



Research article

TaZFP1, a C2H2 type-ZFP gene of *T. aestivum*, mediates salt stress tolerance of plants by modulating diverse stress-defensive physiological processes

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ABSTRACT

Salt stress suppresses plant growth, development, and crop productivity. In this study, we characterized the role of *TaZFP1*, a C2H2 type-zinc finger protein family member of *T. aestivum*, in salt stress tolerance. *TaZFP1* possesses a conserved C2H2 motif (CX₂₋₄CX₁₂HX₃₋₅H) shared by plant ZFP proteins, translocates to the nucleus after endoplasmic reticulum (ER) assortment, and displays a ZF 3-D structure similar to its eukaryote homologs. The transcripts of *TaZFP1* were upregulated during salt stress condition and this effect was restored under normal conditions. Compared to wild type (WT), the transgenic lines of *TaZFP1* overexpression or knockdown displayed improved phenotypes, biomass, photosynthesis parameters (Pn, ΨPSII, and NPQ), osmolytes contents (i.e. proline and soluble sugar), and enhanced antioxidant enzyme (AE) activity following salt stress treatment. A set of genes associated with proline synthesis (i.e., *NtP5CS1* and *NtP5CS2*) and encoding AEs (i.e., *NtSOD2*, *NtCAT1*, and *NtPOD4*) were upregulated in the salt-challenged transgenic lines of *TaZFP1* expression. Additionally, the transgenic lines exhibited similar stomata movement patterns and leaf water retention properties under salinity conditions compared to those induced by exogenous abscisic acid (ABA) treatment, suggesting that the *TaZFP1*-mediated salt response is dependent on the ABA signaling. High throughput RNAseq analysis revealed significant alteration of gene transcription in transgenic lines upon salt stress. Among them, the differentially expressed genes (DEGs) represented by the gene ontology (GO) terms were associated with organic acid, carboxylic acid, carbohydrate, and coenzyme as well as organonitrogen compounds, translation, peptide metabolism, and peptide biosynthesis. A set of upregulated DEGs were found to be thylakoid- and photosystem-associated, which is consistent with the *TaZFP1*-mediated improvement in photosynthesis in salt-stressed transgenic lines. Our investigation indicated that the *TaZFP1*-mediated salt tolerance is ascribed to the regulation of gene functions related to photosynthesis, osmolytes metabolism and ROS homeostasis mediated by ABA signaling.

1. Introduction

Salt stress is one of the main factors that limit plant growth and development, which negatively affects crop productivity (Zhou et al., 2018). Over the past two decades, secondary salinization of large areas of arable soils in the semi-arid and arid regions due to irrigation and unsuitable management has resulted in drastic reduction of yields from cultivated crops which has posed a threat to the sustainable crop production (Munns and Tester, 2008; Flowers, 2004; Farooq et al., 2017).

Dissection of the molecular mechanisms underlying plant salt stress response may help improve salt tolerance in crops. So far, it has been shown that the salt stress response pathway regulated by the salt overly sensitive (SOS) genes is essential to the maintenance of cellular ion

homeostasis under salt stress state. The secondary messenger calcium triggers the activation of plasma membrane Na⁺/H⁺ antiporter SOS1 by the SOS3-SOS2 complex, there extruding excess sodium ions from the cytosol (Zhu, 2001; Han et al., 2014; Wang et al., 2018). Moreover, distinct mitogen-activated protein kinase (MAPK) cascades and the other protein kinase members are involved the salt stress sensing or signaling in plants via the abscisic acid (ABA)-dependent or -independent pathways, which modulate the biological processes associated with salt stress response (Han et al., 2014).

Transcription factors (TF) function as the key regulatory components of the salt stress signaling pathway (Schmidt et al., 2012; Pang and Wang, 2008). The expression of several members of the TF family, such as HSF, WRKY, zinc-finger, bZIP, MYB, and AP2/ERF, have been

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found to be modified under salt stress condition (Miao et al., 2004; Nishizawa et al., 2006; Davletova et al., 2005; Sun et al., 2010; Yun et al., 2010), suggesting that they are involved in the salt signal transduction and salt stress adaptation.

Zinc finger proteins (ZFP) belong to one of the largest plant TF families. According to the number and arrangement of cysteine (C) and histidine (H) groups as well as their interaction properties with the zinc finger. ZFP are categorized into various subfamilies, such as Cys2/His2-type (C₂H₂), C₃H, C₂C₂, C₃HC₄, C₂HC₅ (Miller et al., 1985; Klug, 2010). Among the subfamilies, C2H2, also known as the TFIIIA-type finger based on its typical feature, CX₂₋₄CX₁₂HX₃₋₅H (C represents cysteine, H indicates histidine, X stands for any amino acid residue), contains two antiparallel β hairpin and one α helix, in which, two pairs of the conserved cysteine and histidine residues combine tetrahedrally with a zinc ion (Sakamoto et al., 2000; Kodaira et al., 2011).

C2H2 members of the ZFP family play critical roles in regulating various plants physiological processes, such as growth, development, and osmotic stress responses (Sakamoto et al., 2000; Xu et al., 2007; Kodaira et al., 2011). For example, Arabidopsis *Zat10* acts as a regulator of high salinity adaptation and is responsible for increased osmotic and salt stress tolerance in plants (Mittler et al., 2006). Overexpression of *T. hispida* *ThZFP1* in Arabidopsis enhances the activities of peroxidase (POD) and superoxide dismutase (SOD) and promotes osmotic stress adaptation in plants (Zang et al., 2015). Four C2H2 subfamily genes in Arabidopsis, *AZF1*, *AZF2*, *AZF3* and *STZ*, interact with the EP2 motif located downstream of the genes to regulate plant salt response (Sakamoto et al., 2004). Likewise, other molecules related to the AtZat7-mediated salt adaptation associate with the EAR motif. This motif which interacts with the *cis*-acting regulatory elements located downstream of the stress-defensive gene promoters (Ciftci-Yilmaz et al., 2007). Overexpression of rice (*O. sativa*) *OSISAP1* and *OSZFP179* improves plant tolerance to high salinity, low temperature, and dehydration by enhancing oxidative stress response (Mukhopadhyay et al., 2004; Sun et al., 2010). Collectively, these findings suggest that the C2H2 subfamily members play important functions in mediating salt stress response in plants.

Wheat (*T. aestivum* L.) is one of the cereal crops that is globally cultivated. To date, although the physiological and biochemical properties of plant salt stress response has been extensively studied in *T. aestivum* species (Kumar et al., 2017; Abhinandan et al., 2018; Zou et al., 2018), the salt signal transduction mechanisms regulated by the C2H2 subfamily genes are not fully understood. In this study, we characterized the role of *TaZFP1*, a C2H2 subfamily gene of *T. aestivum* (GenBank accession No. AK334138 and its name designated based on its *B. distachyon* homolog), in plant salt stress response. We found that *TaZFP1* was highly responsive to salt stress and improved plant salt tolerance by modulating photosynthesis, osmolytes biosynthesis and cellular ROS homeostasis.

2. Materials and methods

2.1. Characterization of *TaZFP1*

Analysis of sequence similarity between *TaZFP1* and its homologous genes was performed at amino acid level using the MEGA7 software (<https://www.mega.com>). The conserved C2H2 motifs in *TaZFP1* (i.e., CX₂₋₄CX₁₂HX₃₋₅H) were specified as previously described (Sun et al., 2010). The phylogenetic relationships among *TaZFP1* and its plant homologous genes were established at cDNA level using the DNASTar software (<https://www.dnastar.com>).

2.2. Prediction of 3-D structure and analysis of subcellular localization of *TaZFP1*

The three-dimensional (3-D) structure of *TaZFP1* was established based on an online tool referred to as the SWISS-MODEL algorithm

(<https://swissmodel.expasy.org/interactive>), using zinc finger protein 568 (designated as 5wj1.1.C) as the model template. The subcellular localization of *TaZFP1* after it is sorted from the endoplasmic reticulum (ER) was predicted using an online tool referred to as TargetP server 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>). In addition, experiments were performed to determine the location of *TaZFP1*. In the experiments, the open reading frame (ORF) of *TaZFP1* was amplified by PCR using specific primers (Table S1) and a green fluorescence protein encoding gene (*GFP*) was integrated in frame under the control of the constitutive CaMV35S promoter. The expression cassette was then subjected to genetic transformation to *N. tabacum* using *A. tumefaciens* (strain EHA105)-mediated approach. The subcellular localization of *TaZFP1* was determined based on the detection of GFP signals from the transformed tobacco epidermis cells as described by Guo et al. (2013).

2.3. Expression analysis of *TaZFP1*

Shimai 22, an elite high-yielding cultivar of *T. aestivum* introduced in Northern China in recent years, was selected to characterize the expression patterns of *TaZFP1* in response to salt stress. Briefly, wheat seeds were germinated in darkness at 25 °C, and the young seedlings were then cultured hydroponically using standard Murashige and Skoog (MS) solution to the third leaf growth stage. The nutrient solution was air-circulated using a mini pump and renewed twice per week during the culture. Seedlings were cultured under the following conditions: photoperiod of 16 h/8 h (light/dark) with a light intensity of 230 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the light phase, the temperature of 22/20 °C (light/dark), and air humidity ranging from 65 to 75%. At the third-leaf stage, the wheat seedlings were subjected to salt stress treatment by growing in modified MS solution containing 200 mM NaCl. To explore the recovery expression pattern of *TaZFP1*, aliquots of the 27 h-salt challenged seedlings were re-subjected to standard MS solution culture. At 0 h (prior to salt stress), 1, 3, 9, and 27 h during salt stress, and at 3, 9, and 27 h during normal recovery treatment, roots and leaves were collected for qRT-PCR analysis of *TaZFP1* transcripts, using the first-strand cDNA synthesized from total RNA as templates. qRT-PCR analysis was performed in a total volume of 25 μL containing the following constituents: 12.5 μL ExTaq (TaKaRa, Dalian, China), 0.5 μL each of forward and reverse primers, 1 μL cDNA and 10.5 μL of nuclease-free water. *Tatubulin*, a constitutive gene of *T. aestivum*, was used to normalize the expression of the target transcripts. The gene-specific primers used for qRT-PCR are shown in Table S1.

