arabidopsis* lines showed significantly increased expression of genes related to salt stress, including *SOS1*, *SOS2*, *SOS3*, *KIN2*, *RD29A*, and *HKT1*, compared to the wild-type. Additionally, these transgenic plants exhibited an enhanced antioxidant defense system characterized by elevated activities of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), GST, and glutathione reductase (GR), and higher levels of glutathione (GSH). In summary, our results suggest that overexpressing *MsGSTU17* in transgenic plants can mitigate salt-caused damage by scavenging reactive oxygen species and increasing GSH levels via upregulation of GST activity.

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Introduction

Alfalfa (*Medicago sativa* L.) is a perennial legume that is widely recognized for its rich nutritional profile and exceptional quality as a fodder crop. It has a well-developed root system, vigorous tillering, and abundant foliage, along with key advantages such as high yield, excellent quality, strong adaptability, and broad distribution. It has become a widely utilized forage species with the largest planted area in China^[1]. However, the development and yield of alfalfa are limited by extreme environmental stressors, and soil salinization is particularly detrimental^[2]. Therefore, deciphering how plants cope with salt stress is a crucial step in breeding salt-tolerant crops, which is essential for satisfying the steadily rising global food demand.

Glutathione S-transferases (GSTs), an ancient family of proteins, play crucial roles in various catalytic and regulatory functions. These enzymes are instrumental in the S-conjugation process, which involves the attachment of the thiol group of glutathione (GSH, composed of glutamic acid, cysteine, and glycine) to the electrophilic groups of hydrophobic and potentially harmful substrates^[3]. Their principal role involves detoxifying noxious compounds of exogenous origin, such as herbicides and foreign pathogens, or endogenous stress-induced metabolites. GSTs can be induced by a variety of stresses and play a direct role in reducing oxidative damage or in detoxification^[4,5]. Furthermore, GSTs can be activated by various phytohormones such as abscisic acid (ABA), auxin, ethylene, salicylic acid, cytokinins, and methyl jasmonate, which are essential for plant growth and development^[6,7]. GSTs are key regulators of signaling pathways and are vital for numerous developmental processes, including apoptosis and growth modulation^[8]. In photosynthetic eukaryotes, the GST family is categorized into 14 distinct classes, including tau (GSTU), phi (GSTF), zeta (GSTZ), theta (GSTT), and tetrachlorohydroquinone dehydrogenase (TCHQD), which feature GSTs that possess serine at their active sites. Iota (GSTIs), DHARs, hemerythrins (GSTHs), GHRs, lambda (GSTLs), mPGES-2s,

and metaxins are categorized as Cys-GSTs, while the catalytic residues in the EF1B γ and Ure2p classes remain undefined^[9,10]. Specifically, GSTUs, GSTFs, GSTLs, and TCHQDs are related to plants^[11].

In plants, the function of GSTs is closely related to stress response. For instance, the BES/BZR family transcription factor TaBZR2 enhances the drought resistance of wheat (*Triticum aestivum*) through the activation of TaGST1 and mediates the crossover between the brassinosteroid (BR) and drought signaling pathways^[12]. In poplar (*Populus alba* \times *Populus glandulosa*), salt stress markedly upregulates the expression of *PtrGSTF8*, and the overexpression of *PtrGSTF8* enhances salt tolerance in tobacco by reducing reactive oxygen species (ROS) levels^[13]. GSTU is crucial for scavenging hydrogen peroxide (H₂O₂) and superoxide anions (O₂⁻) to maintain ROS homeostasis under various stresses^[14]. *Arabidopsis* plants with increased expression of *AtGSTU19* demonstrate enhanced resistance to salt, drought, and methyl viologen stress^[15]. Ectopic expression of rice *OsGSTU4* in *Arabidopsis* can enhance the tolerance to salt and oxidative stress at different developmental stages^[16]. MsCML10 interacts with MsGSTU8 and MsFBA6, activating them to maintain ROS homeostasis at low temperatures, promote sugar accumulation, and ultimately enhance the cold tolerance of alfalfa^[17]. Overexpression of tomato (*Lycopersicon esculentum*) *LeGSTU2* enhances the resistance to NaCl and mannitol-induced salt and osmotic stress in transgenic *Arabidopsis*^[18]. In poplar, *PtrGSTU23/40* overexpression regulates the activity of GST enzymes to modulate ROS scavenging capacity, ultimately enhancing salt tolerance^[19]. The jasmonic acid signaling pathway mediated by CsJAZ1-CsMYC2.2 regulates tea plant (*Camellia sinensis* [L.] O. Kuntze) sensitivity to *Colletotrichum camelliae* by modulating the accumulation of H₂O₂ through CsGSTU45^[20]. Overexpression of *Glycine soja* *GsGSTU13* enhances salt tolerance in alfalfa^[21]. In *Medicago truncatula*, an expression profile analysis of GST family genes shows that salt stress significantly induces or inhibits the expression of GST genes, indicating that GST family genes are closely related to salt stress reactions^[22]. However, the

extent to which the GST family genes in salt stress tolerance in alfalfa remains unclear.

Salt-responsive genes in alfalfa have been explored using transcriptomics^[23]. *MsGSTU17* is upregulated in the highly salt-tolerant cultivar 'Magnum Salt' following salt treatment^[23]. However, the salt tolerance function of *MsGSTU17* in alfalfa has not been confirmed. In the present study, we identified *MsGSTU17* as a gene that responds to salt stress and encodes a protein belonging to the GST family. Furthermore, we studied the salt tolerance of transgenic *Arabidopsis* plants overexpressing *MsGSTU17* and its role in regulating salt tolerance by modulating salt-response genes and the antioxidant defense system.

