

# MicroRNA Member TaMIR5062 in *T. Aestivum* Involves Plant Drought and Salt Acclimation via Regulating Physiological Processes Associated with Water Retention and ROS Homeostasis

Yuanjinzi Qiao

Hebei Agricultural University

Ling Wang

Hebei Agricultural University

Zidi Yu

Hebei Agricultural University

Chenyang Ni

Hebei Agricultural University

Tianjiao Li

Hebei Agricultural University

Chengjin Guo

Hebei Agricultural University

Kai Xiao (✉ [xiaokai@hebau.edu.cn](mailto:xiaokai@hebau.edu.cn))

Hebei University of Architecture

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## Research Article

**Keywords:** wheat (*Triticum aestivum* L.), P deprivation, miRNA member, drought and salt stresses, plant growth, physiological traits, antioxidant enzymes

**Posted Date:** December 16th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1137207/v1>

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# Abstract

microRNA members negatively regulate target genes via posttranscriptional cleavage or translation repression mechanisms, impacting on plant growth, development, and stress response. In this study, we characterized TaMIR5062, a miRNA member in *T. aestivum*, in mediating drought and salt responses. TaMIR5062 interacts with six target genes, including two encoding calmodulins, three coding for 4-oxalocrotonate tautomerase, and one for pumilio-family RNA binding domain protein. The TaMIR5062 transcripts were gradually upregulated in plants upon 27-h drought and salt treatments, whose induced expression under stress treatments was restored following the normal recovery condition. Tobacco (*N. tabacum*) lines transformed with TaMIR5062 modified growth traits under drought and salt treatments; the lines overexpressing miRNA (i.e., Sen 1 and Sen 2) improved growth traits (i.e., biomass, leaf area, and root length) whereas those with knockdown (Anti 1) alleviated growth features compared with wild type. These results suggested the critical role of TaMIR5062 in improving plant drought and salt tolerance. In line with growth traits in stress-challenged lines, improved leaf water retention (i.e., promoted stomata closing, water losing rate, and osmolytes) and ROS-associated parameters (higher SOD, CAT, and POD activities, etc.) were shown in Sen 1 and Sen 2 under stress conditions. Antioxidant enzyme (AE) genes *NtMnSOD1*, *NtCAT*, and *NtPOD9* encoding SOD, CAT, and POD, respectively, enhanced transcription in Sen 1 and downregulated expression in Anti 1 challenged with drought and salt stress. These results suggested the improved ROS homeostasis mediated by TaMIR5062 associates modified expression of distinct AE genes. Quantities of genes functional into categories “*biological process*”, “*cellular component*”, and “*molecular function*” contribute to TaMIR5062-mediated osmotic stress adaptation by regulating distinct biological pathways (i.e., protein folding) and metabolisms (i.e., photosynthesis and isoprenoid biosynthesis), which impact on plant osmotic-regulation, ROS homeostasis, and stress defensiveness underlying miRNA regulation. TaMIR5062 is a valuable target for molecular breeding of drought-tolerant crop cultivars.

## Key Message

TaMIR5062 confers plants drought and salt stress adaptation via modulation of physiological processes associated with stomata movement, osmolyte biosynthesis, and ROS homeostasis.

## Introduction

As the frequently occurred stresses to plants, drought and high salinity alleviate drastically cell function, negatively regulating plant growth, development, and the yield formation capacity for cereal crops (Cattivelli et al. 2008, Maiti et al. 2014). Under osmotic stress conditions (i.e., drought and salt stress), a large set of physiological processes associated with plant growth behavior including root system architecture (RSA) establishment, protein biosynthesis metabolism, photo-chemical efficiency and assimilate production in photosystem, biosynthesis of osmolytes, and differentiation and establishment of tissues are altered, which act with synergistic manner in regulating the growth traits and yield formation potential of crop plants (Benjamin and Nielsen 2006; Zhao et al. 2019; Naheed et al. 2021).

Elite cultivars sharing strong capacities to cope with drought and salt stresses have been potential in improving productivity of the crop plants cultivated under water-saving and high salinity conditions.

Plants have evolved a suite of strategies to acclimate to adverse osmotic stress conditions through various mechanisms initiated at physiological and molecular levels (Chaves et al. 2009; Fang and Xiong 2015; Yang and Gao 2018). Thus far, the knowledge as to the molecular networks mediated by drought and salt signaling has been extensively developed, given the advanced research approach established in past several decades. A quantity of critical regulators functional in plant response to osmotic stressors has been identified, which has benefited the elucidation of biochemical pathways and action mechanisms underlying drought and salt stress stressors. Among them, signaling receptors, kinases in the families of mitogen-activated protein kinases (MAPK) and calcium-dependent protein kinases (CDPK), and transcriptional regulators (i.e. calmodulin-binding proteins, phosphatases, and distinct transcription factors), act in a cooperate mode in defining plant stress response, given their crucial roles in mediating transduction of the stress signaling (Danquah et al. 2014; Delormel and Boudsocq 2019; Yoshida et al. 2014). Therefore, plant responses to osmotic stresses are determined largely by the signaling transduction system. Functional characterization on the critical regulators constituting the system can help depth dissection of the molecular mechanisms underlying plant osmotic stress responses.

Members of the microRNA (miRNA) family are non-coding RNA molecules with nucleotide acids from 20 to 24 nt in length. They are involved in the modulation of quantities of physiological processes through regulating target genes at posttranscriptional or translational level with a negative regulation mechanism (Ferdous et al. 2015; Sun et al. 2019). Thus far, characterization on miRNA members in plant species has validated their function as critical mediators in diverse biological pathways, being associated with growth and development, such as growth phase transition, leaf morphogenesis, floral organ identity, and root architecture establishment (Lu and Huang 2008; Rubio-Somoza and Weigel 2011). Additionally, a large set of miRNA members also act as crucial modulators in plant responses to adverse abiotic stresses, through regulating transcription of the stress defensiveness-associated genes (Phillips et al. 2007; Lu and Huang 2008; Mazzucotelli et al. 2008; Shukla et al. 2008; Lewis et al. 2009). For instance, member miR319 in *Arabidopsis* confers plant responses to a set of stress conditions, including water deficit, high salinity, and extreme low temperature, which is largely ascribed to its modified transcription efficiency upon these stressors (Sunkar and Zhu 2004; Liu et al. 2008; Lv et al. 2010; Thiebaut et al. 2012; Zhou et al. 2010; Kar and Raichaudhuri 2021). Altered expression of this miRNA leads to significant variation on transcripts of genes in the TCP transcription factor (TF) family encoding basic helix-loop-helix (bHLH) proteins, endowing plants increased adaptation to above stress conditions (Palatnik et al. 2003; Ori et al. 2007; Nag et al. 2009). In cereal crops, a subset of miRNA members is responses to drought stress, contributing to improved adaptation of plants to this stressor (Zhou et al. 2010; Qiu et al. 2020). Likewise, the miRNA members involving plant salt stress have also been documented extensively in various plant species to date, including miR393, miR319c, miR389a.1 in *A. thaliana* (Sunkar and Zhu 2004), miR169, miR319, miR393, miR396c, miR398, miR528, miR820, miR1848 in *O. sativa* (Haldar and Bandyopadhyay 2021), mi408 in *T. aestivum* (Bai et al. 2018), miR156j, miR156k, miR159a, miR159b, miR162a, miR162b, miR167a, miR168a, miR169g, miR169f, etc. in *Z. mays* (Ding et al. 2009; Zhao et al. 2009), and miR156d,

miR164a, miR393a, miR319a and miR172b in barley (Lv et al. 2012). However, although the miRNA members and characterization on them were extensively investigated, the mechanisms underlying miRNA-mediated responses to osmotic stresses (i.e., drought and salt stress) are needed to be further characterized in plant species, especially in cereal ones.

Wheat (*T. aestivum*) acts as one of the important cereal crops and is extensively cultivated around the world, contributing greatly to the food supply security worldwide. However, the cultivation process of wheat is frequently challenged by the shortage of water resource, leading to drought stress for the crop plants. Additionally, large area for wheat planting is also grown in the environment with high salt contents, resulting in the alleviated productivity of crops due to high Na<sup>+</sup> toxicity (Terletskaya et al. 2013). Therefore, improvement of plant adaptation to drought and salt stress can benefit promotion of the sustainably agricultural development in developing countries. Thus far, although the miRNA database in *T. aestivum* (www.mirbase.org, release 21) deposits a large set of members of the miRNA family and a suite of investigations have reported the characterization of miRNAs, target genes, and putative miRNA/target interaction behaviors (Sun et al. 2014; Wang et al. 2014; Bakhshi et al. 2017), the mechanisms as to how distinct miRNA members of wheat mediate plant osmotic stress responses are largely to be determined. In this study, we characterized TaMIR5062, a miRNA member in *T. aestivum*, in mediating plant adaptation to drought and salt stresses. Our results suggested that TaMIR5062 functions a crucial mediator in regulating above stressors through regulating the physiological processes associated with osmotic stress response.