2.4. Generation of transgenic tobacco lines

Transgenic tobacco lines subjected to *TaZFP1* overexpression or knockdown were used to characterize the function of target genes in salt stress response. Briefly, RT-PCR was performed to amplify the ORF of *TaZFP1* in both sense- and antisense-orientation, using gene-specific primers (Table S1). The PCR products were then separately inserted into the *NcoI/BstEII* restriction sites in the binary vector pCAMBIA3301 at a position downstream of the CaMV35S promoter. Genetic transformation of the expression cassettes harboring sense or antisense ORF of *TaZFP1* into *A. tumefaciens* (strain EHA105) and further generation of the tobacco lines were performed as described previously (Sun et al., 2012). The abundance of the transcripts of the target gene in the lines of *TaZFP1* overexpression (Sen 1 to Sen 6) or knockdown (Anti 1 to Anti 4) were evaluated by qRT-PCR assay using gene-specific primers.

2.5. Assays of phenotypes, biomass, and photosynthetic parameters in transgenic lines under salt stress

Three T3 lines with high expression of the target gene (Sen 2, Sen 3, and Sen 5) and one being a transgenic knockdown (Anti 2) were selected to address the *TaZFP1*-mediated salt stress response. Briefly, the

transgenic and wild type (WT) seeds were germinated and cultured in soil mix (half of rich soil and another half of vermiculite) under the following conditions: photoperiod of 14 h/10 h (light/dark) with light intensity of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the light phase, temperature of 28/24 °C (light/dark), and the air humidity ranging from 65 to 75%. At the 5th-leaf stage, the transgenic and WT plants were subjected to normal growth treatment (supplied by standard MS solution) and salt stress treatment (provided by modified MS solution containing 200 mM NaCl). After five weeks of treatments, the transgenic and WT phenotypes were recorded using a digital camera. In addition, the biomass of the transgenic and WT plants was obtained from the oven-dried representative samples. A set of photosynthetic parameters, including photosynthetic rate (Pn), photosystem II efficiency (ΨPSII), and non-photochemical quenching (NPQ), were also assessed using representative leaves as previously described (Guo et al., 2013).

2.6. Assays for proline and soluble contents and ROS-associated parameters

To determine whether the *TaZFP1*-mediated salt stress response was associated with the osmolytes behavior, the contents of proline and soluble sugar that affect plant osmotic regulation in transgenic and WT plants were evaluated after salt treatment as described previously (Du et al., 2013). Cellular reactive oxygen species (ROS) initiated by osmotic stress affects plant stress tolerance. To define the ROS homeostasis status mediated by *TaZFP1*, the activities of a set of antioxidant enzymes (AE), including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), and the contents of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and superoxide anion in the salt-stressed transgenic lines were assessed. The ROS homeostasis-associated parameters mentioned above were determined as described by Huang et al. (2010).

2.7. Analysis of the expression profile of P5CS and AE encoding genes

To explore the effects of the osmolytes and AE encoding genes on proline contents and AE activities in the salt-challenged transgenic lines, qRT-PCR was performed to measure the expression patterns of *NtP5CS1-4*, the delta-1-pyrroline-5-carboxylate synthase (P5CS) encoding genes in *N. tabacum* and a subset of tobacco AE genes, including five SOD genes (i.e., *NtSOD1*, *NtSOD2*, *NtSOD3*, *NtMnSOD1*, and *NtMnSOD2*), six CAT genes (i.e., *NtCAT*, *NtCAT1*, *NtCAT3*, *NtCAT1;1*, *NtCAT1;2*, and *NtCAT1;3*), and eleven POD genes (i.e., *NtPOD1;1* to *NtPOD1;7*, *NtPOD2;1*, *NtPOD2;2*, *NtPOD4*, and *NtPOD9*) in the salt-stressed transgenic lines (i.e., Sen 2 and Anti 2). Gene accession numbers and specific primers used for amplification of the above osmolytes and AE genes are shown in Table S1.

2.8. Assay of stomata closing rate and cell water retention ability

Osmotic stress induces the closing of stomata and modifies the cell water retention ability mainly via an ABA-dependent pathway, thereby protecting plants from stress injury. To understand whether the *TaZFP1*-improved salt tolerance was associated with an ABA-dependent pathway, the stomata aperture and leaf water loss rate (WLR) of transgenic (Sen 2) and WT plants were assessed along the stress progression timeline. To this end, Sen 2 and WT seeds were germinated in darkness. The young seedlings were then hydroponically cultured in standard MS solution to the 5th leaf stage similar to the culture of the wheat seedlings mentioned above. At the 5th stage, the plants were subjected to salt stress treatment (cultured by standard MS solution supplemented with 200 mM NaCl) and ABA treatment (cultured by MS solution containing $2 \mu\text{M}$ ABA). At time points of 0 (prior to treatment) and 0.5, 1, and 3 h after treatments, representative fresh leaves detached from the transgenic line and WT were weighed to calculate leaf WLR as described by Yang et al. (2017). The stomata widths at the time points mentioned above in transgenic and WT under both salt stress and

ABA treatments were recorded as described previously (Pei et al., 1997; Yang et al., 2017).

2.9. High-throughput transcriptome analysis and differential genes identification in transgenic lines

To define the expression profile of genes modulated by *TaZFP1*, high-throughput transcriptome analyses were performed for the salt stress-challenged transgenic lines. Briefly, seedlings from Sen 2 and wild type plants were cultured in standard MS solution for two weeks and then subjected to salt stress treatment (200 mM NaCl) for another one week. Root tissues of the transgenic and WT seedlings were then collected for high throughput RNA-Seq analysis. Briefly, total RNA was isolated using TRIzol reagent (Invitrogen). Three micrograms of total RNA were used to construct strand-specific RNA-seq libraries using procedures described previously with three replicates (Zhong et al., 2011). The RNA-seq libraries were then sequenced on Illumina HiSeq 2500 system using single-end mode. Illumina raw reads were processed using Trimmomatic to remove adaptor and low-quality sequences (Bolger et al., 2014). Reads shorter than 40 bp were discarded. The resulting reads were aligned to *N. tabacum* transcript database (Novogene Co, LTD, Beijing) allowing three mismatches. Differentially expressed genes (DEGs) were identified using edgeR with raw count data (Robinson et al., 2010). Of which, the raw P values were corrected using false discovery rate (FDR) of less than 0.05 (Benjamini and Hochberg, 1995). The genes in Sen 2 showing 2-fold variation relative to WT were regarded as DEGs.

Plant MetGenMap (<http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi>), an online tool for web-based analysis, was used to identify the enriched GO terms of DEGs. The overrepresented (enriched) GO terms were identified based on CPAN pearl module (Boyle et al., 2004) using the hypergeometric distribution to determine the GO term significance. The functional classification of DEGs was established based on GO annotations.

2.10. Statistical analysis

Averages of plant biomass, photosynthetic parameters, osmolytes contents, AE activities, MDA contents, stomata width, and leaf WLR in the transgenic and WT plants were derived from results of three replicates. The upregulated or downregulated DEGs were identified based on triplicate data sets. Standard errors of averages and significant difference tests were analyzed using the Statistical Analysis System software (SAS Corporation).

3. Results

3.1. The molecular characterization of *TaZFP1*

The cDNA sequence of *TaZFP1* is 1612 bp-long in length, encoding a 383-aa long polypeptide (Fig. S1) with a molecular mass of 44.09 kD and isoelectric point (pI) of 7.54. *TaZFP1* contains eight specific C2H2 motifs (each specified by CX₂₋₄CX₁₂HX₃₋₅H) that bind zinc ion and participate in protein-DNA interaction (Fig. S1). At the nucleic acid level, *TaZFP1* shows high similarities to its homologous genes in diverse plant species, with the highest similarities being that of *H. vulgare* (AK374527), *O. sativa* (NM_001052211), *P. trichocarpa* (XM_002325505), and *B. distachyon* (XM_003573216) (Fig. 1). These results suggest a similar evolution pattern between *TaZFP1* and the homologous C2H2 subfamily genes in plants.

The 3-D structure of *TaZFP1* was established using the model target, named mouse ZFP568, based on SWISS-MODEL online tool (Fig. 2A). The assignment coefficients of *TaZFP1* based on TargetP server 1.1 analysis displayed low output scores on cTP (chloroplast transit peptide, 0.086), mTP (mitochondrial targeting peptide, 0.142), and SP (signal peptide, 0.102), and a high output score on other organelles