Materials and methods

Plant material and growth conditions

Medicago sativa (Zhongmu No.1) and *Nicotiana benthamiana* were grown in a growth medium consisting of fertile soil and perlite in a 2:1 volumetric ratio. They were cultivated under controlled light conditions, with an irradiance of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16 h/8 h (light/dark) cycle in a greenhouse maintained at 23 °C. Seeds from the Columbia ecotype (Col-0) of *Arabidopsis thaliana* were disinfect with immersion in a 10% (v/v) solution of sodium hypochlorite for 16 min to eliminate surface contaminants, followed by three rinses with sterile water. Sterilized seeds were sown on a medium containing 1/2 Murashige and Skoog (MS) nutrients and stored at 4 °C for 3 d to promote germination. Seedlings (7 days old) were replanted into a soil matrix that included a blend of nutrient-rich soil and perlite at a volume ratio of 2:1, and accommodated in a growth chamber at 23 °C with an irradiance of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16 h/8 h (light/dark) cycle.

Sequence analysis

The sequences of the MtGST gene family, used as baits to search for the target sequence of alfalfa, were obtained from Hasan et al.^[22]. **Supplementary Table S1** provides information on MsGST proteins. The phylogenetic tree was generated using MEGA X software, employing the maximum-likelihood (ML) method with parameters set to JTT + G + F and 1,000 bootstrap replicates. The amino acid sequences of *MsGSTU17* homologs, MtGSTU17, and MtGSTU18 from *Medicago truncatula* and AtGSTU25 from *Arabidopsis*, were aligned using DNAMAN software. The sequences of MtGSTU17, MtGSTU18, and AtGSTU25 were obtained from Phytozome (<https://phytozome.jgi.doe.gov/>). The 2,000 bp genomic DNA sequence upstream of the transcription start site of *MsGSTU17* was retrieved from the *Medicago sativa* genome^[24]. The *cis*-acting elements in the promoter region of *MsGSTU17* were identified through the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The transmembrane domains and signal peptide of the *MsGSTU17* protein were predicted by analyzing its protein sequence using the TMHMM Server v. 2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) and SignalP-5.0 (<http://www.cbs.dtu.dk/services/SignalP/>), respectively.

Subcellular localization of MsGSTU17 protein

The coding region of *MsGSTU17*, excluding the termination codon, was amplified and inserted into the pCAMBIA-1305 vector to create a fusion protein with a green fluorescent protein (GFP) driven by the CaMV 35S promoter. Using *Agrobacterium*-mediated transformation, the recombinant construct, along with an empty vector as a control, was co-transformed into the abaxial side of four-week-old *Nicotiana benthamiana* leaves, together with the 35S::AtAKT1-mCherry vector. After 48–72 h of transformation, fluorescence was observed using a confocal laser scanning microscope (Carl Zeiss SAS, Jena, Germany).

Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was extracted using the RNAPrep Pure Plant Kit (Tiangen Inc., Beijing, China), and cDNA was synthesized using HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, China). The qRT-PCR was conducted using the Thermal Cycler Dice Real-Time System from Takara (Otsu, Japan) with diluted cDNA as the template. The alfalfa β -actin gene (*JQ028730.1*) served as the internal control for normalizing template quantities and *Arabidopsis actin* gene was similarly utilized for the normalization of template amounts^[25]. The normalized ($2^{-\Delta\Delta\text{CT}}$) method was utilized to measure the relative gene expression levels. **Supplementary Table S2** provides a comprehensive list of the primers used.

Generation of transgenic plants

We inserted the coding DNA sequence (CDS) of *MsGSTU17* into the pGAMBIA3301 vector driven by the CaMV 35S promoter. *Arabidopsis thaliana* (Col-0) plants at four weeks of age were genetically transformed using the *Agrobacterium tumefaciens* strain EHA105 harboring the recombinant plasmid used for gene expression by employing a modified flower dipping method. We subjected the progeny to Basta selection and collected homozygous T3 transgenic seeds for further analysis.

Salt tolerance assays

Sterile seeds of both wild type (WT) and transgenic *Arabidopsis* were transferred onto 1/2-strength MS medium. Seeds were subjected to 3 d of cold stratification at 4 °C, followed by a 4-d incubation at 22 °C for further growth. The germinated seeds were transferred to fresh medium with varying concentrations of NaCl (0, 100, and 125 mM), and the seedlings were cultivated vertically for 12 d in a growth chamber to measure root elongation and fresh weight. To determine salt tolerance, plants grown on 1/2 strength MS medium were transferred to soil. They were then allowed to grow for 16 additional days in a growth chamber at 22 °C. Irrigation of the plants commenced with 3-d treatments with 200 and 250 mM NaCl solutions, followed by a subsequent 6-d treatment with 300 mM NaCl solution. Electrolyte leakage was assessed as previously described^[26]. The survival rate was calculated by counting the surviving plants after a 12-d treatment with 300 mM NaCl^[27].

Measurements of enzyme activities

To extract superoxide dismutase (SOD) and catalase (CAT), 0.2 g of fresh leaf material was flash-frozen in liquid nitrogen, then ground into a fine powder in 3 mL of ice-cold 50 mM phosphate buffer (pH 7.8) using a mortar and pestle. For ascorbate peroxidase (APX) extraction, leaves were homogenized in 3 mL of 50 mM phosphate buffer (pH 7.0), cooled on ice, and enriched with 1 mM EDTA and 1 mM ascorbic acid (AsA). Both homogenates were centrifuged at 12,000 \times g for 20 min at 4 °C and the supernatants were collected for subsequent enzyme activity and protein concentration assays following the method described by Guo et al.^[28]. To determine the GST and glutathione reductase (GR) activities and GSH content, 0.2 g of the sample was homogenized in sodium phosphate buffer and centrifuged, then the absorbance of the supernatant was measured using a spectrophotometer at specific wavelengths using commercial kits as per the manufacturer's instructions (Grace Biotechnology, Suzhou, China)^[15].