## Materials And Methods

### Characterization of TaMIR5062

Our previous investigation with an aim to identify wheat miRNA family members in response drought stress revealed that TaMIR5062 (accession number MI0030391) was upregulated on transcripts in plants (cv. Shimai 22) challenged with this stressor. This finding prompted us to further characterize it in mediating plant osmotic stress response in more detail. Sequences of TaMIR5062 at precursor and mature conditions and structure of stem-loop feature initiated by the miRNA precursor were derived from the miRNA information deposited in database of *T. aestivum* (www.mirbase.org).

### Prediction of the target genes of TaMIR5062

An online tool psRNATarget (Plant microRNA Potential Target Finder; <http://plantgrn.noble.org/psRNATarget/>) was used to predict the target genes interacted by TaMIR5062 using the default parameters suggested. The target genes were identified by search against the cDNA deposits referred to as cDNA databases of *T. aestivum* (bread wheat), cDNA, EnsemblPlant, release 43. Putative biological roles of the target genes were determined through BLASTn search analyses supplemented in National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/), with cDNA sequences of target genes used as queries

## Expression analysis of TaMIR5062

The seedlings of wheat (cv. Shimai 22) cultivated under different conditions, including normal growth, drought, and salt stress were used to determine the expression patterns of TaMIR5062. In brief, the seedlings after germination were firstly cultured in a standard Murashige and Skoog (MS) solution to the third leaf stage under following growth condition: a photoperiod range of 12 h/12 h (day/night) with light intensity of  $300 \mu\text{mol E m}^{-2} \text{s}^{-1}$  during light phase, temperature range of  $28^{\circ}\text{C}/23^{\circ}\text{C}$  (day/night), and relative humidity from 65 to 70%. At that time, they were subjected to treatments of simulated drought and salt stress. Of which, the drought treatment was established by growing an aliquot of seedlings in a modified MS solution supplemented with PEG-6000 (10%, w/v) whereas salt treatment was initiated by culturing seedlings in a MS solution containing 200 mM NaCl. Tissues of leaf and root were sampled at time points of 0 h (prior to treatment), and 3 h, 6 h, 9 h, 12 h, and 27 h after the treatments. In addition, aliquots of the seedlings after 27 h of drought and salt treatments were re-subjected to the normal growth condition to understand regular recovery response of TaMIR5062. The normal recovery treatments were established by transferring the drought- and salt-stressed seedlings (27 h after treatments) into standard MS solution. Likewise, tissues mentioned were sampled at time points of 3 h, 6 h, 9 h, 12 h, and 27 h following recovery treatments. qRT-PCR was performed to evaluate the transcripts of TaMIR5062 in samples collected as previously described (Guo et al. 2013). With this purpose, total RNA in samples was extracted using TRIzol reagent (Invitrogen, USA). The first-strand cDNA was synthesized using RT-AMV transcriptase (TaKaRa, Dalian, China) from total RNA (2  $\mu\text{g}$ ) after removal of putative genomic DNA treated with RNase-free DNase (TaKaRa, Dalian, China). qRT-PCR was conducted in a volume of 25  $\mu\text{L}$  which contained following components: 12.5  $\mu\text{L}$  of SYBR Premix ExTaq<sup>TM</sup> (TaKaRa, Dalian, China), 0.5  $\mu\text{L}$  each for forward and reverse primers, 1  $\mu\text{L}$  cDNA and 10.5  $\mu\text{L}$  nuclease-free water. The transcripts of TaMIR5086 were calculated according to the  $2^{-\Delta\Delta\text{CT}}$  formula, using a constitutive gene *Tatubulin* in *T. aestivum* as an internal standard to normalize the target expression levels. Specific primers used for amplification of TaMIR5062 and *Tatubulin* are shown in Table S1.

## Assays of the growth traits in transgenic lines

Tobacco (*N. tabacum*) lines with overexpression or knockdown expression of TaMIR5062 were established to characterize the role of this miRNA in mediating drought and salt responses. With this purpose, we performed RT-PCR to amplify the precursor of TaMIR5062 using specific primer pair (Table S1), then inserted it into the restriction sites *NcoI/BstEII* in binary vector pCAMBIA3301 under the control of the CaMV35Spromoter. The expression cassettes harboring TaMIR5062 in sense and antisense orientations were separately integrated into *A. tumefaciens* strain EHA105 using heat-shock approach and subjected to genetic transformation onto *N. tabacum* (cv. Wisconsin 35) as described previously (Shi et al. 2020). Transcripts of the target miRNA in transgenic lines were evaluated based on qRT-PCR performed to be similar for evaluating expression levels of TaMIR5062 upon stress conditions.

Three lines at T3 generation, including two with more TaMIR5062 transcripts (Sen 1 and Sen 2) and one less transcript of TaMIR5062 homolog (Fig. S1), were selected to be subjected to various growth

treatments, including normal growth condition, drought stress, and salt treatment. Of which, the normal growth condition was established by growing plants of the transgenic lines (i.e., Sen 1, Sen 2, and Anti 1) together with wild type (control without transformed with TaMIR5062, WT) in the standard MS solution; drought treatment was initiated by culturing the transgenic and WT plants in a modified MS solution supplemented with PEG-6000 (5%, w/v); salt treatment was established by culturing transgenic and WT plants in MS solution containing 150 mM NaCl. After five weeks of treatments, phenotypes, biomass in aerial and root tissues, leaf area, root lengths and root fresh weights were assessed. Of which, phenotypes were recorded as images taken by a digital camera; biomass in aerial tissues, roots, and plants were obtained from the representative samples after oven-drying; leaf area, root length, and root fresh weights in plants were determined following conventional approach.

### **Assays of stomata stomatal behavior and leaf water retention capacity in transgenic lines**

The stomata closing feature and water retention capacity of leaf that impact largely on plant response to osmotic stresses were assessed in the transgenic lines (Sen 1, Sen 2, and Anti 1) and WT under drought and salt stress conditions. To characterize stomata movement, the plants of Sen 1, Anti 1, and WT grown under normal condition were subjected to drought and salt treatments mentioned above. At 0 h (prior to treatment), and 0.25 h, 0.5 h, and 1 h following stress conditions, leaf samples in transgenic and WT plants were collected. Stomata nature in sampled leaves was observed under microscope after fixation using nail polish oil as described previously (Ding et al. 2014). At least fifty of representative stomata were observed in each sample. Stomata aperture properties were observed under a microscope at the indicated times. Water retention capacities were analyzed using detached representative leaves of transgenic (Sen 1 and Anti 1) and WT plants collected after 0 h, 2 h, 4 h, and 6 h during drought and salt treatments. Fresh weights of the transgenic and WT plants under various conditions were obtained using an electronic balancer. Water losing rates (WLR) of leaves were calculated based on decreases of fresh weights at indicated times with respect to that at 0 h.

### **Assays of osmolyte contents in transgenic lines**

To evaluate the osmo-regulatory function mediated by TaMIR5062, the contents of soluble sugar and soluble protein were assessed in transgenic lines (Sen 1, Sen 2, and Anti 1) and WT under stress treatments, using the leaves of transgenic and WT plants treated with drought and salt as samples. The soluble sugar contents were analyzed as suggested by Hu et al. (2016) whereas the soluble protein contents were evaluated according to the approach described previously (Read and Northcote 1981).

### **Assessments of reactive oxygen species (ROS)-associated parameters and AE gene expression in transgenic lines**

A set of reactive oxygen species (ROS)-associated parameters, including activities of antioxidant enzyme (AE, i.e., superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), contents of MDA, and membrane electric conductance (MEC), were assessed in the transgenic and WT plants after normal growth and stress treatments as aforementioned. Of which, the AE activities and MDA contents were

analyzed as described previously (Huang et al. 2010). The MEC values in samples were determined using an electric conductance analyzer (DDS-307A, Shanghai, China) following the manufacturer's suggestion. To understand the molecular processes as to the TaMIR5062-mediated AE activities with expression patterns of AE family genes, six genes in SOD family, six genes in CAT family, and eleven genes in POD family in *N. tabacum* identified in NCBI GenBank database were subjected to evaluation of expression levels under various growth conditions. To this end, the transcripts of these AE genes were assessed using the transgenic and WT plants grown under normal condition, drought, and salt stress based on qRT-PCR analysis, which was performed to be similar for characterization of TaMIR5062 expression mentioned previously. The genes and the gene specific primers used in qRT-PCR are shown in Table S1. *Nttubulin* was used as internal standard to normalize the target transcripts.