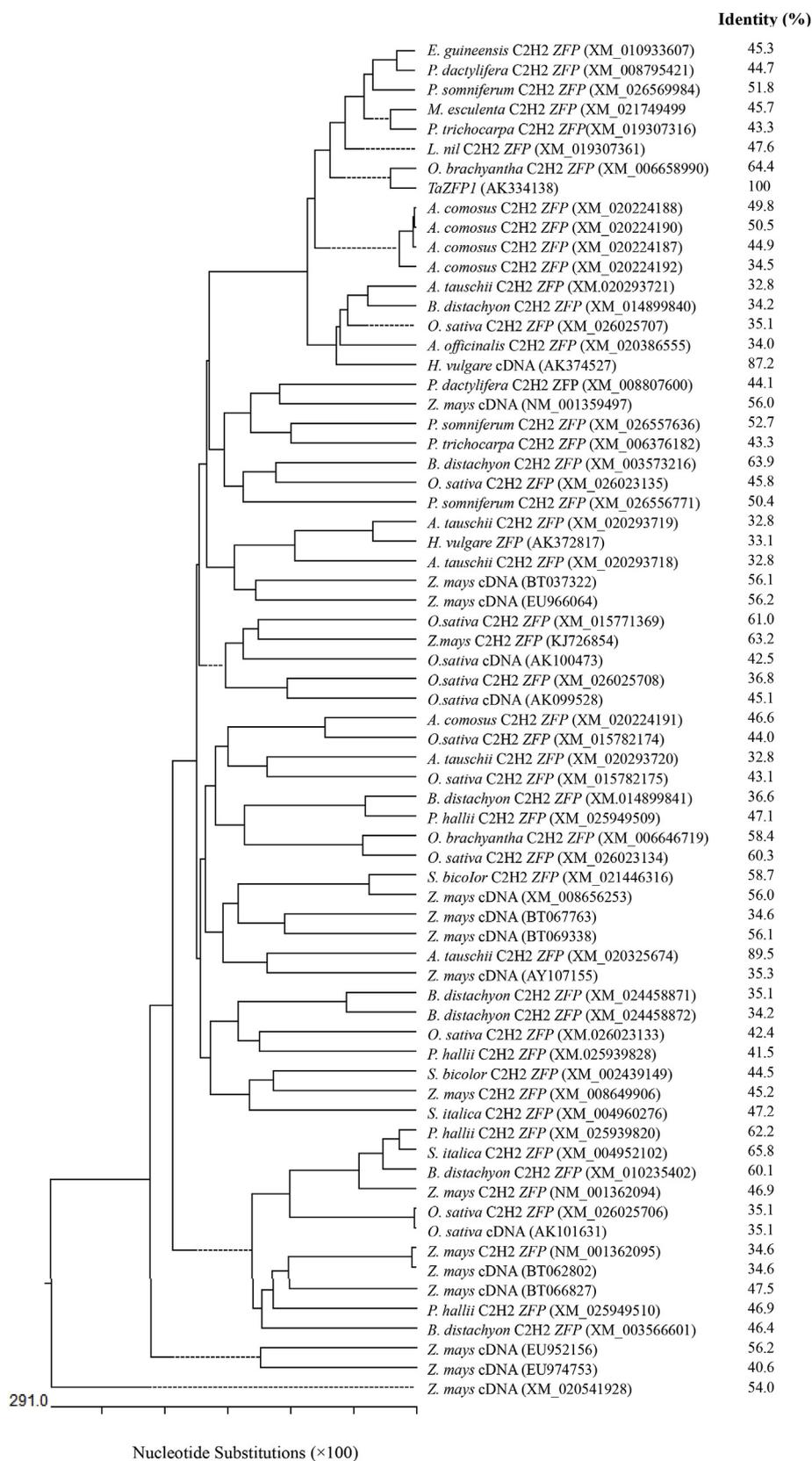


Fig. 1. Phylogenetic relations between *TaZFP1* and its homologous genes from various plant species.

(0.949) (Fig. 2B), suggesting that *TaZFP1* targets many organelles except chloroplast, mitochondrion, and cytoplasm membrane. To experimentally characterize the subcellular location of *TaZFP1*, the GFP signal derived from *TaZFP1-GFP* in transformed *N. tabacum* epidermal

cells was detected. The results indicated that the GFP signals were concentrated at the nucleus (Fig. 2C), suggesting a nuclear localization of this C2H2 member of *T. aestivum* after ER assortment. This finding is consistent with the nature of TF proteins to be functional in the nucleus

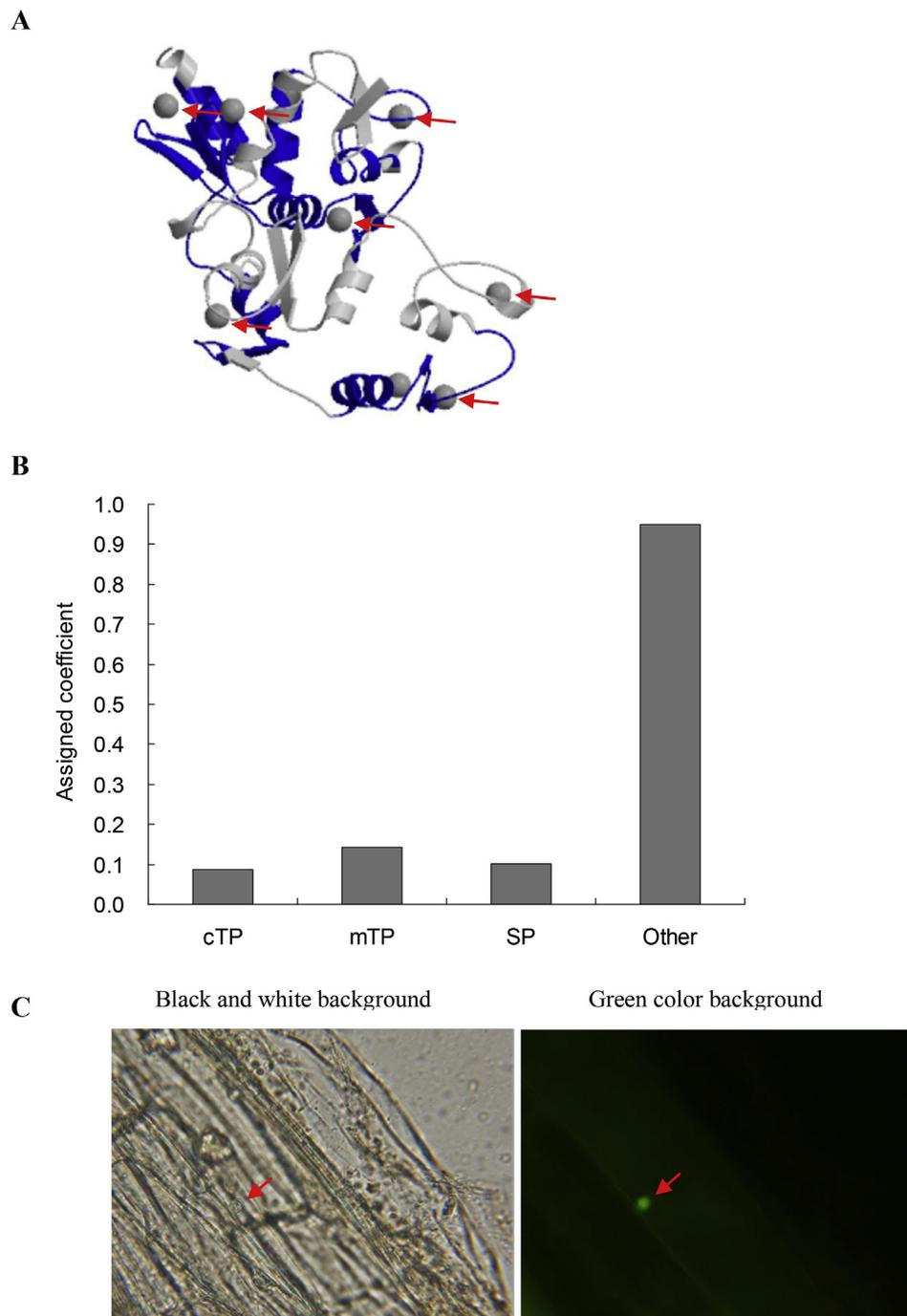


Fig. 2. 3-D structure, predicted subcellular localization, and target defined based on fusion detection of TaZFP1-GFP. **A**, simulated 3-D structure of TaZFP1; **B**, assigned coefficients to various organelles. **C**, subcellular localization of TaZFP1-GFP fusion under fluorescence microscope. In **A**, red arrows point to zinc fingers interacted by eight neighboring C2H2 domains. In **B**, cTP, mTP, and SP represent chloroplast transit peptide, mitochondrion targeting peptide, and signal peptide, respectively. In **C**, arrow points to nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

where they regulate transcription of their target genes.

3.2. The expression pattern of TaZFP1

The expression pattern of *TaZFP1* was found to be associated with salt stress response in roots and leaves of the *T. aestivum* plants. Under normal growth conditions, negligible transcripts of *TaZFP1* were detected in tissues of the roots and leaves. Upon salt stress treatment, the *TaZFP1* expression in both organs was gradually upregulated over a 27 h treatment period, reaching peak values after 27 h of the salt treatment (Fig. 3A). In addition, the high *TaZFP1* transcripts abundance

in tissues caused by salt treatment was gradually decreased over a 27 h normal recovery treatment (Fig. 3B). These results suggested that *TaZFP1* expression is sensitive to external salt signaling.

3.3. TaZFP1 regulates growth and photosynthetic parameters of plants under salt stress

Three T3 lines with *TaZFP1* overexpression, Sen 2, Sen 3, and Sen 5, and Anti 2, a T3 line with target knockdown (Fig. S2), were used to evaluate the plant salt stress response. Under normal growth conditions, the phenotypes of all transgenic lines were comparable to WT (data not

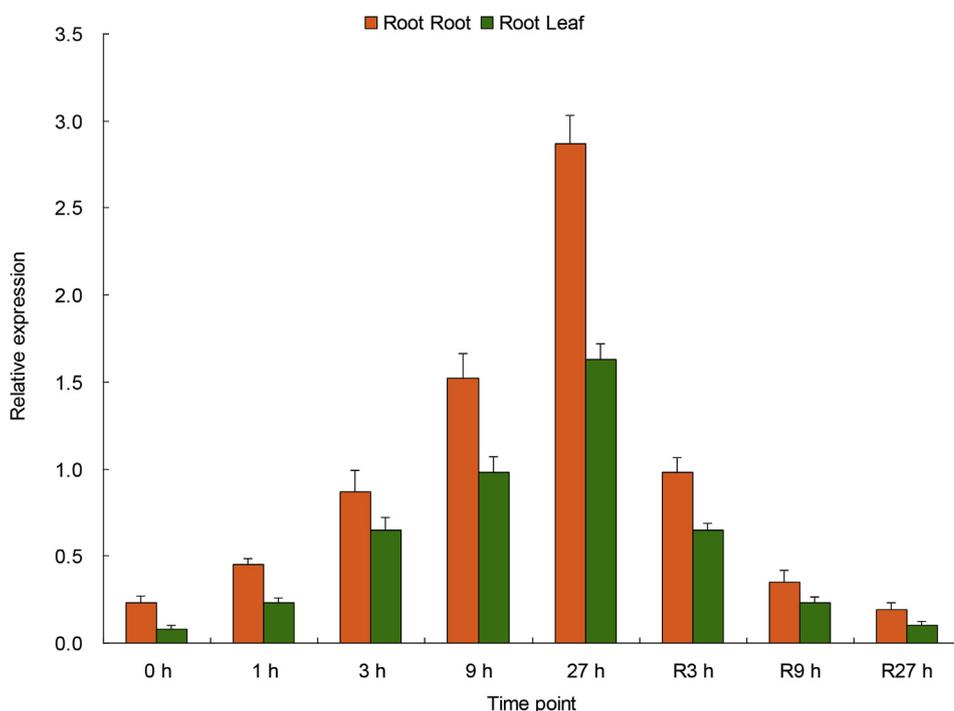


Fig. 3. Expression patterns of *TaZFP1* in roots and leaves upon salt stress and normal recovery condition. 0 h, time point prior to salt stress. 1 h, 3 h, 9 h, and 27 h, time points after salt stress. R3 h, R9 h, and R27 h, time points after normal recovery treatment. Error bar represents SE and symbol * indicates significant differences between transgenic lines and wild type calculated using one-way ANOVA with significance level of 0.05.

shown). However, under salt stress treatment, Sen 2, Sen 3, and Sen 5 showed an enlarged phenotype whereas Anti 2 displayed stunted growth feature with respect to WT (Fig. 4A), suggesting that *TaZFP1* plays a crucial role in regulating plant salt response. In addition, Sen 2, Sen 3, and Sen 5 exhibited increased plant biomass whereas Anti 2 experienced a reduction in dry matter production after salt stress treatment relative to WT. Analysis of the photosynthetic parameters indicated a higher Pn and Ψ_{PSII} , and lower NPQ in Sen 2, Sen3, and Sen 5 but a lower Pn and Ψ_{PSII} , and high NPQ in Anti 2 compared to WT (Fig. 4C–D), suggesting that *TaZFP1* mediates plant salt tolerance by modifying the photosynthetic function that affects plant biomass production upon salt stress.