3,3'-diaminobenzidine (DAB) staining and nitroblue tetrazolium (NBT) staining

To detect H₂O₂, leaf samples were incubated for 1 h in DAB solution at a concentration of 1 mg·mL⁻¹. To identify O₂⁻, the leaflets were treated with a 5 mg·mL⁻¹ NBT solution for 12 h in darkness^[29]. To facilitate imaging and deplete chlorophyll, the leaflets were immersed in a 95% (v/v) ethanol solution.

Statistical analysis

All data were analyzed using a completely randomized design model with the SPSS software (SPSS Inc., Chicago, IL, USA). To determine the significance of mean differences among treatments or plant lines, ANOVA was implemented, followed by Duncan's test to ascertain significance at the 0.05 level.

Results

Isolation and characterization of *MsGSTU17*

To explore the role of *MsGSTU17*, a 675 bp open reading frame (ORF) encoding a 224-amino acid peptide (GenBank accession No. PQ816732) was isolated from the salt-tolerant alfalfa cultivar

'Zhongmu No.1'. It was highly homologous to *MtGSTU17* based on the phylogenetic analysis of *Medicago truncatula* (Fig. 1). Multiple sequence alignments of *MsGSTU17* with *MtGSTU17* (Medtr3g467420), *MtGSTU18* (Medtr3g467430), and *AtGSTU25* (At1g17180) revealed high sequence identities of 94%, 89%, and 65%, respectively (Fig. 1). The Pfam database revealed that *MsGSTU17* contains two conserved domains, GST-N and GST-C, which are characteristic of the GST family (Supplementary Fig. S1a). Structural modeling of *MsGSTU17* predicted seven distinct protein folds (Supplementary Fig. S1b). The 2,000 bp promoter segment of *MsGSTU17* was scrutinized for *cis*-regulatory elements using the PlantCARE platform. The existence of four ABA-responsive, one MeJA-responsive, and five low-temperature-responsive elements suggests that *MsGSTU17* is involved in stress response mechanisms (Supplementary Fig. S1c).

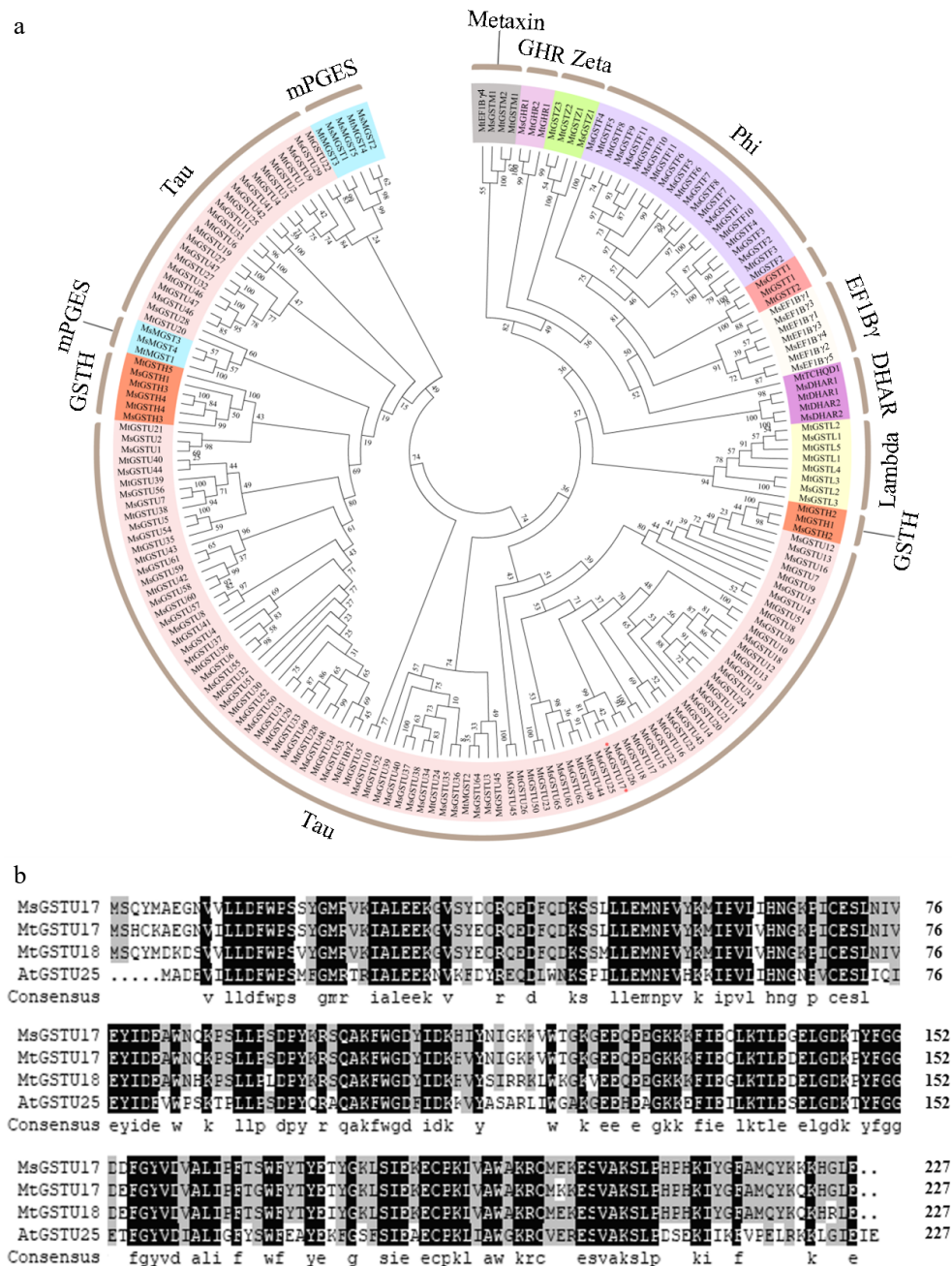


Fig. 1 Phylogenetic evaluations and alignments of multiple GST sequences from various plant species were performed. (a) Phylogenetic tree of GSTs constructed based on *Medicago truncatula* and alfalfa. (b) Multiple sequence alignment of *MsGSTU17*, *MtGSTU17*, *MtGSTU18*, and *AtGSTU25* was performed. Those amino acids which were the same are distinguished by a black background, while those with a similarity of $\geq 75\%$ are represented in gray.