### **RNA-seq analysis for drought-challenged transgenic lines**

The transcriptome profile upon drought underlying modulation of TaMIR5062 was investigated based on high-throughput RNA-seq analysis. With this purpose, Sen 1 and WT were cultured regularly in a standard MS solution as aforementioned. The plants of them at the fifth leaf stage were subjected to drought treatment by culturing in a MS solution supplemented with PEG-6000 (w/v, 10%) for three days. After drought treatment, total RNA in samples was extracted using TRIzol reagent (Invitrogen). RNA-seq libraries were constructed for the drought-challenged Sen 1 and WT plants following the procedure as described previously (Zhong et al. 2011) and subjected to generation of transcripts using Illumina HiSeq 2500 system. Putative valuable transcripts in the libraries were obtained after removal of adaptors in the reads, the transcripts with cDNA length less than 40 bp, and those being low quality based on software referred to as Trimmomatic (Bolger et al. 2014). Alignment analysis using the generated transcripts was performed by searching against the cDNA database of the reference genome (*N. tabacum*, Novogene Co, LTD, Beijing). The genes were defined differentially expressed (DE) when they exhibited 2-fold variation on transcripts between transgenic and WT plants (Robinson et al. 2010), using a false discovery rate (FDR) less than 0.05 (Benjamini and Hochberg 1995). GO terms and biochemical pathways of the DE genes were categorized using the online tool referred to as Plant MetGenMap (<http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi>), using a CPAN pearl module during which applied as described previously (Boyle et al. 2004)

### **Statistical analysis**

Averages of gene expression levels in qRT-PCR analysis, growth traits such as leaf area, biomass in aerial and root tissues, root fresh weights and root lengths, stomata closing rates, AE activities, MDA contents, osmolyte contents, MEC, and RNA-seq analysis in transgenic lines and WT were derived from results of four replicates. Standard errors of averages and significant differences were analyzed using the Statistical Analysis System software (SAS Corporation, Cary, NC, USA).

## **Results**

### **The targets genes interacted with TaMIR5062**

In total of six genes are suggested to be the targets of TaMIR5062, with regulation mechanism of them underlying miRNA at posttranscriptional level. Of which, two encode calmodulin proteins, three code for 4-oxalocrotonate tautomerase, and one encode pumilio-family RNA binding domain protein (Fig. 1). That half of target genes involving signaling transduction (those encoding calmodulin) and transcriptional regulation (the gene encoding RNA binding domain protein) suggests that TaMIR5062 is involved in the modulation of distinct biological processes by regulating the target genes through a cleavage mechanism.

### **Expression patterns of TaMIR5062 under drought and salt stress conditions**

The transcripts of TaMIR5062 in aerial and root tissues were modified dramatically upon both drought and salt stresses, displaying patterns to be gradually upregulated over a 27 h-regime of stress treatments (Fig. 2). Additionally, the stress-regulated expression of TaMIR5062 under both drought and salt treatments was restored following progression of normal recovery condition (Fig. 2). These results suggested that TaMIR5062 sensitively responds to drought and salt stress signaling in plants. That TaMIR5062 displays modified expression patterns upon stressors mentioned suggests its putative involvement in regulating plant responses to drought and salt stresses by impacting on modifying the transcripts of target genes.

### **Growth behaviors of the TaMIR5062 transgenic lines under drought and salt treatments**

The growth traits of two lines with strong expression of TaMIR5062 (i.e., Sen 1 and Sen 2) and one with knockdown expression of target miRNA (i.e., Anti 1) (Fig. S1) were assessed under drought and salt treatments. Under normal growth condition, similar phenotypes, biomass in aerial tissue and roots, plant biomass, leaf areas, root fresh weights, and root lengths were observed among the transgenic lines and WT (Figs. 3a-3g). In contrast, under treatments of drought and salt, the transgenic lines modified the growth traits mentioned, with those shown to be drastically improved on phenotype (Fig. 3a), biomass of aerial tissue and root, and plant (Figs. 3b to 3d), leaf area (Fig. 3E), root fresh weight (Fig. 3f) and root length (Fig. 3g) in Sen 1 and Sen 2 whereas significantly alleviated on growth traits mentioned in Anti 1 compared to WT plants (Figs. 3a-3g). These results suggested that TaMIR5062 plays critical roles in mediating plant tolerance to drought and salt stresses.

### **Stomata movement feature and water retention capacity of transgenic lines**

The stomata movement property mediated by TaMIR5062 was analyzed by assessing stomata aperture property in Sen 1 and Anti 1 treated with 1 h regime of drought and salt stresses. Upon these stressors, the stomata closing rates (SCR) in detached leaves were all lowered in the transgenic and WT plants; however, the decrease on closing speeds was much faster in Sen 1 and lower in Anti 1 than WT (Figs. 4a and 4b). In lines with behavior of leaf SCR shown in transgenic and WT plants, the leaf water losing rates (WSR) were in contrast to SCR values among the lines with modified TaMIR5062 expression and WT. Sen 1 showed lower whereas Anti 1 higher WSR values following progression of 6 h drought and salt treatments than WT plants (Figs. 4c and 4d). These results suggested that the TaMIR5062-mediated

plant adaptation to osmotic stress is associated closely with its role in regulating water retention capacity through modulating stomata movement that affects leaf WSR behavior.

### **Contents of soluble sugar and protein in transgenic lines under drought and salt treatments**

The contents of osmolyte (i.e., soluble sugar) and soluble protein were evaluated in stress-treated transgenic lines to address whether modified osmolytes associate with the TaMIR5062-mediated drought and salt stress responses, using transgenic lines (Sen 1, Sen 2, and Anti 1) and WT after stress treatments as samples. As expected, no variations were found on contents of soluble sugar and protein in transgenic and WT plant under normal growth condition (Figs. 5a and 5b). Under treatments of drought and salt stress, higher levels of soluble sugar and soluble protein were found in lines overexpressing TaMIR5062 than WT treated with above stressors. In contrast, the contents of soluble sugar and protein were decreased in Anti 1 compared with WT under drought and salt treatments (Figs. 5a and 5b). These results suggested that TaMIR5062 modifies osmotic stress response partly by its role in regulating accumulation of the osmolytes in plants.

### **Behaviors of ROS-associated parameters in transgenic lines under drought and salt treatments**

The ROS-associated parameters in transgenic and WT plants were measured after the drought and salt treatments. Under normal growth condition, comparable behaviors on antioxidant enzyme (AE) activities and over-oxidation product on membrane system were observed among the transgenic lines and WT plants (Figs. 6a to 6e). Under drought and salt stress treatments, compared with WT, Sen 1 and Sen 2 were enhanced on activities of SOD, CAT, and POD, decreased on contents of MDA, and lowered on membrane electric conductance (MEC) whereas Anti 1 lowered on AE activities, increased on MDA contents, and elevated on MEC under the stress conditions (Figs. 6a to 6e). These results indicated that the cellular ROS homeostasis was modified underlying TaMIR5062 regulation, namely, this miRNA member exerts positive roles in regulating cellular ROS homeostasis by which to contribute to plant adaptation to drought and salt stresses.

### **Expression patterns of AE family genes in transgenic lines**

Expression patterns of a subset of AE family genes were analyzed in the transgenic and WT plants after drought and salt treatments to characterize the molecular processes associated with TaMIR5062-mediated ROS homeostasis. Among the genes in SOD family, *NtMnSOD1* modified transcripts in transgenic lines (Sen 1, Sen 2, and Anti 1) with respect to WT. Likewise, the CAT family gene *NtCAT* and the POD family gene *NtPOD9* displayed modified expression in transgenic lines compared with those shown in WT plants (Figs. 7a to 7f). Of which, all of the differentially expressed genes in AE families were significantly upregulated in lines overexpressing TaMIR5062 (i.e., Sen 1 and Sen 2) and downregulated in line with knockdown expression of the miRNA (Anti 1) (Figs. 7a to 7f). These results together suggest that distinct genes in AE families, including *NtMnSOD1*, *NtCAT*, and *NtPOD9*, respond to drought and salt signaling underlying regulation of TaMIR5086 at the transcriptional level. They contribute to the improved cellular ROS homeostasis in TaMIR5062 overexpression lines under the osmotic stress conditions.

## The transcriptome profile modified by TaMIR5062 under drought stress condition

Transcriptome profile upon drought stress underlying modulation of TaMIR5062 was investigated at global level based on RNA-seq analysis, using the drought challenged line (Sen 1) and WT as samples. In total of 3126 genes with differentially expressed (DE), including 1650 with patterns of upregulation and 1467 of downregulation, were identified (Fig. 8a). Gene ontology (GO) analysis on upregulated DE genes suggested that they are enriched into GO terms associated with “*biological process*”, “*cellular components*”, and “*molecular function*”, especially that of the first one. The DE genes in GO term “*biological process*” associates with processes of photosynthesis, oxidation-reduction process, metabolic process, single-organism metabolic process, organonitrogen, organonitrogen compound metabolic process, organonitrogen compound biosynthetic process, protein folding, isoprenoid metabolic process, isoprenoid biosynthetic process, lipid metabolic process, oxoacid metabolic process, organic acid metabolic process, lipid biosynthesis process, small molecule metabolic process; the DE genes in GO term “*cellular components*” impacts on constitutions of photosystem, photosynthetic membrane, thylakoid, thylakoid part, photosystem II oxygen evolving complex, thylakoid membrane, photosystem II, photosystem I, oxidoreductase complex, photosystem I reaction center, extrinsic component of membrane, membrane protein complex; the DE genes in GO term “*molecular function*” exerts role in regulation of oxidoreductase activity, cofactor binding, coenzyme binding, and fructose-biophosphate aldolase activity (Fig. 8b). KEGG analysis on the DE genes identified in the drought-challenged TaMIR5062 overexpression lines suggests the modified biochemical pathways under the control of miRNA, including metabolic pathways, biosynthesis of secondary metabolites, carbon metabolism, carbon fixation in photosynthetic organisms, photosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, glyoxylate and dicarboxylate metabolism, nitrogen metabolism, etc. (Fig. 8c). These findings together suggested that a large set of genes in plants are underlying regulation of TaMIR5062 at transcriptional level, which are involved in modifying diverse physiological processes associated with plant response to osmotic stress.