3.4. *TaZFP1* modulates osmolytes biosynthesis and cellular ROS homeostasis

To explore whether the *TaZFP1*-mediated salt adaptation was associated with alteration in osmolytes synthesis and ROS homeostasis, the contents of proline and soluble sugar, activities of SOD, CAT, and POD, and the amounts of MDA, superoxide anion and peroxide hydrogen (H_2O_2) in transgenic and WT plants were assessed after the salt stress treatment. Compared with WT, the lines with *TaZFP1* over-expression (Sens 2, 3 and 5) exhibited increased proline contents (Fig. 5A), soluble sugar contents (Fig. 5B), activities of SOD (Fig. 5C), CAT (Fig. 5D), and POD (Fig. 5E), and decreased amounts of MDA (Fig. 5F), superoxide anion (Fig. 5G) and H_2O_2 (Fig. 5H). In contrast, Anti 2 displayed decreased proline contents (Fig. 5A), soluble sugar contents (Fig. 5B), activities of SOD (Fig. 5C), CAT (Fig. 5D), and POD (Fig. 5E), and elevated amounts of MDA (Fig. 5F) relative to WT. These results indicated that *TaZFP1*-mediated improvement in salt tolerance is associated with osmolytes biosynthesis and cellular ROS homeostasis, which affect the photosynthetic capacity and biomass production of plants once challenged by salt stress.

3.5. *TaZFP1* modulates the expression of genes related to osmolytes synthesis and AE activities

To determine whether *TaZFP1* modulates the expression of genes involved in osmolytes biosynthesis and ROS in the transgenic lines, the

expression patterns of genes encoding P5CS, a key enzyme catalyzing proline biosynthesis and those encoding AE in the salt-stressed transgenic lines were analyzed. Among the genes examined, two P5CS genes including *NtP5CS1* and *NtP5CS2* and three AE genes including *NtSOD2*, *NtCAT1*, and *NtPOD4* were upregulated in Sen 2 and downregulated in Anti 2 relative to WT (Fig. 6A–D). The expression pattern suggested that P5CS and AE genes modulate proline accumulation and activities of SOD, CAT, and POD in the transgenic lines.

3.6. *TaZFP1* modifies stomata closing and leaf water retention ability in plants

Plant response to osmotic stress is associated with ABA signaling which initiates stress adaptation in plants by modulating stomatal movement and LWR capacity. To understand whether *TaZFP1* influence on salt tolerance is associated with ABA signaling pathway, the stomata aperture and WLR in salt-stressed transgenic (Sen 2) and WT plants were assessed over 3-h regimen involving salt stress and exogenous ABA treatments. Compared with WT, Sen 2 showed enhanced stomata closing rate following salt stress and ABA treatment, with a similar closing pattern under these two treatments (Fig. 7A–C). This finding suggested the involvement of ABA signaling in the regulation of stomata movement in the salt-stressed transgenic lines. Consistent with the stomata closing properties, Sen 2 displayed decreased leaf WLR over the 3-h regimen of salt stress and ABA treatment relative to WT (Fig. 7D). Taken together, these results suggested that *TaZFP1* mediates stomata closing under salt stress via an ABA-dependent mechanism, which influenced leaf WLR and salt stress response-associated biological processes.

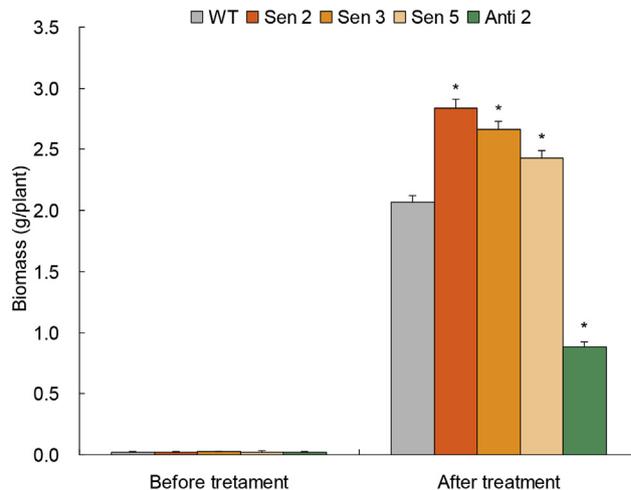
3.7. Transcriptome patterns of salt-stressed transgenic lines

To determine the transcription profile of genes related to *TaZFP1*, high throughput RNA-Seq analyses were performed to globally characterize the DEGs and KEGG pathways regulated by this C2H2 sub-family gene of *T. aestivum*. A total of 2926 genes were identified to be differentially expressed in Sen 2. Of which, 1370 were upregulated and 1556 were downregulated (Data sets S1–S2). These results demonstrated the modulatory role of *TaZFP1* on gene expression at the

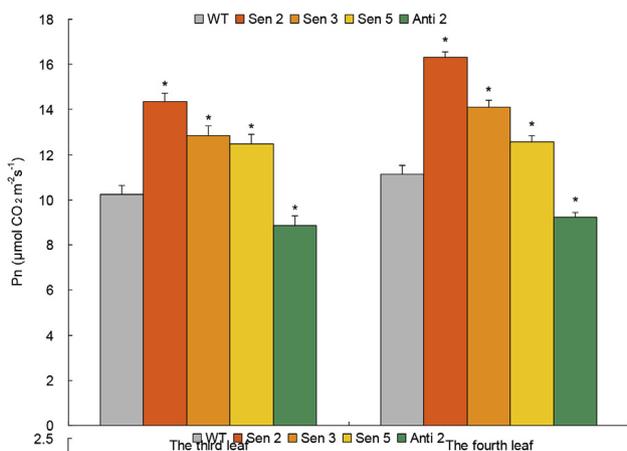
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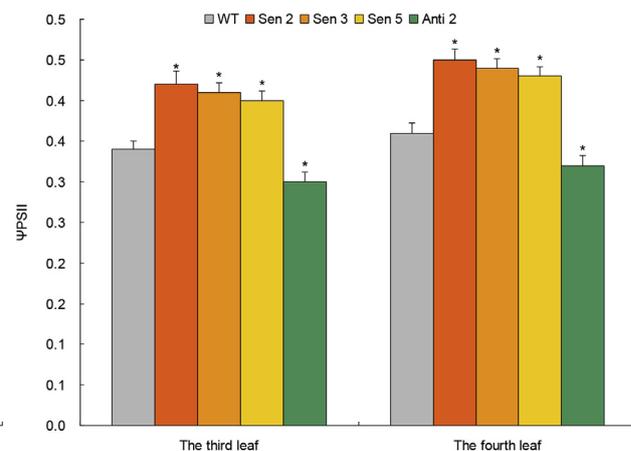
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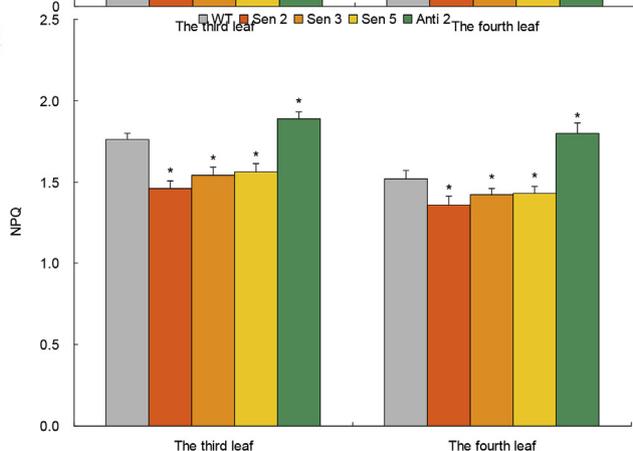


Fig. 4. Phenotypes, biomass, and photosynthetic parameters in *TaZFP1* transgenic lines under salt stress treatment. **A**, phenotypes. **B**, biomass. **C**, photosynthetic rate (Pn). **D**, photosystem II efficiency (ψPSII), **E**, nonphotochemical quenching (NPQ). WT, wild type. Sen 2 and Sen 3, lines with *TaZFP1* overexpression. Anti 2, line with *TaZFP1* knockdown. In **B** to **E**, bar plots represent average values each derived from triplicates. Error bar represents SE and symbol * indicates significant differences between transgenic lines and wild type calculated using one-way ANOVA with significance level of 0.05.

transcription level.