Subcellular localization of MsGSTU17 and its expression pattern

Analysis of MsGSTU17 revealed the absence of transit peptides, signal peptides, and transmembrane domains (Supplementary Fig. S2), and evidence strongly suggested that MsGSTU17 is localized to the cytoplasm. To validate this hypothesis, the cellular localization of MsGSTU17 was assessed by co-expressing MsGSTU17-GFP and AtAKT1-mCherry in tobacco leaves. The data revealed that the MsGSTU17 fluorescence signal did not coincide with the AtAKT1 fluorescence signal (Fig. 2), confirming that MsGSTU17 was localized in the cytoplasm.

The tissue-specific expression pattern of *MsGSTU17* was examined using qRT-PCR. The findings revealed that *MsGSTU17* was expressed in all tissues tested, including the leaves, stems, flowers, roots, nodules, and seeds of alfalfa. Notably, its expression was highest in the leaves, whereas lower levels were observed in the roots and stems (Fig. 3a). After salt treatment, *MsGSTU17* was expressed in both the roots and leaves. Transcription levels of *MsGSTU17* increased in both the roots and leaves after 2 h of salt stress and decreased after 24 h of salt stress (Fig. 3).

Overexpression of *MsGSTU17* augmented the salinity endurance of transgenic *Arabidopsis*

Transgenic *Arabidopsis* plants overexpressing *MsGSTU17* were successfully created, and the presence of *MsGSTU17* transcripts was confirmed in two homozygous lines (OE6 and OE13). In contrast, no transcripts were detected in the wild-type (WT) plants (Fig. 4a). The transgenic lines had significantly longer roots than the WT plants under salt stress. Despite the general growth inhibition caused by salt stress, transgenic plants sustained a higher fresh weight than WT plants (Fig. 4). To investigate the involvement of *MsGSTU17* in salt tolerance, plants that had been cultivated in soil for three weeks underwent irrigation with 300 mM NaCl solution for 12 d. Under salt

stress, the survival rate of the transgenic lines ranged from 66.67%–70.83%, which was significantly higher than that of the wild type (33.33 %), and their ion leakage rates were also notably lower than that of the WT plants (Fig. 5). These results suggest that *MsGSTU17* overexpression significantly improved the salt tolerance of transgenic plants.

ROS accumulation and antioxidant enzyme activities

The NBT and DAB staining results showed that under control conditions, there were no differences in O_2^- and H_2O_2 levels between transgenic and WT plants. The relative increase in O_2^- and H_2O_2 levels in transgenic plants was lower than that in WT plants under salt stress. The activities of SOD, CAT, and APX were similar between the WT and transgenic plants under control conditions. However, transgenic plants exhibited a more substantial increase in enzyme activity than WT plants under salt stress (Fig. 6).

Changes in contents of GSH and related enzymes under salt stress

Compared to the WT plants, transgenic *Arabidopsis* exhibited markedly higher levels of GST activity and GSH content under control conditions, without notable variation in GR activity. After salt treatment, GST and GR activities increased in all genotypes, whereas the GSH content decreased. A comparative analysis of WT and transgenic plants under salt stress revealed that transgenic *Arabidopsis thaliana* exhibited significantly elevated GST and GR activities, as well as increased GSH levels, compared to those in WT specimens (Fig. 7).

Transcriptional analysis of gene expression patterns under salt stress conditions

To investigate how *MsGSTU17* responds to salt stress, we analyzed the expression levels of the salt stress-related genes *SOS1*, *SOS2*, *SOS3*, *RD29A*, *KIN2*, and *HKT1* in 3-week-old plants treated with