## Discussion

The members of miRNA families involve modulation of diverse physiological processes of growth, development, and stress responses through regulating target genes via posttranscriptional cleavage or translation repression mechanisms (Ayushi et al. 2015). In this study, our prediction analysis on TaMIR5062, a miRNA member in *T. aestivum*, indicated that it interacts with six target genes. Of which, two encode calmodulins, three code for 4-oxalocrotonate tautomerase, and one encodes pumilio-family RNA binding domain protein. Thus far, extensive investigations have been performed to validate the function of genes encoding calmodulin, a critical component of the signaling pathways sensing modified levels of  $Ca^{2+}$  at cellular or subcellular level, in modulating wide range of stress defensive-associated biological processes (Scholz et al. 2015; Wang et al. 2016; Kim et al. 2017; Raina et al. 2021). Likewise, distinct members of RNA binding domain protein are involved in plant stress response through modulating assembly, activity, and efficiencies of transcription and translation of mRNA members

involving plant acclimation to various abiotic stressors (Mahmood et al. 2020; Muthusamy et al. 2021; Raina et al. 2021). Therefore, most of the target genes encoding signaling and regulation-associated proteins underlying TaMIR5062 regulation suggested that this miRNA impacts on modulation of diverse biological processes, given the wide range of function mediated by target genes. Further characterization on the target genes of TaMIR5062 can help to establish the function module (i.e., TaMIR5062/calmodulin) underlying this miRNA and its target genes.

Similar to that of mRNA molecules, the transcription mechanism of miRNA members in plants species is comprehensively regulated by a subset of regulatory factors, such as enzyme Pol II proteins recruiting to promoter region of miRNA via actions of a set of transcriptional coactivators (Kim et al. 2011), TATA box acting as conserved motif involving transcription that is situated at promoter region (Xie et al. 2005), and the cis-acting regulatory elements locating at distinct positions of promoter involving DNA-protein interaction (Megraw et al. 2006; Hajdarpašić and Ruggenthaler 2012; Liang et al. 2012). To date, a subset of miRNA members in plant species has been documented with modified transcription efficiency upon osmotic stresses (Leung and Sharp 2010). In this study, our expression analysis on TaMIR5062 revealed that the transcripts of this miRNA modify sensitively upon osmotic stresses (i.e., drought and salt stress), displaying significantly upregulated under stress treatments and gradually recovered in aerial and root tissues along with normal recovery condition. These results suggest that TaMIR5062 is response to drought and salt stresses at transcription level, whose modified transcripts upon stresses impact on modulation of physiological processes via its role in regulation of its target genes. Previously, a cis-regulatory motif referred to as CRE (with motif CCGCGT, CACGTGT, and AAGTCAA) present frequently in gene promoters has been verified to playing critical roles in regulating transcription efficiency of the osmotic stress-responsive genes, via an interaction mechanism between the motif with the DNA binding domain harboring bZIP transcription factors (Ma et al. 2012). Therefore, further characterization on the cis-regulatory elements, such as CRE and other ones exerting roles in modulating miRNA transcription under drought and salt stresses, can provide insights into novel understanding for transcription mechanisms of miRNA members of plant species in response to osmotic stresses.

miRNA members have been documented in mediating plant adaption to various abiotic stresses. For example, member miR319 in *Arabidopsis* displayed increased transcripts upon drought stress. The transgenic lines overexpressing miR319 conferred plants improved growth traits and enhanced drought tolerance by regulating leaf wax biosynthesis and water retention capacity, given that it downregulates expression of target genes *AsPCF5*, *AsPCF6*, *AsPCF8*, and *AsTCP14*, the members in TCP transcription factor family (Nag et al. 2009). In this study, our transgene analysis on TaMIR5062 confirmed its positive function in mediating adaptations to drought and salt stresses, given findings that the lines overexpressing this miRNA improved drastically phenotypes, plant biomass, and photosynthetic function under the osmotic stress conditions. Therefore, our results suggested that TaMIR5062 can be used as a useful molecular index for evaluation of drought and salt adaptation in *T. aestivum*. It is also a valuable target for molecular breeding of the wheat cultivars to be elite in drought and salt tolerance.

Stomata movement sensitively responds to environmental cues, which acts as one of the critical adaptation mechanisms of plants to osmotic stress conditions (Kollist et al. 2014). In this study, our analysis on stomata closing rates (SCR) in lines with overexpression or knockdown expression of TaMIR5062 revealed that the miRNA exerts large role in regulating stomata movement; the lines overexpressed the miRNA (i.e., Sen 1 and Sen 2) displayed faster on decrease of SCR whereas that with knockdown expression of miRNA (Anti 1) slower on SCR reduction under treatments of drought and salt stress, with respect to WT plants. Additionally, TaMIR5062 also confers plants enhanced osmo-regulatory ability, based on our osmolytes assay results in the stress-challenged transgenic lines. These results suggested that TaMIR5062 functions as a mediator in improving osmotic stress tolerance through the mechanism to enhance water retention capacity via regulating stomata movement and osmolyte biosynthesis. Thus far, extensive literatures have reported the internal relation between stomata movement upon osmotic stressors and the induced abscisic acid (ABA) accumulation (Boursiac et al. 2013; Song et al. 2014). Moreover, the ABA signaling pathways associated with stomata movement under stress signaling, such as ABA receptors (i.e., RCAR, PYR1, and PYL), type 2C protein phosphatases (PP2C), and SNF1-related protein kinase OPEN STOMATA1 (OST1)/SnRK2 that function as essential members in pathways, have been extensively characterized in the model plants (Park et al. 2009; Ma et al. 2009). Therefore, further investigation of the ABA signaling pathway regulating stomata movement underlying TaMIR5082 modulation can provide insights into plant osmotic stress responses mediated by miRNA members.

Plant tissues display induced ROS upon abiotic stresses, which negatively impact on plant stress adaptations due to its injury on molecule structures of protein, lipid, and nucleic acid and cell function (Gill and Tuteja 2010). Acting as effective agents for ROS scavenging, the AE enzymes SOD, CAT, and POD alleviate osmotic stress effects and contribute to cellular ROS homeostasis (You and Chan 2015). For example, overexpression of *N. plumbaginifolia* MnSOD gene in alfalfa significantly improved growth traits and yield potential of plants treated with drought stress (McKersie et al. 1996). The *O. sativa* lines overexpressing *A. marina* cytosolic *CuZnSOD* display enhanced ability to osmotic stress (Prashanth et al. 2008). In this study, our assay on SOD, CAT, and POD activities in lines with TaMIR5062 overexpression or knockdown expression validated the improvement and alleviation of them under osmotic stress conditions, which impact on behavior of contents of MDA, membrane electric conductance (MEC), and finally cellular ROS homeostasis. Moreover, based on expression analysis, we identified distinct genes to be differentially expressed in the TaMIR5062 overexpression line and the line with miRNA knockdown expression, including SOD gene *NtMnSOD1*, CAT gene *NtCAT*, and POD gene *NtPOD9* under stress treatments. Our findings thus suggested that the TaMIR5062-mediated ROS homeostasis is associated with the modified transcription of distinct AE genes mentioned above that enhance cellular AE activities. Further characterization on the relations among these differential AE genes and ROS homeostasis can help the understanding of plant drought response underlying the miRNA member-ROS pathways in plant species.