Based on functional characterization, the upregulated DEGs were categorized into two large functional types: (i) biological process and

(ii) molecular function. The DEGs involved in biological process had the following enriched GO terms: metabolic processes of single-organism, small molecule, oxoacid, organic acid, carboxylic acid, oxidation-

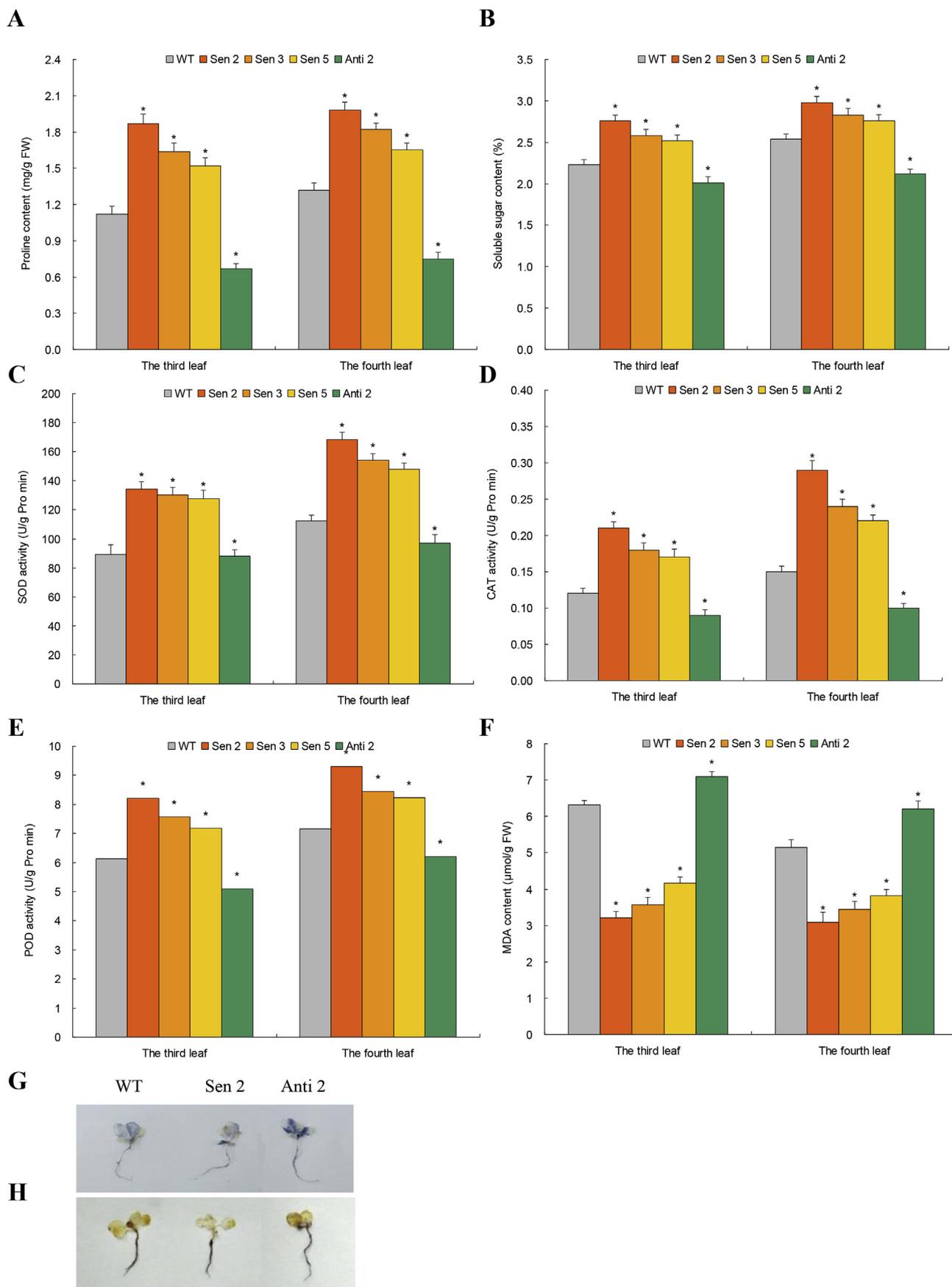


Fig. 5. Osmolytes contents and ROS-associated parameters in *TaZFP1* transgenic lines under salt stress treatment. **A**, proline contents. **B**, soluble sugar contents. **C**, SOD activities. **D**, CAT activities. **E**, POD activities. **F**, MDA contents. **G**, superoxide anion amounts. **H**, H_2O_2 amounts. WT, wild type. Sen 2 and Sen 3, lines with *TaZFP1* overexpression. Anti 2, line with *TaZFP1* knockdown. In **A** to **F**, bar plots represent average values each derived from triplicates. Error bar represents SE and symbol * indicates significant differences between transgenic lines and wild type calculated using one-way ANOVA with significance level of 0.05.

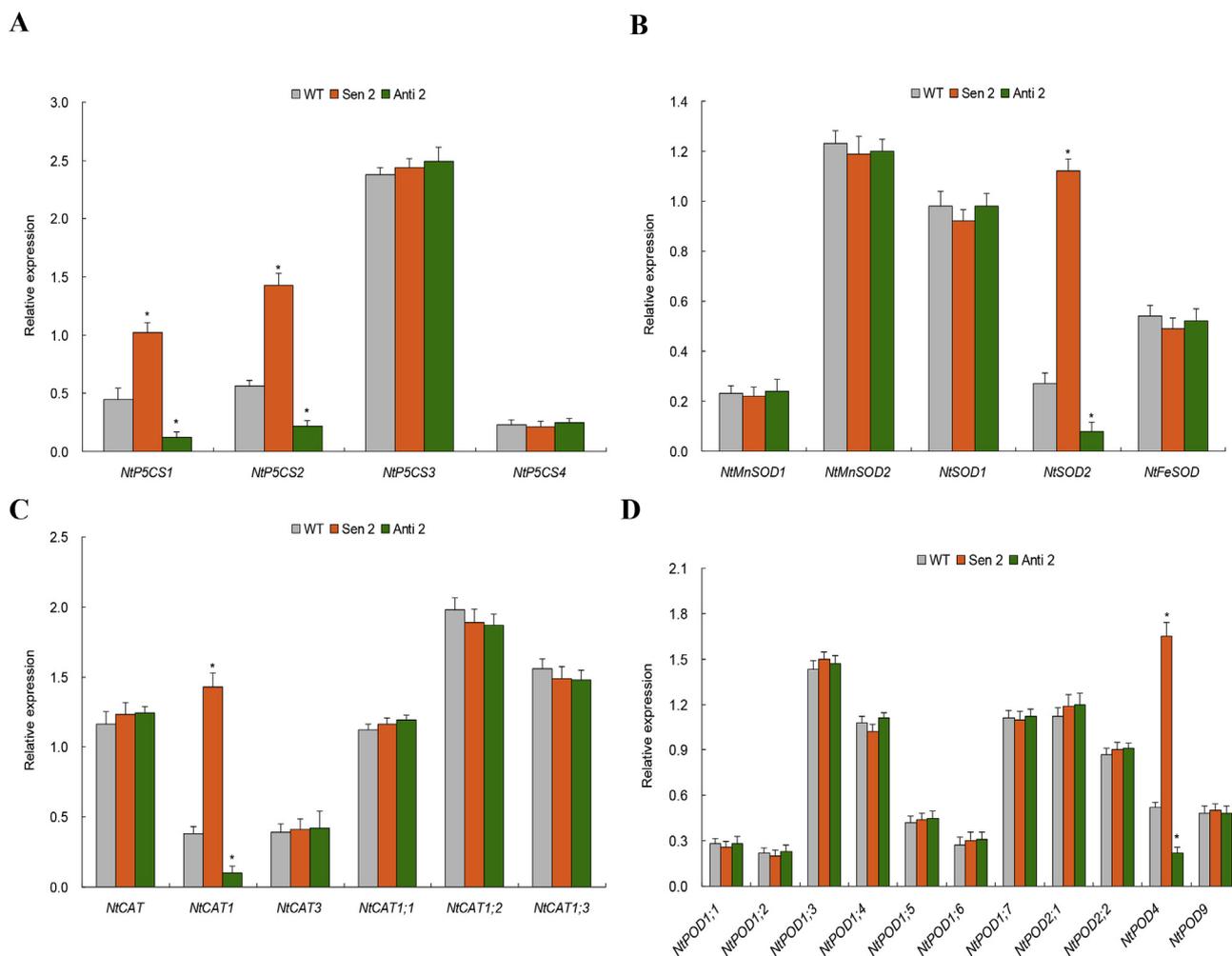


Fig. 6. Expression patterns of genes for delta-1-pyrroline-5-carboxylate synthase and AE proteins in salt stressed *TaZFP1* transgenic lines. **A**, expression patterns of genes for delta-1-pyrroline-5-carboxylate synthase. **B**, expression patterns of SOD genes. **C**, expression patterns of CAT genes. **D**, expression pattern of POD genes. WT, wild type. Sen 2, line with *TaZFP1* overexpression. Anti 2, line with *TaZFP1* knockdown. In **A** to **D**, bar plots represent average values each derived from triplicates. Error bar represents SE and symbol * indicates significant differences between transgenic lines and wild type calculated using one-way ANOVA with significance level of 0.05.

reduction, carbohydrate, coenzyme, carbohydrate catabolism, pyridine-containing compound, organonitrogen compound, monocarboxylic acid, pyridine nucleotide, nicotinamide nucleotide, spermine biosynthesis, spermine metabolism, cellular carbohydrate, and pyruvate. The DEGs involved in molecular function (ii) had the following enriched GO terms: activities of fructose-bisphosphate aldolase, lyase, oxidoreductase, aldehyde-lyase, carbon-carbon lyase, oxidoreductase, glutamate synthase, and adenosylmethionine decarboxylase (Fig. 8A; Data set S3). Likewise, the downregulated DEGs were categorized into three functional types: (i) biological process, (ii) cellular component, and (iii) molecular function. The DEGs involved in biological process (i) had the following enriched GO terms: metabolism associated with organonitrogen compound, translation, peptide metabolism, peptide biosynthesis, amide biosynthesis, cellular amide, organonitrogen compound biosynthesis, biosynthesis, organic substance biosynthesis, cellular nitrogen compound biosynthesis, DNA-template transcription, metabolism, cellular biosynthesis, nitrogen compound, gene expression, cellular macromolecule biosynthesis, cellular nitrogen compound, organic substance, primary metabolism, and nucleoside diphosphate metabolism. The DEGs involved in cellular component (ii) had the following enriched GO terms: ribosome, ribonucleoprotein complex, intracellular non-membrane-bounded organelle, cytoplasmic part, and macromolecular complex. The DEGs involved in molecular function (iii) had the following enriched GO terms: structural constituent of

ribosome, structural molecule activity, and fructose-bisphosphate aldolase activity (Fig. 8B; Data set S4). These results indicated that there was modification of the transcription of genes related to *TaZFP1* involved in diverse biological processes, especially those that modulate growth, biomass production, photosynthesis behavior, osmolytes biosynthesis, and ROS homeostasis in plants during salt stress.