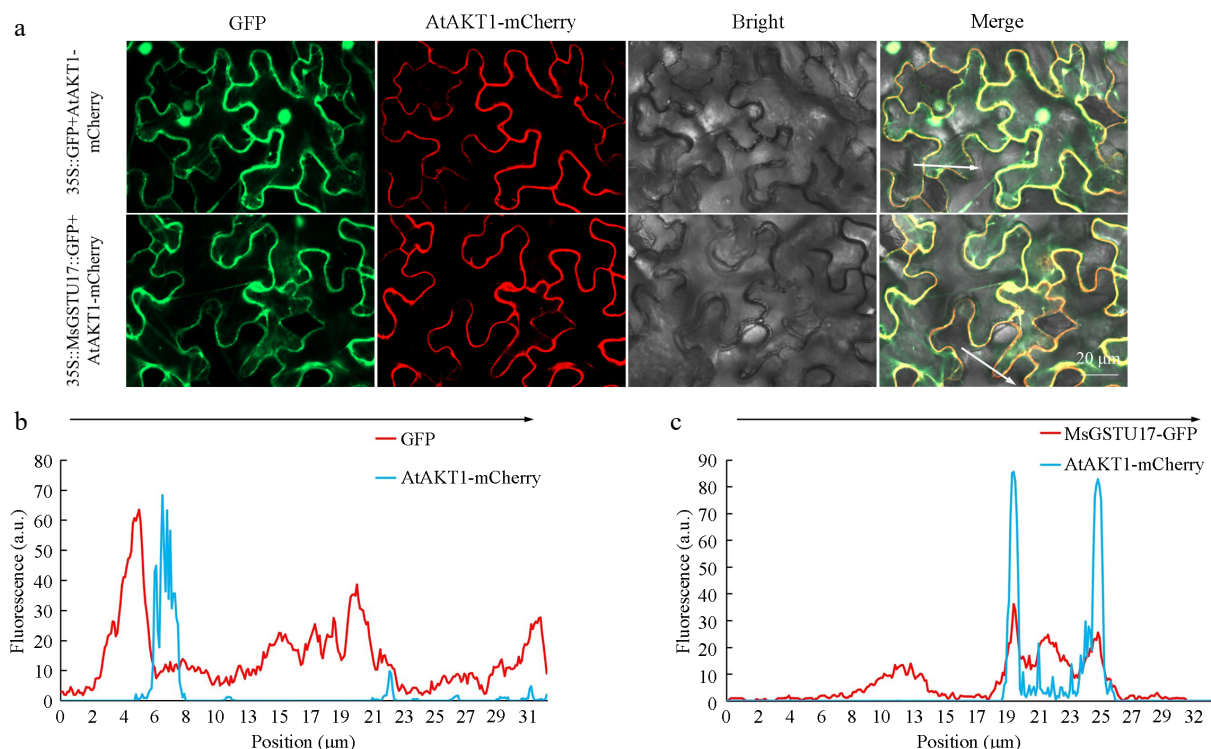


Fig. 2 Subcellular localization analysis of MsGSTU17. (a) The study evaluated the colocalization of MsGSTU17-GFP and AtAKT1-mCherry in the context of tobacco leaf tissue. Presented are representative laser-scanning photographs illustrating the fluorescence of GFP, the fluorescence of mCherry, the bright field, and an overlay combining all three. The fluorescence intensities (in arbitrary units) that crossed the tobacco leaf cell (indicated by the white line) along the arrow in (b) 35S::GFP, and (c) MsGSTU17-GFP were analyzed using ImageJ software. Scale bars = 20 μm.

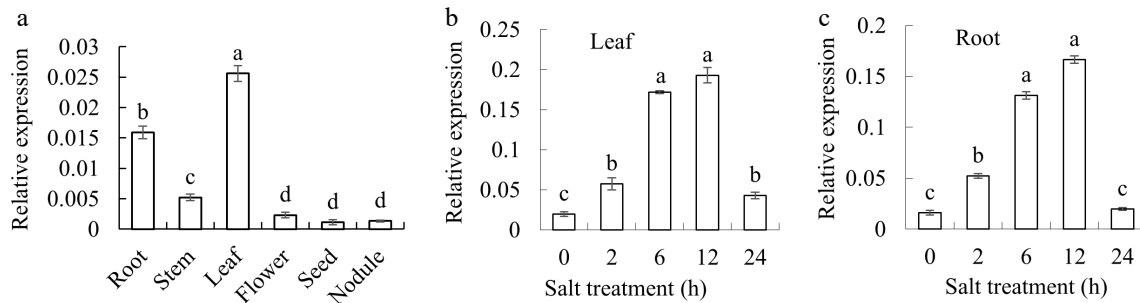


Fig. 3 Analyzing the expression profile of *MsGSTU17* gene. (a) Investigating the expression patterns of *MsGSTU17* across diverse tissues of *Arabidopsis*, encompassing roots, stems, leaves, flowers, seeds, and nodules. Under conditions of salt stress, a quantitative assessment of *MsGSTU17* expression was conducted in both (b) leaves, and (c) roots. The mean values with their standard deviations are depicted in the bar charts ($n = 3$), at the significance level of $p < 0.05$, bars on the chart that bear the same letter indicate no statistically differences.