High throughput RNA-seq analysis can identify genes that are modified on transcripts upon environmental stressors at global level, which provides novel understanding of the molecular processes

as to plant stress responses (Kreps et al. 2002; Metzker 2010; Zhao et al. 2021). In this study, our RNA-seq analyses revealed that in total of 3126 genes displaying significantly modified expression (i.e., 1650 upregulated and 1476 downregulated) in the drought-challenged lines with TaMIR5062 overexpression. That the DE genes are categorized into diverse GO terms referred to as “*biological process*”, “*cellular components*”, and “*molecular function*” and they are overrepresented by a subset of biochemical pathways suggest the complicate nature in miRNA-mediated plant drought and salt stress response. Based on GO terms enriched and KEGG pathways overrepresented by the drought upregulated DE genes, we depicted a diagram to illustrate plant responses to osmotic stress (i.e., drought) underlying TaMIR5062 regulation (Fig. 9). TaMIR5062 mediates plant drought adaptation to be accomplished through three sequential biochemical pathways: (i) the pathway is constituted by biological process (GO:0008150), cellular process (GO:0009987), and protein folding (GO:0006457); (ii) pathways are consisted of biological process (GO:0008150), signal-organism process (GO:0044699), cellular lipid metabolism (GO:0044255), isoprenoid metabolic process (GO:0006720), which impact on isoprenoid biosynthesis (GO: 0008299); (iii) pathways are composed of biological process (GO:0008150), metabolic process (GO:0008152) modulating cellular metabolic process (GO:0044237), biosynthetic process (GO:0009058), signal-organism metabolic process (GO:0044710), primary metabolic process (GO: 0044238), organic substance metabolic process (GO:0071704), and nitrogen compound metabolic process (GO:0006807); these processes further modulate following biochemical metabolisms, including cellular biosynthesis metabolism (GO:0044249), signal-organism biosynthesis metabolism (GO:0044711), oxidation-reduction metabolism (GO:0055114), lipid metabolic process (GO:0006629), organic substance biosynthesis metabolism (GO:1901576), and orantrogen compound metabolism (GO:1901564), and finally the isoprenoid biosynthesis metabolism (GO:0008299). In addition, for the third pathway, an important one that involves photosynthetic function modulation needs to be mentioned, which is constituted by biological process (GO:0008150), metabolic process (GO:0008152), cellular metabolic process (GO:0044237), photosynthesis (GO:0015979). These metabolism- and biosynthesis-associated biological processes modulated by DE genes comprehensively impact on water retention, ROS homeostasis, and plant osmotic stress response underlying the miRNA regulation

## Conclusion

TaMIR5062 targets six genes that are functional in various biological processes. The transcripts of TaMIR5062 were upregulated upon drought and salt stress conditions. TaMIR5062 conferred plants improved drought and salt adaptations, which are closely associated with its function in modulating water retention capacity and cellular ROS homeostasis. Distinct AE genes, including SOD family member *NtMnSOD1*, CAT family member *NtCAT*, and POD family one *NtPOD9* that are upregulated on expression in the TaMIR5062 lines treated with drought and salt stress. TaMIR5062 modifies transcription of a large set of genes in osmotic stress-challenged plants, which improve the acclimation of plants to stress by modulating biological processes associated with distinct biological pathways (i.e., protein folding) and biosynthesis metabolisms (i.e., photosynthesis and isoprenoid biosynthesis). TaMIR5062 can be a useful

index for evaluation of plant osmotic stress tolerance in *T. aestivum* and one valuable target for molecular breeding of elite crop cultivars for resistance of drought and salt stresses.

## Declarations

### Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31872869) and Science and Technology Planning Project of Hebei Province (Local Scientific Research Project supported by Chinese government, No. 216Z6401G).

### Author contributions

Kai Xiao and Chengjin Guo designed the research. Yuanjinzhi Qiao, Ling Wang, Zidi Yu, Chenyang Ni, and Tiaojiang Li conducted the experiment and performed data analysis. Kai Xiao wrote the paper.

### Compliance with ethical standards

**Conflict of interest** All authors declare that they have no conflict of interest.

### Data availability statement

All data generated or used during the study appear in the submitted article.

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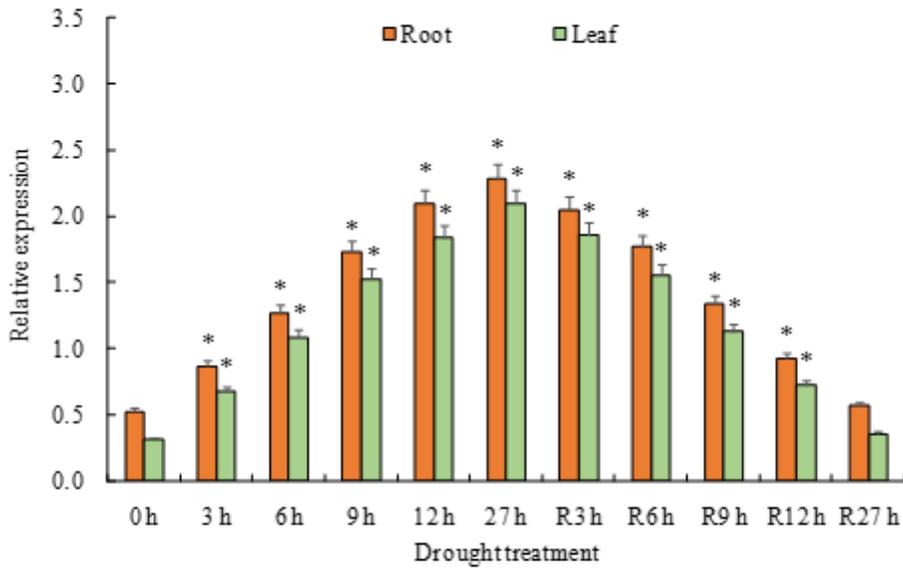
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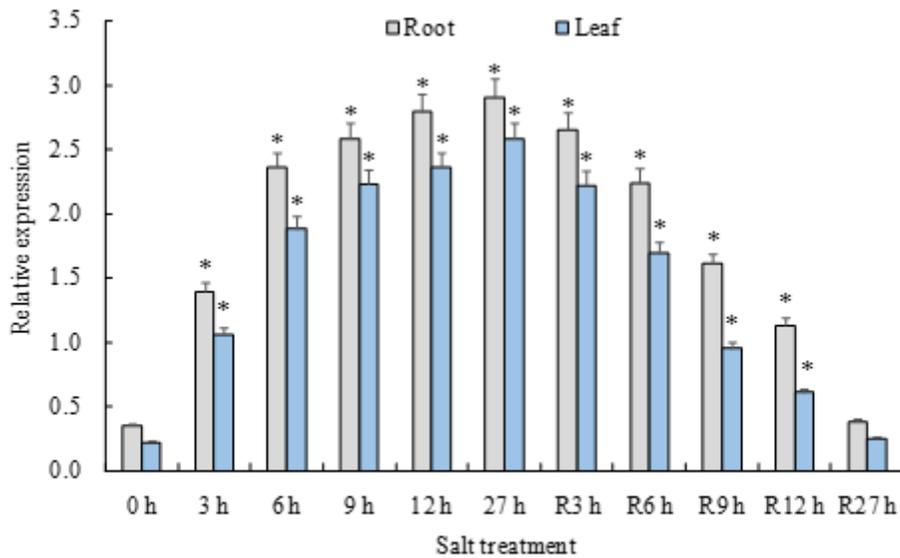
## Figures



**a**



**b**

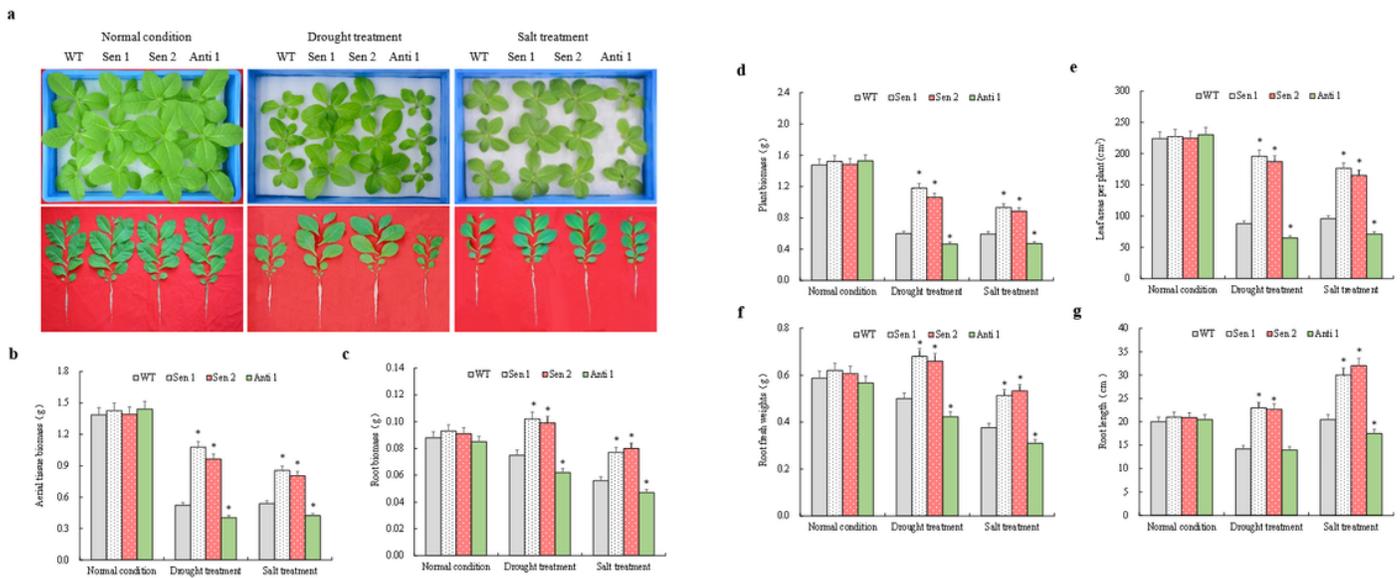


**Figure 2**

Expression patterns of TaMIR5062 upon drought and salt stress treatments

**a**, relative expression under drought stress condition; **b**, relative expression under salt stress condition. 0 h, time prior to treatment; 3 h, 6 h, 9 h, 12 h, and 27 h, time points during drought and salt treatments; R3 h, R6 h, R9 h, R12 h, and R27 h, time points during the normal recovery treatment. Data are normalized by