KEGG analysis of the upregulated DEGs revealed the connection between enriched GO terms and salt stress response. As shown in Fig. 9, the network established by enriched GO terms related to the upregulated DEGs revealed the diverse functions of cellular components, including the membrane constituents, macromolecule, and the cell action of plants. Among them, macromolecules associated with the protein complex, catalytic complex, intracellular part, thylakoid, photosynthetic membrane-bound organelle, photosystem (photosystem II and I), and photosystem II oxygen complex, activated each other in a sequential manner (Fig. 9). These results suggested that the modified photosynthetic function in Sen 2 can be mainly ascribed to the DEGs that modulate photosystem establishment, structure, and behavior. KEGG analysis of the downregulated DEGs identified the following biological processes modulated by *TaZFP1*: the cellular components that modify intracellular part and organelle behavior, processes that modulate the cytoplasmic part and intracellular non-bound organelles, processes that modulate ribosome functions and behaviors of the salt stressed transgenic lines (Fig. 10). Further studies are needed to explore

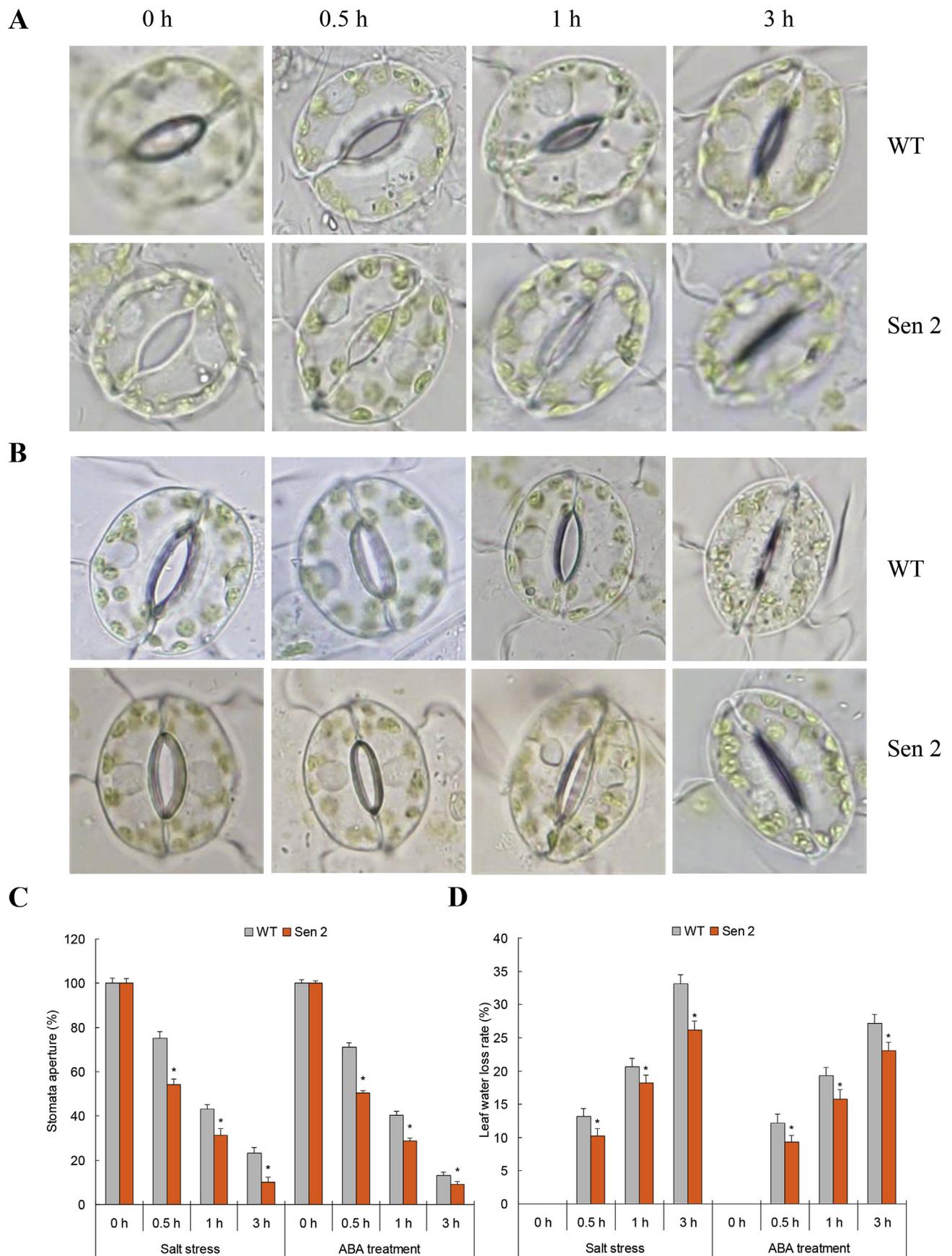
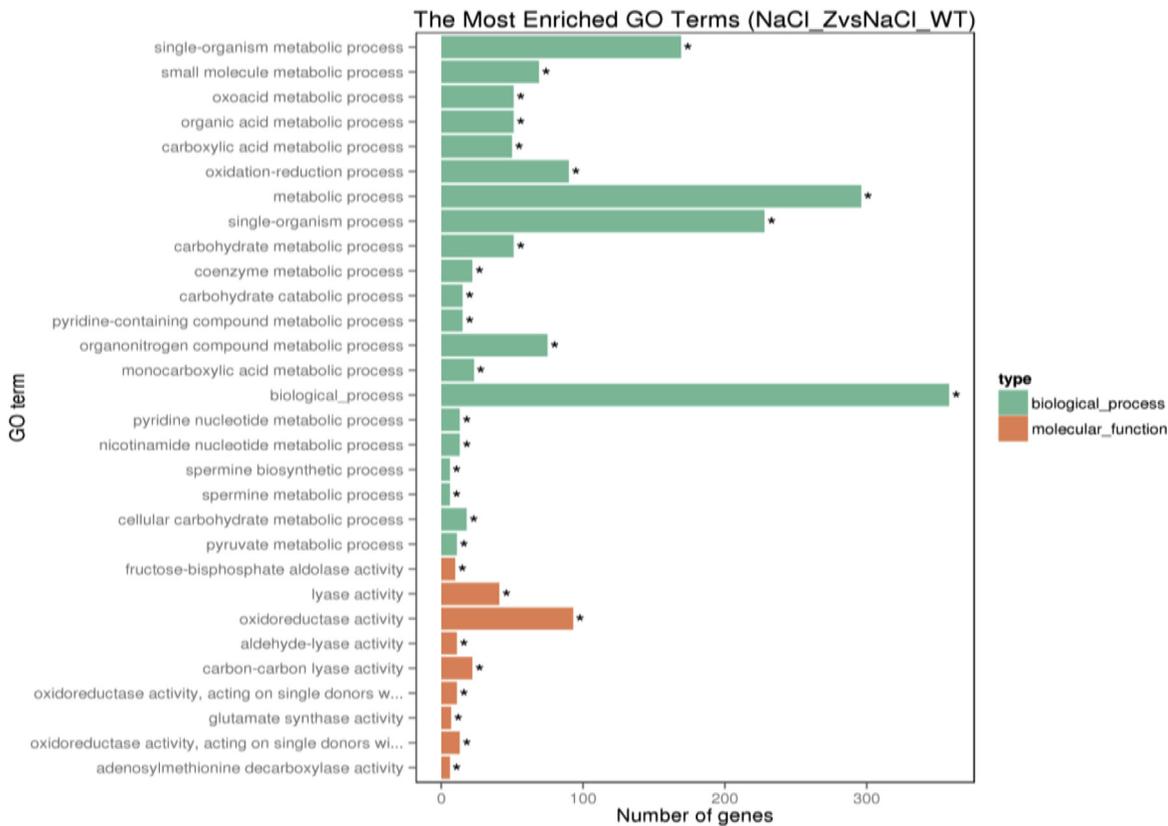


Fig. 7. Stomata movement characterization and leaf water retention ability in salt stressed *TaZFP1* transgenic lines under salt stress and ABA treatments. **A**, stomata aperture properties under salt stress. **B**, stomata aperture properties under ABA treatment. **C**, stomata widths. **D**, leaf water lose rates. 0 h, prior to salt stress and ABA treatments. 0.5 h, 1 h, and 3 h, time points after salt or ABA treatments. WT, wild type. Sen 2, line with *TaZFP1* overexpression.