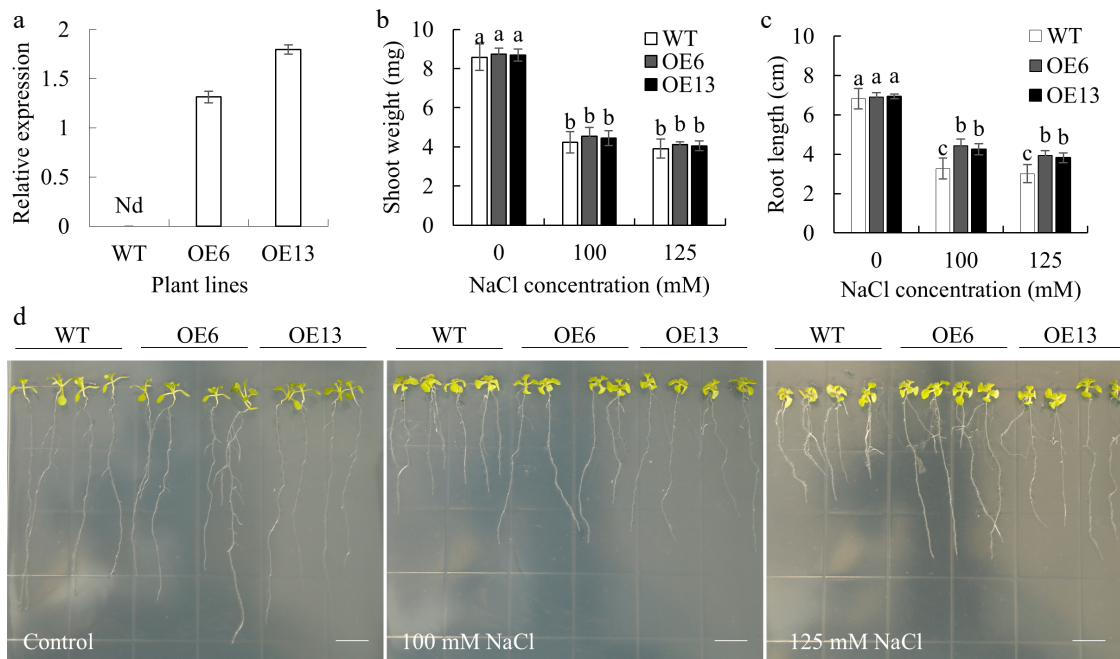


Fig. 4 The root length and shoot fresh weight of transgenic *Arabidopsis* plants overexpressing *MsGSTU17* after salt stress treatment. WT (Col-0) and *MsGSTU17* overexpressing seedlings (OE6 and OE13) were included in the treatment. (a) qRT-PCR analysis of *MsGSTU17* transcription levels in plants. The (b) fresh shoot weight, and (c) root length of each group of seedlings after 12 d of growth in 1/2 MS medium containing different NaCl concentrations. The same letters above the bar indicate no significant difference in the corresponding items ($p < 0.05$). The sample size of seedlings in each experiment is greater than 30. (d) Phenotype of each group of seedlings grown on 1/2 MS medium with different NaCl concentrations for 12 d. Bar = 10 mm.

200 mM NaCl using qRT-PCR. Under normal conditions, the transgenic lines showed no significant difference in the expression of these genes, except for *SOS2*, when compared to wild-type plants. However, under salt stress, the expression of all these genes was markedly higher in the transgenic lines than in the wild-type plants (Fig. 8).

Discussion

Salinity stress is a major abiotic factor affecting plant growth and development. It primarily disrupts the dynamic balance of ions, osmosis, and ROS, severely reducing plant yield and quality^[30,31]. The ascorbate-glutathione (AsA-GSH) cycle exists in various organisms. It plays a vital role in the antioxidant defense systems of plants and in their responses to diverse abiotic stresses. Within the AsA-GSH cycle, GSTs, proteins found extensively across diverse organisms, are crucially involved in detoxification mechanisms^[32]. The tau class is one of the most important and widespread GST classes in plants^[33]. Previous studies have shown that the tau class in

Medicago truncatula consists of 52 members. A previous study reports that the expression levels of *MtGSTU5*, *MtGSTU8*, *MtGSTU17*, *MtGSTU46*, and *MtGSTU47* increase significantly in response to various abiotic and biotic stress conditions. Among the MtGST family, *MtGSTU17* stands out for its exceptional responsiveness to stress, particularly its strong affinity for traditional substrates, such as reduced GSH and 1-chloro-2,4-dinitrobenzene^[23]. In the present study, we characterized a salt-inducible glutathione S-transferase gene, designated *MsGSTU17*, which showed a high degree of sequence similarity to the previously characterized MtGSTU17. The orthologous gene of *MsGSTU17* in *Arabidopsis thaliana*, designated AtGSTU25 (At1g17180), exhibited 65% sequence similarity and was hypothesized to participate in the assimilation of sulfur and possess a spectrum of detoxification functions against environmental contaminants^[34]. Analysis of the 2,000 bp *MsGSTU17* promoter revealed the presence of abscisic acid-responsive elements (ABREs), MeJA-responsive elements, low-temperature response elements, and MYB-responsive elements. Among these, ABREs are primarily involved in regulating the ABA response or drought expression^[35].

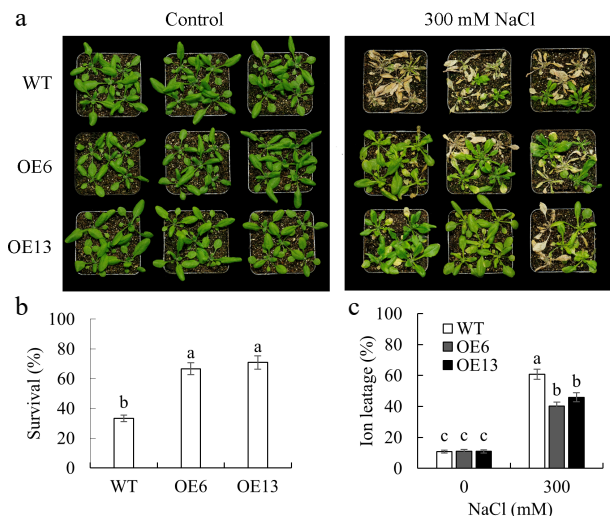


Fig. 5 The survival performance of transgenic *Arabidopsis* plants overexpressing *MsGSTU17* in a salt stress environment was investigated. WT (Col-0) and *MsGSTU17* overexpressing seedlings (three weeks old) were treated with 200, 250, and 300 mM NaCl for 3, 3, and 6 d, respectively. (a) The photos depicting the growth status of seedlings. (b) The survival rate. (c) The degree of ion leakage in the leaves is represented as the mean \pm standard deviation ($n = 3$ replicates, four plants/replicate). The same letters above the bar chart signified that there was no significant difference in the corresponding items ($p < 0.05$).

Based on the obtained data, we speculate that *MsGSTU17* facilitates the conjugation of GSH and ROS in the cytoplasm, contributing to plant defense against abiotic stressors.

Understanding the subcellular localization of proteins is crucial for the functional analysis of genes. *OsGSTU4* is detected in both the cytoplasm and nucleus, where it plays a role in enhancing the expression of genes related to stress responses and cellular

detoxification^[16]. *PeGSTU58* is also observed in these two compartments, contributing to improved salt-alkaline tolerance and increased methionine content^[36]. *MdGSTU12* from *Malus domestica* is localized in the cytoplasm, functioning as a cytoplasmic transporter and promoting anthocyanin accumulation^[37]. *MsGSTU8* from *Medicago sativa* is found in the cytoplasm, where it is involved in salt stress responses^[38]. Like *MdGSTU12* and *MsGSTU8*, *MsGSTU17* was cytoplasmic in location. However, the influence of different subcellular localizations on the functional roles of GSTs remains an area that requires further exploration.