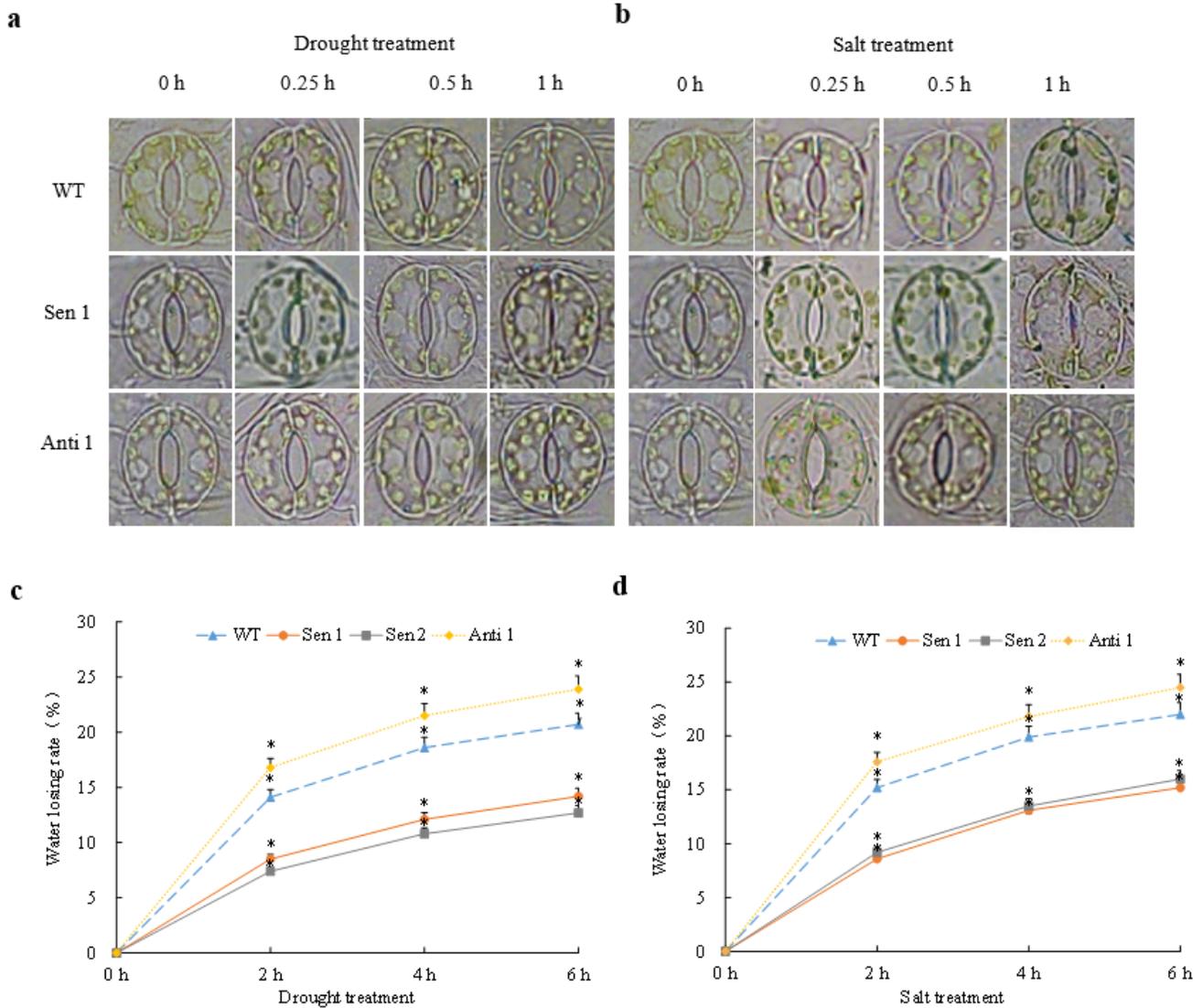
internal standard *Tatubulin* and shown by averages plus the standard errors with symbol \* to represent statistically significant at indicated times with respect to 0 h ( $P < 0.05$ ).



**Figure 3**

Phenotypes and growth traits in transgenic lines of TaMIR5062 under drought and salt treatments

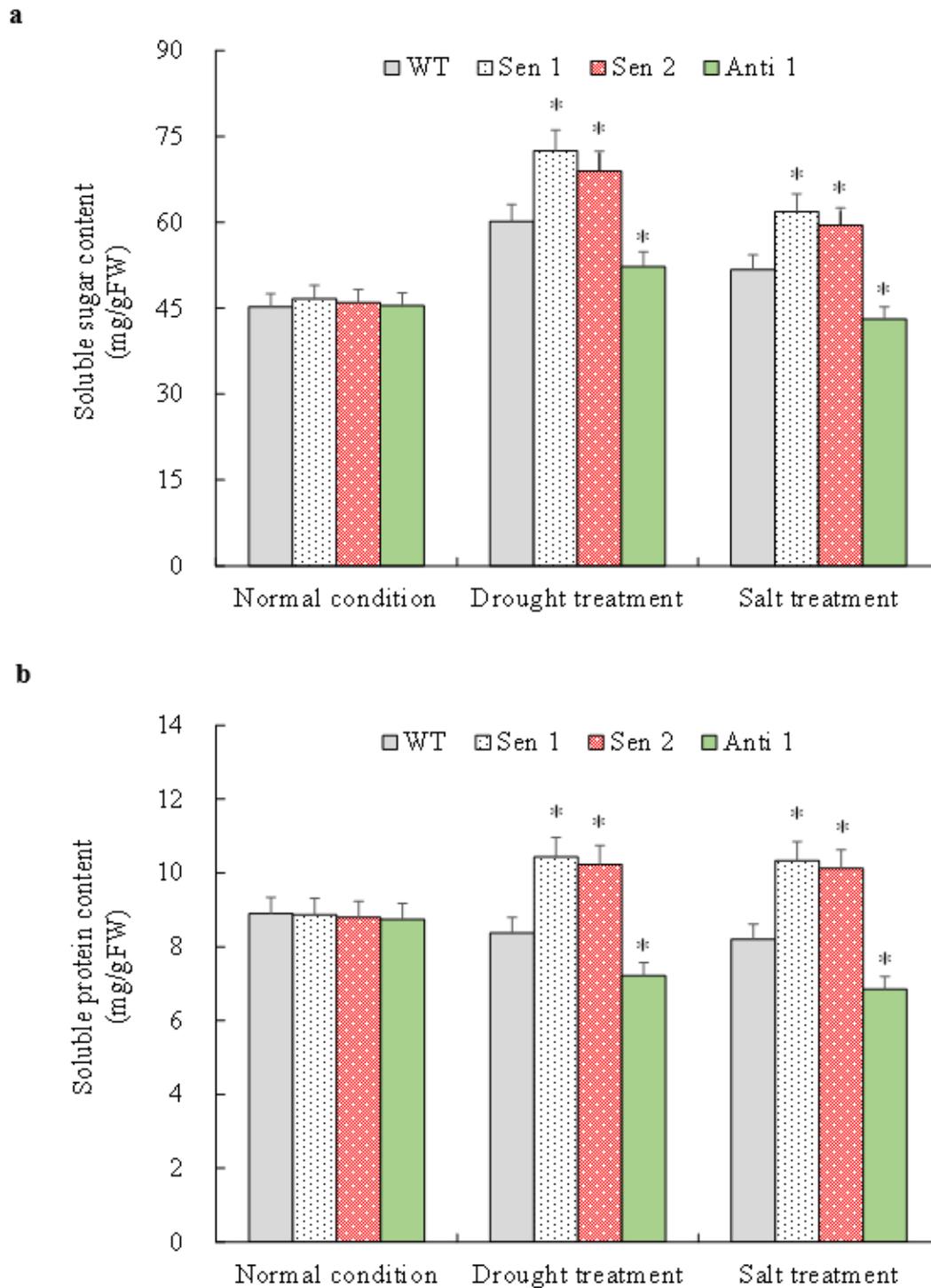
**a**, phenotypes under normal condition, drought, and salt stress conditions; **b**, aerial tissue biomass; **c**, root biomass; **d**, plant biomass; **e**, leaf areas; **f**, root fresh weights; **g**, root lengths. WT, wild type; Sen 1 and Sen 2, two lines overexpressing TaMIR5062. Anti 1, one line with knockdown expression of TaMIR5062. In **b** to **g**, data shown are average plus standard error with symbol \* to represent statistically significant compared with WT ( $P < 0.05$ )



**Figure 4**

Stomata aperture and water retention capacity of leaves in transgenic lines upon drought and salt treatments

**a**, stomatal aperture rates under drought stress condition; **b**, stomatal aperture rates under salt stress condition; **c**, water losing rates under drought stress condition; **d**, water losing rates under salt condition. 0 h, time point for leaves immediately collected; 0.25 h, 0.5 h, and 1 h, time points for leaves detached from plants for water deficit treatment. WT, wild type; Sen 1 and Anti 1, two transgenic lines with TaMIR5062 overexpression and knockdown expression, respectively. In **c** and **d**, data shown are average plus standard error with symbol \* to represent statistically significant compared with WT ( $P < 0.05$ ).