A



B

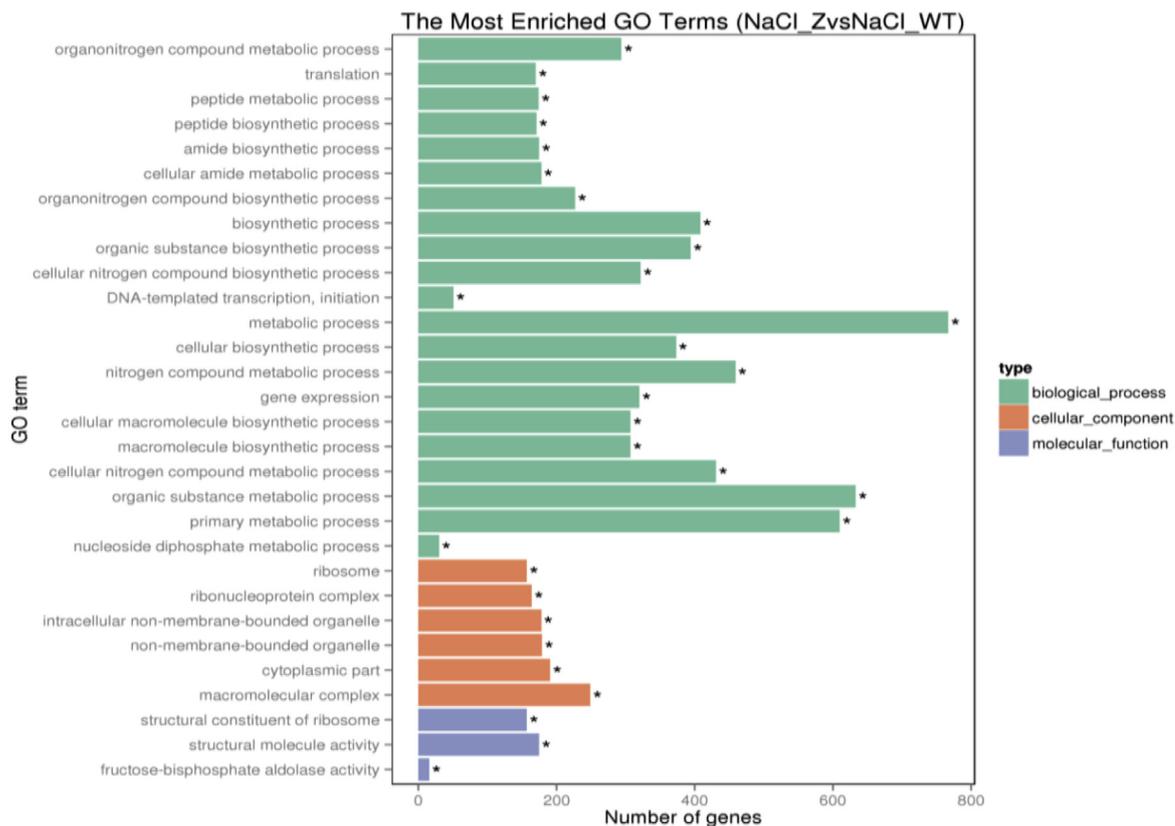


Fig. 8. Functional category groups of enriched GO terms for DEGs in the salt stress-challenged transgenic line with *TaZFP1* overexpression. A, functional category groups for upregulated DEGs; B, functional category groups for downregulated DEGs.

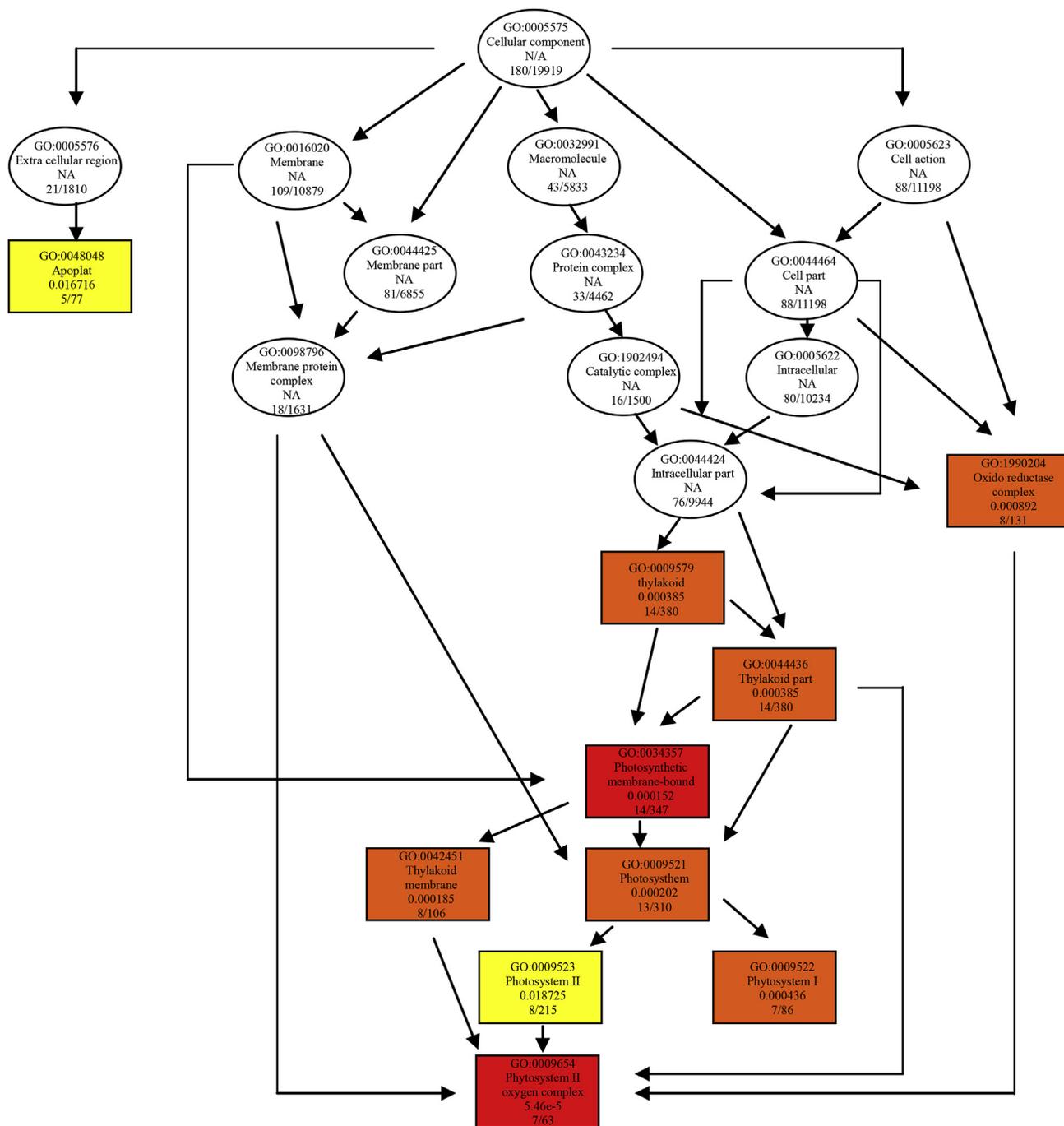


Fig. 9. Concentrated KEGG biological process-associated enriched GO terms for upregulated DEGs in the salt stress-challenged transgenic line with *TaZFP1* over-expression.

the detailed mechanisms by which *TaZFP1* regulates plant salt response and the underlying biological processes.

4. Discussion

Transcription factors (TF) are central mediators in the regulation of transcription of the stress defensive genes of stress adaptation in plants (Chen et al., 2002; Nakashima et al., 2009). A subset of the ZFP family genes, including Arabidopsis *Zat 7* (Ciftci-Yilmaz et al., 2007), *Zat10* (Mittler et al., 2006), *AZF1*, *AZF2*, *AZF3* and *STZ* (Sakamoto et al., 2004), *Tamarix hispida ThZFP1* (Zang et al., 2015), *O. sativa OSISAP1* (Mukhopadhyay et al., 2004) and *ZFP179* (Sun et al., 2010), modulates salt stress response by interacting with the *cis*-acting regulatory

elements of the downstream stress-defensive gene promoters. In this study, we found that *TaZFP1*, a C2H2 subfamily gene of *T. aestivum*, displayed the typical features of this TF subfamily. This is because it contained conserved C2H2 motifs, nuclear localization, and similar 3-D protein structure to ZFP protein. Although the phylogenetic analysis revealed high similarities of *TaZFP1* to a large set of homologous genes in monocotyledonous species (Fig. 1), no homologous genes of *TaZFP1* were identified in the model plant species (i.e., Arabidopsis) based on the NCBI GenBank database using default threshold of low similarity level. This revealed the monocot-specific evolutionary nature of *TaZFP1*.

Members of ZFP family modulate salt stress response in plants by regulating the transcription pattern of specific genes (Sun et al., 2010;

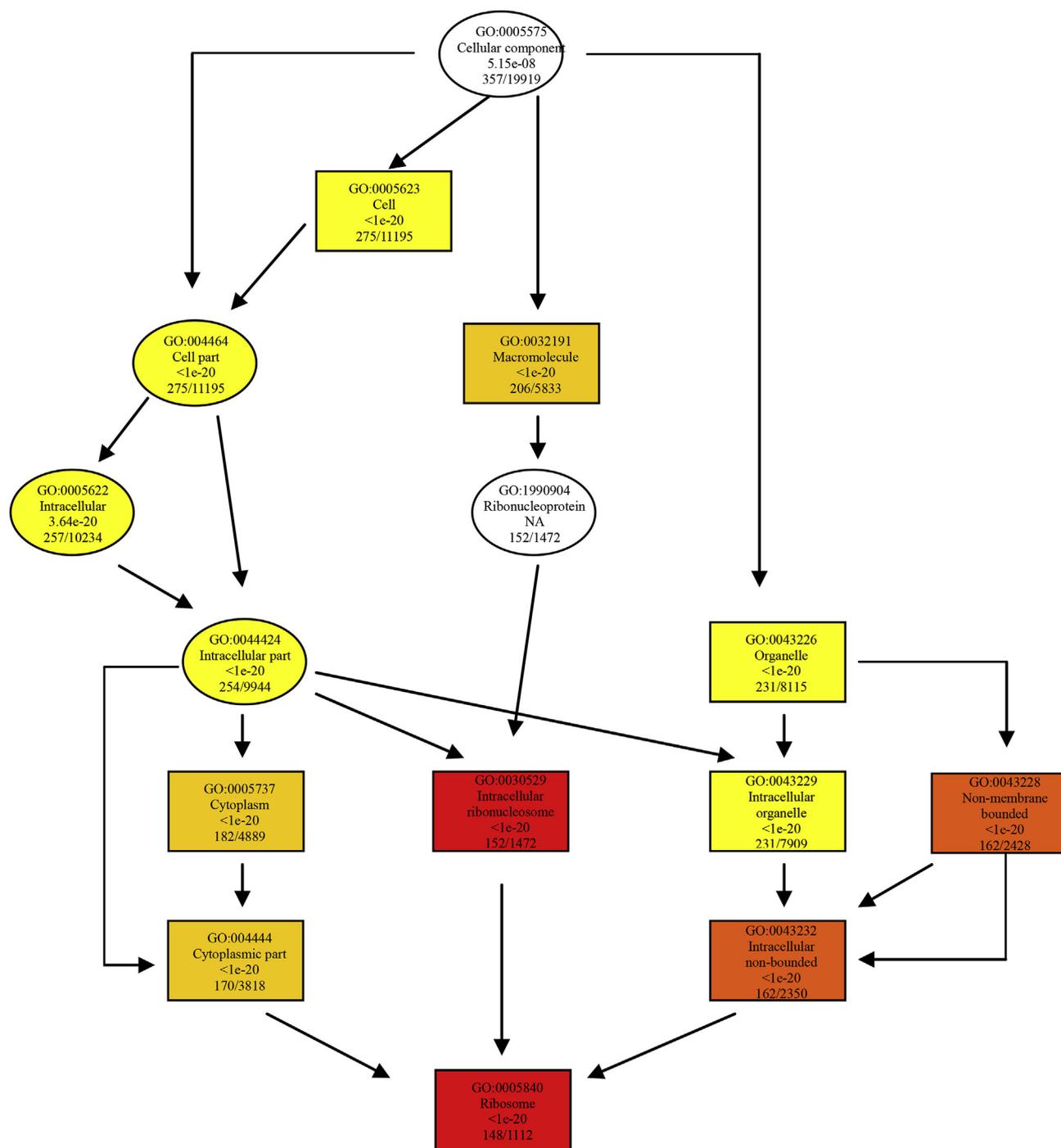


Fig. 10. Concentrated KEGG biological process-associated enriched GO terms for downregulated DEGs in the salt stress-challenged transgenic line with *TaZFP1* overexpression.