Overexpression of the *MsGSTU8* gene from alfalfa in transgenic tobacco enhances saline-alkali stress tolerance by reducing ROS accumulation and boosting antioxidant enzyme activity^[38]. Similar to *MsGSTU8*, *MsGSTU17* overexpression also significantly enhanced plant growth under salt stresses. This finding is also consistent with previous studies that demonstrated the key role of GSTUs in plant responses to environmental stimuli^[9]. Under salt stress, the transcription of *MsGSTU17* was upregulated in both leaf and root tissues. Compared with the WT, overexpression of *MsGSTU17* resulted in increased shoot weight, root length, and survival rate after salt treatment, whereas ion leakage was reduced, indicating that *MsGSTU17* positively regulates salt tolerance. Similarly, the soybean gene *GmGSTU23* improves plant tolerance to salt stress by facilitating the removal of ROS and GSH, as evidenced by increased GST and GR activity and GSH content in transgenic lines exposed to salt stress^[39]. Overexpression of *GmGSTU4* in tobacco enhances salt stress tolerance by regulating metabolic shifts towards protective metabolites and detoxification pathways, including increased proline and trehalose, and activation of the oxidative pentose phosphate pathway^[40].

The salt overly sensitive (SOS) signaling pathway plays a crucial role in regulating the balance of Na^+ and K^+ ions in plants, which is essential for their tolerance to salt stress^[41]. *SOS1* is a Na^+/H^+ exchanger, *SOS3* acts as a Ca^{2+} -binding protein, and *SOS2*, a serine/threonine protein kinase, plays a crucial role in the SOS pathway^[42]. *RD29A*, *KIN2*, and *HKT1* serve as stress- and ABA-responsive marker

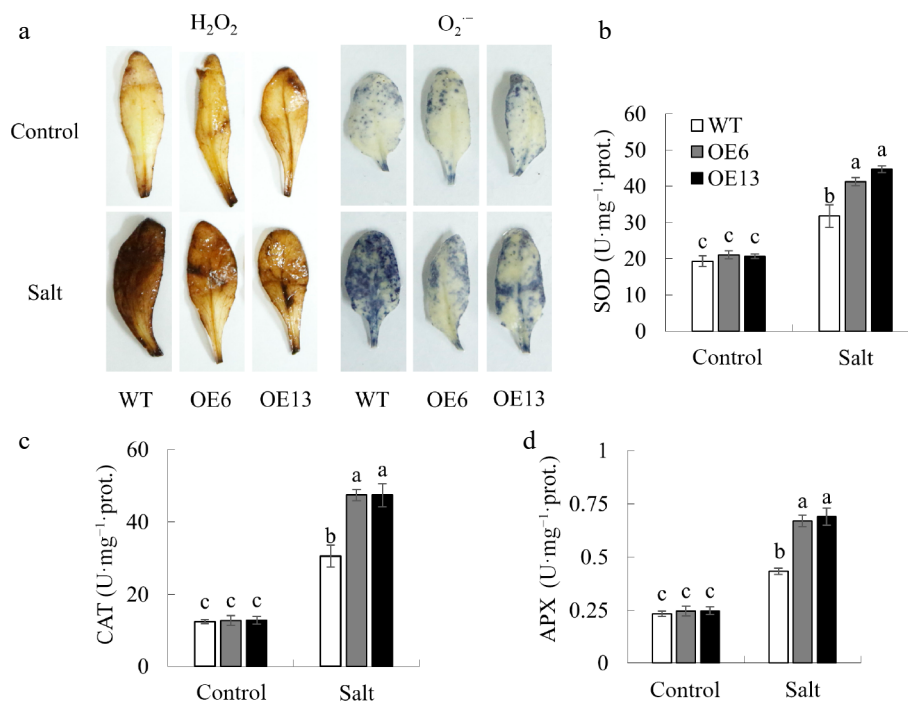


Fig. 6 The impact of *MsGSTU17* overexpression on the activity of antioxidant enzymes and accumulation of ROS. (a) Reactive oxygen species, including O_2^- and H_2O_2 , were detected in leaves. The enzymatic activities of (b) SOD, (c) CAT, and (d) APX were quantified. The data are presented as mean \pm SD based on three independent replicates. The same letter above the bars indicates that there is no significant difference between the groups ($p < 0.05$).

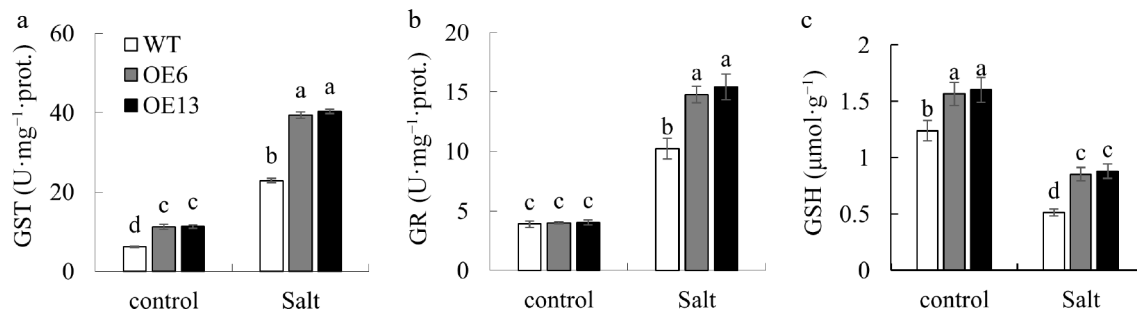


Fig. 7 Changes of GSH content and related enzymes. Variations in (a) GST, and (b) GR activities, along with (c) GSH levels, were observed in 15-day-old wild-type and transgenic *Arabidopsis* plants following a 20-d exposure to 0 or 200 mM NaCl. Distinctive letters above the bars signify statistically significant disparities among the treatments at the $p < 0.05$ level.