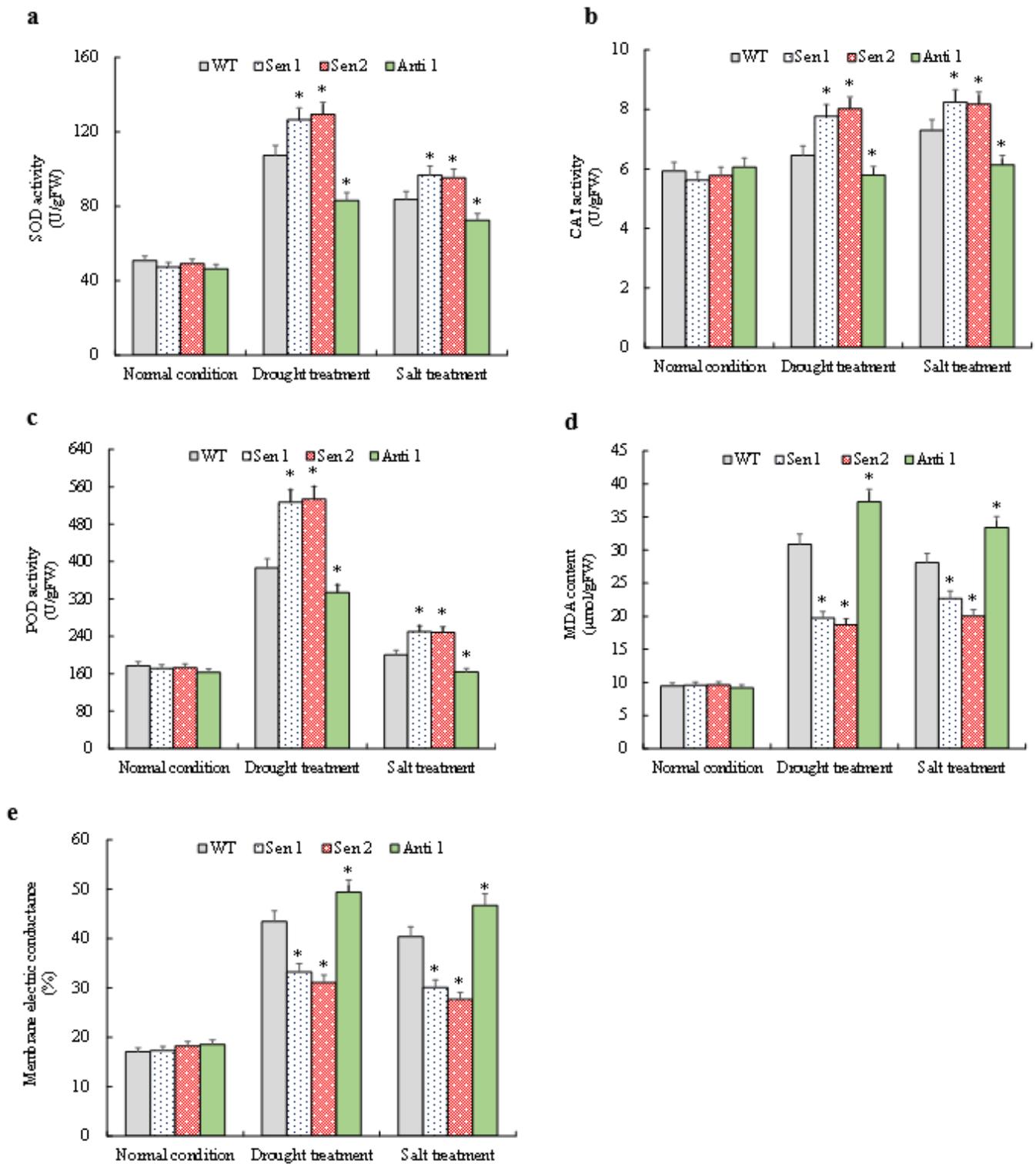


**Figure 5**

Contents of soluble sugar and soluble protein in the transgenic lines under drought and salt treatments

**a**, soluble sugar contents; **b**, soluble protein contents. WT, wild type; Sen 1 and Sen 2, two transgenic lines overexpressing TaMIR5062; Anti 1, one line with knockdown expression of TaMIR5062. Data shown are

average plus standard error with symbol \* to represent statistically significant compared with WT (P<0.05)



**Figure 6**

Cellular ROS-associated parameters and membrane electric conductance in the transgenic lines under drought and salt treatments

**a**, SOD activities; **b**, CAT activities; **c**, POD activities; **d**, MDA contents; **e**, membrane electric conductance. WT, wild type; Sen 1 and Sen 2, two transgenic lines overexpressing TaMIR5062; Anti 1, one line with knockdown expression of TaMIR5062. Data shown are average plus standard error with symbol \* to represent statistically significant compared with WT ( $P < 0.05$ )