Schmidt et al., 2012). In this study, analysis of the *TaZFP1* expression revealed that this C2H2 subfamily gene responds to salt stress in a temporal-dependent manner, indicating its function in salt stress response regulation. Furthermore, transgenic lines i.e. overexpression or knockdown of *TaZFP1* confirmed its positive role in salt stress tolerance regulation. Compared with wild type plants, the *TaZFP1* overexpressing lines exhibited improved phenotype, biomass, and physiological and biochemical processes, including elevated photosynthetic function, osmolytes (i.e., proline and soluble sugar) amounts, and activities of SOD, CAT, and POD together with decreased MDA content, a marker of cellular membrane over-oxidation degree during salt stress condition. Collectively, these results demonstrated that *TaZFP1* modulates salt

stress adaptation via influencing the corresponding physiological processes. Previously, the signaling module mitogen-activated protein kinase (MAPK) cascade was found to associate with distinct TFs during osmotic signaling (Skopelitis et al., 2006; Pang and Wang, 2008), which potentiates plant adaptation to high salinity in various species (Xiong and Yang, 2003; Nakagami et al., 2005; Teige et al., 2004). Whether *TaZFP1* interacts with members of the MAPK cascade to participate in the *TaZFP1*-mediated salt response requires further characterization.

Induced accumulation of osmolytes (i.e., proline and soluble sugar) positively affects the osmotic stress tolerance in plants (Xiong et al., 1999). In addition, accumulated osmolytes, protective solutes, and proteins may also promote plant osmotic stress response (Xiong et al.,

1999; Verslues et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). These induced molecules help stabilize the cellular structure in the presence of abiotic stress (Hoekstra et al., 2001). It has also been confirmed that the transitory starch degradation caused by osmotic stresses is initiated by glucan-hydrolyzing enzymes (including β -amylases [BAM], α -amylases [AMY]) which regulate the levels of osmolytes and stress response in plants (Kötting et al., 2009; Edner et al., 2007; Valerio et al., 2011; Streb and Zeeman, 2012; Zanella et al., 2016). In this study, elevated osmolytes (i.e., proline and soluble sugar) contents were found in the transgenic lines carrying *TaZFP1* overexpression relative to WT following salt stress treatment. Analysis of the expression of P5CS encoding genes revealed that the expression of two genes, *NtP5CS1* and *NtP5CS2*, was significantly increased in the salt-stressed transgenic lines (Sen 2 and Anti 2). Thus, these two genes which regulate proline biosynthesis affect the cellular osmotic potential, membrane stabilization, and protein conformation retention of the salt-challenged transgenic lines.

Osmotic stress induces accumulation of cellular ROS, such as H₂O₂ (hydrogen peroxide), O₂⁻ (superoxide), O₂ (singlet oxygen) and OH (hydroxyl) radicals, causing the leakage of electrons to molecular oxygen (Bowler et al., 1992; Munns and Tester, 2008; Xiong et al., 2003) and oxidative damage of lipids, proteins, and nucleic acids. This finally leads to injury of the plant tissues (Fridovich, 1986; Wu et al., 2018). On the other hand, plants have evolved distinct ROS scavenging mechanisms. SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and POD (EC 1.11.1.7) are critical scavengers of ROS which protects plants from oxidative stress damage (Miller et al., 2010). In this study, analysis of AE activities and ROS levels (i.e., MDA, superoxide anion, and H₂O₂) in the salt-stressed *TaZFP1* overexpression lines indicated that the activities of SOD, CAT, and POD were increased while the level of ROS was decreased relative to wild type, suggesting that improved ROS homeostasis contributes to the *TaZFP1*-mediated salt response. Moreover, analysis of the expression of AE encoding genes revealed that three of them, including *NtSOD2*, *NtCAT1*, and *NtPOD4*, were significantly up-regulated in Sen 2 and Sen 3 compared to WT. This demonstrated that these differentially expressed genes improved the activities of AE and the cellular ROS homeostasis in the salt-stressed transgenic lines. Previously, the ZFP gene-mediated osmotic stress adaptation in plants was attributed to the modifications of genes that alter the activity of AE and ROS homeostasis. For example, *O. sativa ZFP179* improved plant salt tolerance due to enhanced oxidative stress tolerance (Sun et al., 2010). *Tamarix hispida ThZFP1* promoted salt stress adaptation via enhancement of POD and SOD activities (Zang et al., 2015). Further characterization of the cis-regulatory elements in AE gene promoters which interact with *TaZFP1* may provide novel insights into the transcription mechanism of the AE genes.

Stress signaling transduction integrates various biological pathways. Among them, the ABA-dependent signaling pathway plays a central role in modulating stomata closing, LWR capacity, and osmolytes biosynthesis (Xiong et al., 2003; Silva et al., 2018). The biosynthesis of ABA is promoted in root tissues by osmotic stress, leading to the reduction in stomata aperture and decreased leaf water loss rate (Xiong et al., 2003; Mahajan and Tuteja, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007; Acharya and Assmann, 2009; Hao et al., 2011). In the past two decades, the ABA receptor referred to as PYR/PYL/RCAR was identified (Leung et al., 1994; Meyer et al., 1994; Saez et al., 2004; Ma et al., 2009; Park et al., 2009; Lim et al., 2012). The mechanisms of ABA receptor-ABA interaction, as well as its interaction with type 2C protein phosphatases (PP2Cs), such as ABI1, HAB1 and AIP1, inactivation of PP2C located downstream of SnRK2 proteins, and activation of ABF/AREB bZIP family TFs under osmotic stress have been characterized (Umezawa et al., 2009; Santiago et al., 2012; Uno et al., 2000; Antoni et al., 2011; Fujita et al., 2013). In this study, we found that stomatal closing and leaf WLR behavior in transgenic lines under salt stress treatment were comparable with those induced by exogenous ABA treatment. These results suggested that *TaZFP1* mediates plant salt

response via an ABA-dependent mechanism. Further characterization of the ABA signaling components and their interaction mechanisms in *T. aestivum* may boost our understanding on the role of C2H2 subfamily genes in the ABA signaling pathway.

RNA-Seq analysis provides global transcriptional data and novel insights into the molecular processes governing plant stress response (Kreps et al., 2002; Metzker, 2010; Imadi et al., 2015). In this study, based on high throughput RNA-Seq analyses, we identified a total of 2926 DEGs in the *TaZFP1* overexpressing lines, among which 1370 were upregulated and 1556 were downregulated (Tables S1–S2). The upregulated and downregulated DEGs were categorized into various functional groups, such as biological process, cellular component, and molecular function. Further analysis indicated that the DEGs were predominantly high in GO terms associated with metabolic processes of carbohydrate, coenzyme, carbohydrate catabolism, and stress defensiveness. These findings suggested that the *TaZFP1*-modulated salt response is closely associated with the global modification of transcription of genes related to diverse physiological processes. The enriched GO terms from the upregulated DEGs were predominant in the photosystem organelles based on KEGG pathway analyses, confirming that *TaZFP1* improved the photosynthetic functions of plants under salt stress conditions. Altogether, our results indicate that *TaZFP1* regulates transcription of genes related to diverse biological processes, which synergistically regulate plant growth, biomass production, photosynthetic function, osmolytes biosynthesis, and cellular ROS homeostasis in plants during salt stress conditions.

5. Conclusion

TaZFP1 transcripts are responsive to salt stress. *TaZFP1* promotes salt tolerance in plants by improving phenotypes, biomass production, photosynthetic function, osmolytes biosynthesis, and ROS homeostasis. Compared to wild type plants, the stomata closing rate and LWR capacity of the transgenic lines with *TaZFP1* overexpression were enhanced by salt stress and exogenous ABA treatment. Moreover, similar behaviors in terms of stomata closing rate and LWR capacity were observed in the transgenic line under the above treatments, suggesting that the *TaZFP1*-mediated salt stress response was dependent on the ABA signaling pathway. High throughput RNA-Seq analysis indicated that several genes were altered by salt stress in the salt-stressed transgenic lines, with the key enriched GO terms being associated with biological processes, cellular components, and molecular function that participate in salt stress response. In addition, the upregulated DEGs were highly associated with GO terms related to thylakoid and photosystem, which is consistent with the *TaZFP1*-mediated improvement in photosynthetic function. Our findings indicate that *TaZFP1* is essential in salt stress tolerance as it modulates global gene transcription that affects photosynthetic organelles, osmolytes biosynthesis, and ROS homeostasis via the ABA signaling-dependent pathway.

Contributions

Kai Xiao designed the research. Binggao Sun, Yingjia Zhao, Shuya Shi, and Mengya Yang conducted the experiment and performed data analysis. Kai Xiao wrote the paper. All authors contributed to the paper and approved the final manuscript.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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