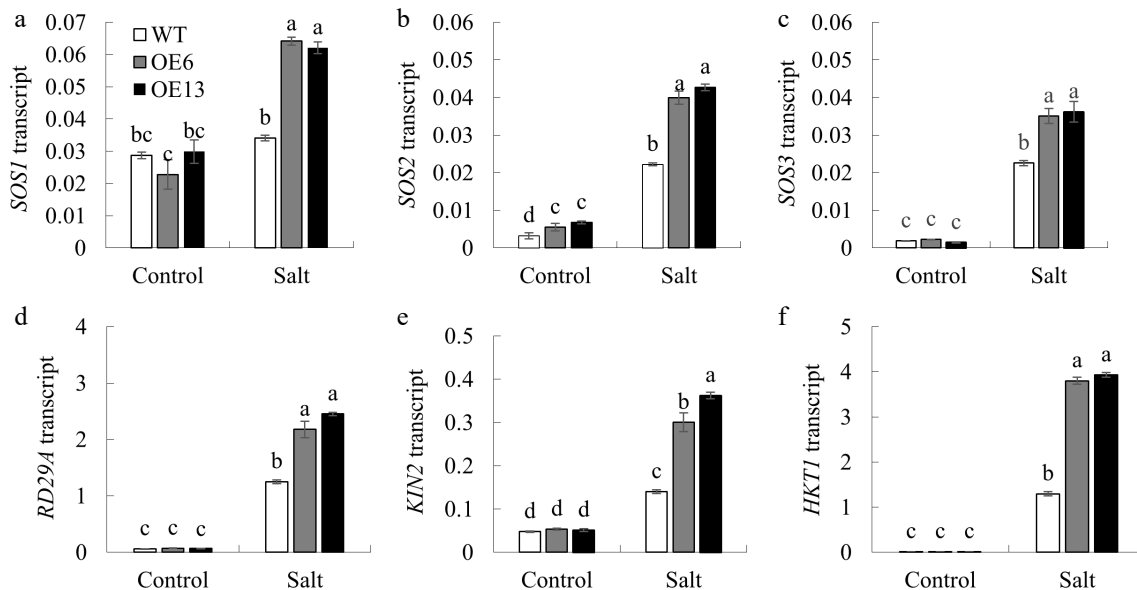


Fig. 8 The impact of salt stress on the expression of genes involved in ion homeostasis and stress response in *Arabidopsis*. After treating 7-day-old *Arabidopsis* seedlings with 125 mM NaCl for 6 h, the expression of (a) *SOS1*, (b) *SOS2*, (c) *SOS3*, (d) *RD29A*, (e) *KIN2*, and (f) *HKT1* was assessed. Values are presented as mean \pm SD ($n = 3$ replicates). Data are shown as the mean \pm standard deviation ($n = 3$ replicates). Groups with the same letter above the bars are not significantly different at the $p < 0.05$ level.

genes that contribute to salt tolerance in plants^[43]. Among the absence of stress, the transcript levels of *SOS1*, *SOS3*, *RD29A*, *KIN2*, and *HKT1* in the transgenic lines were comparable to those in WT plants, transcript level of *SOS2* significantly increased. Under salt stress, the stress-responsive genes *SOS1*, *SOS2*, *SOS3*, *RD29A*, *KIN2*, and *HKT1* were transcriptionally upregulated in both wild-type (WT) and transgenic plants. However, the expression levels in the transgenic plants were higher than those in the WT plants, which corresponded to the enhanced salt tolerance observed in the transgenic lines.

Exposure to abiotic stresses, such as salinity, leads to a substantial increase in ROS levels within plants, potentially inducing oxidative damage. The antioxidant defense system maintains a steady state between ROS production and scavenging, thereby preventing excessive ROS accumulation during stress^[44]. When exposed to salt stress, significant accumulation of $O_2^{\cdot-}$ and H_2O_2 was observed in WT plants compared to that in the transgenic strains. This phenomenon was consistent with the increased enzyme activities of SOD, CAT, and APX detected in transgenic plants, indicating that their antioxidant responses were more effective. SOD is responsible for the detoxification of $O_2^{\cdot-}$, whereas CAT and APX are involved in scavenging H_2O_2 ^[45]. These findings imply that the enhanced salt tolerance in transgenic plants is linked to elevated antioxidant enzyme activities. Compared to WT plants, the transgenic lines exhibited

significant increases in GST and GR activities, as well as a significant increase in the prototype GSH content. These data suggested that MsGSTU17 directly boosts GST activity and engages in ROS scavenging via GSH, thereby improving plant tolerance to salt stress. Nevertheless, there is limited knowledge regarding the interactions between MsGSTU17 and other transcription factors, necessitating further studies. Consequently, future investigations should employ techniques such as yeast two-hybrid screening and RNA sequencing to explore the role of MsGSTU17, to enhance our understanding of the mechanisms underlying salt tolerance in plants.

Conclusions

In this study, a novel MsGSTU17 gene was found to positively regulate salt tolerance. The elevated expression of MsGSTU17 improved salt tolerance in *Arabidopsis* and this overexpression induced alterations in the transcription levels of genes responsive to salt stress. The process mitigated the accumulation of reactive ROS by augmenting enzymatic antioxidant defenses, specifically by upregulating SOD, CAT, APX, GST, and GR catalytic activities, and by elevating intracellular GSH concentrations. Genes homologous to MsGSTU17 in crops may serve as potential targets for improving salt tolerance.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Yu S, Zhang L; data collection: Yu S, Zhuang X; analysis and interpretation of results: Yu S, Chen S, Xie Y, Zhang L; draft manuscript preparation: Yu S, Zhuang X, Chen S, Xie Y, Zhang L. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary information

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