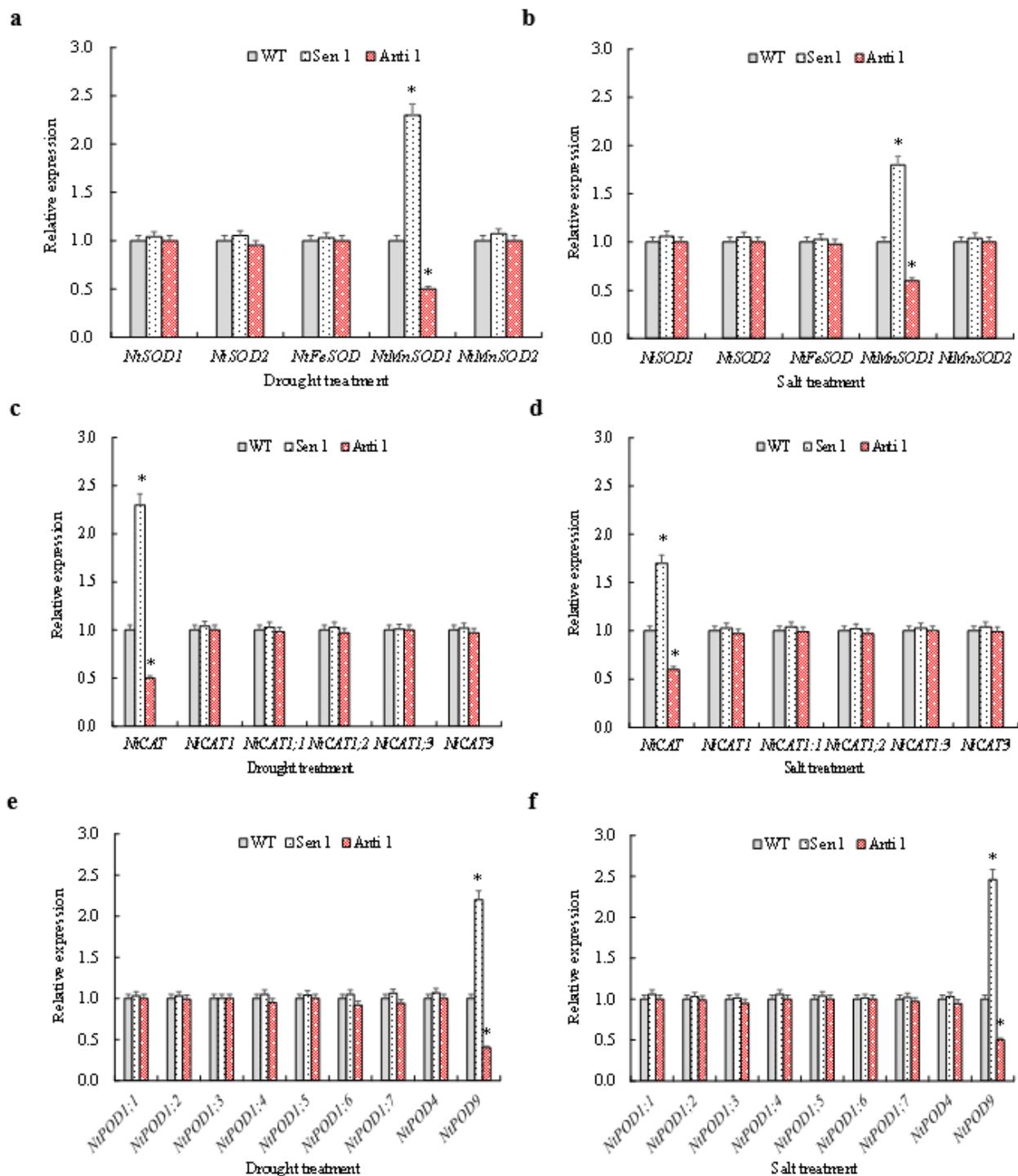
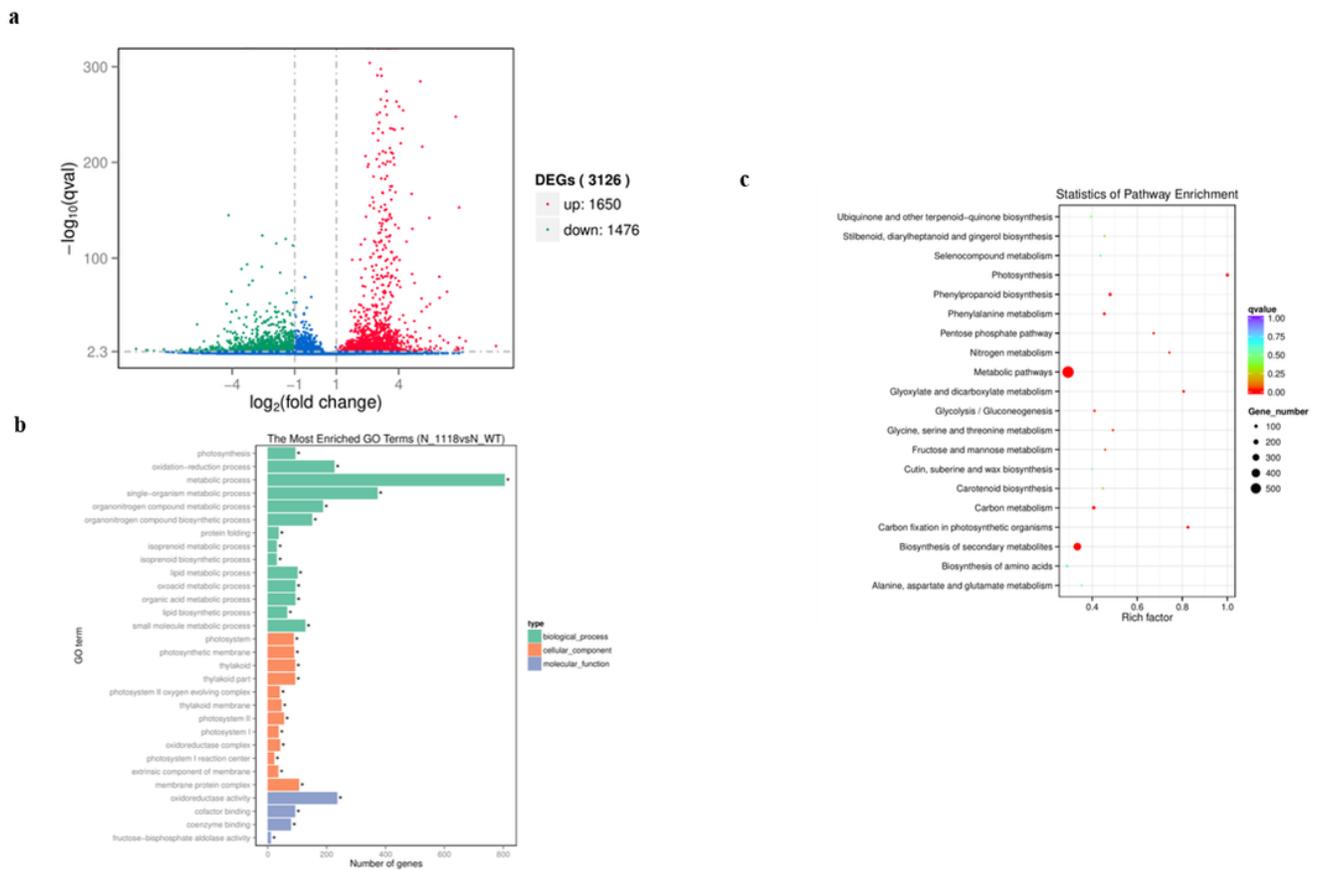


Figure 7

Expression patterns of the genes in antioxidant enzyme families in the transgenic lines under drought and salt treatments

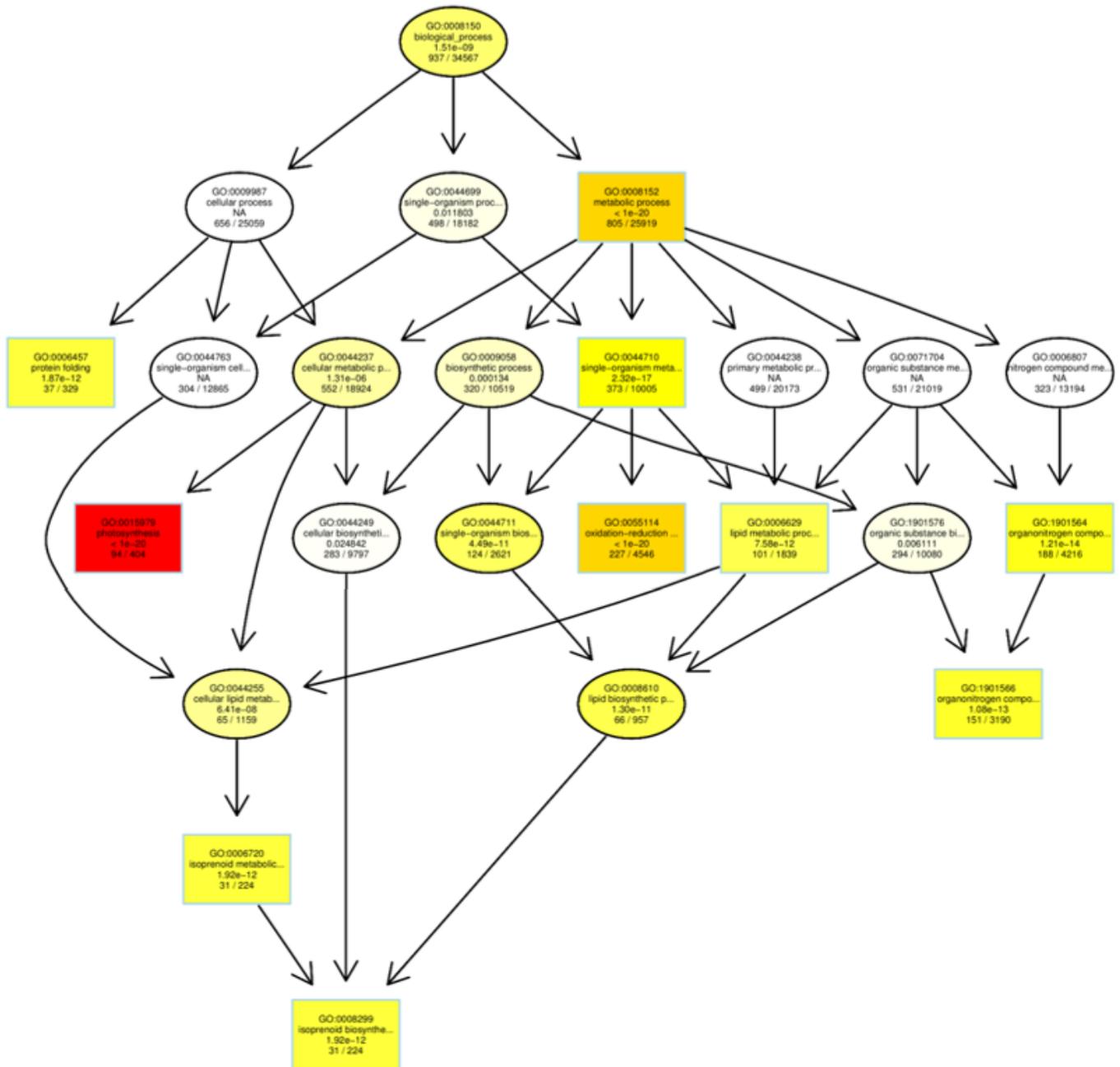
**a**, transcripts of SOD family genes under drought condition; **b**, transcripts of SOD family genes under salt condition; **c**, transcripts of CAT family genes under drought condition; **d**, transcripts of CAT family genes under salt condition; **e**, transcripts of POD family genes under drought condition; **f**, transcripts of POD family genes under salt condition. Data shown are average plus standard error with symbol \* to represent statistically significant compared with WT ( $P < 0.05$ )



**Figure 8**

Characterization of the differentially expressed genes in drought-challenged transgenic lines

**a**, volcano map showing the upregulated and downregulated DE genes; **b**, GO terms over represented by the upregulated DE genes; **c**, Biochemical pathways enriched by the upregulated DEGs.



**Figure 9**

Putative diagram for biological pathways associated with plant drought response underlying modulation of TaMIR5062

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaldata.docx](#)