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GhTZF1 regulates drought stress responses and delays leaf senescence by inhibiting reactive oxygen species accumulation in transgenic *Arabidopsis*

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Abstract Redox homeostasis is important for plants to be able to maintain cellular metabolism, and disrupting cellular redox homeostasis will cause oxidative damage to cells and adversely affect plant growth. In this study, a cotton CCCH-type tandem zinc finger gene defined as *GhTZF1*, which was isolated from a cotton cell wall regeneration SSH library in our previous research, was characterized. *GhTZF1* was predominantly expressed during early cell wall regeneration, and it was expressed in various vegetative and reproductive tissues. The expression of *GhTZF1* was substantially up-regulated by a variety of abiotic stresses, such as PEG and salt. *GhTZF1* also responds to methyl jasmonate (MeJA) and H_2O_2 treatment. Overexpression of *GhTZF1* enhanced drought tolerance and delayed drought-induced leaf senescence in transgenic *Arabidopsis*. Subsequent experiments indicated that dark- and MeJA-induced leaf senescence was also attenuated in transgenic plants. The amount of H_2O_2 in transgenic plants was attenuated under both drought conditions and with MeJA-treatment. The activity of superoxide dismutase and peroxidase was higher in transgenic plants than in wild type plants under drought conditions. Quantitative real-time PCR analysis revealed that overexpression of *GhTZF1* reduced the expression of oxidative-related senescence-associated genes (*SAG*s) under drought conditions. Overexpression of *GhTZF1* also enhanced oxidative stress tolerance, which was determined by measuring the expression of a set of antioxidant genes and *SAG*s that were altered in transgenic plants during H_2O_2 treatment. Hence, we conclude that *GhTZF1* may serve as a regulator in mediating drought stress tolerance and subsequent leaf senescence by modulating the reactive oxygen species homeostasis.

Keywords *GhTZF1* · Drought tolerance · Delayed leaf senescence · Oxidative stress · H_2O_2

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Introduction

Reactive oxygen species (ROS) as unavoidable byproducts of plant metabolism is tightly regulated in organic cells under natural conditions (Gill and Tuteja 2010). The steady state production of ROS is perturbed by a variety of environmental factors, such as drought, salt, and subsequent oxidative stresses (Foyer and Noctor 2005). Drought stress may disturb the redox homeostasis and even lead to oxidative stress, which could cause leaf wilting, cell membrane damage, and, eventually, premature leaf senescence under severe conditions due to excessive ROS generation (Khanna-Chopra and Selote 2007). The influence of drought stress upon leaf physiology could be mediated by the enhanced formation of toxic reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), and hydroxyl radicals (HO^-), which could cause membrane lipid peroxidation, protein degradation, and nucleic acid damage (Irigoyen et al. 1992; Khanna-Chopra 2012; Munné-Bosch et al. 2001). However, plant cells contain an array of protective and repair systems that alleviate the occurrence of oxidative damage. These protective systems include enzymes that react with the active forms of oxygen to detoxify O_2^- and H_2O_2 to prevent the formation of HO^- and enzymes that regenerate oxidized antioxidants to remove H_2O_2 from different cellular

compartments using the ascorbate—glutathione pathway (Porcel et al. 2003).

Leaf senescence is an oxidative process involving ROS outburst, which leads to lipid peroxidation and membrane leakiness and, eventually, cell death (Guo and Gan 2005; Khanna-Chopra 2012). Leaf senescence is also closely correlated with resistance to oxidative stress (Kurepa et al. 1998; Navabpour et al. 2003). The initiation and progression of leaf senescence could be influenced by internal and external environmental factors, such as drought and a variety of plant hormones (Guo 2013; Lim et al. 2003, 2007). Jasmonates (JAs) are known to play a role in promoting the process of leaf senescence and to interact closely with redox processes (He et al. 2002; Shan and Liang 2010). There is a link between delayed leaf senescence and drought adaptation (Rivero et al. 2007; Valente et al. 2009; Yan et al. 2004). Overexpression of an ER-resident molecular chaperone BiP (binding protein) in soybeans and tobacco conferred drought resistance and showed delayed leaf senescence during drought conditions (Valente et al. 2009). Cotton transformed with an *Arabidopsis* 14-3-3 protein GF14λ demonstrated a 'stay-green' phenotype and improved the water-stress tolerance in moderate drought conditions (Yan et al. 2004). Strategies incorporating the expression of an isopentenyl transferase (*IPT*) gene, which encodes an enzyme that catalyzes the rate-limiting step in cytokinin (CK) synthesis under different promoters to delay leaf senescence and enhance drought resistance, have been applied in many species (Rivero et al. 2007; Zhang et al. 2010). For instance, transgenic tobacco expressing an *IPT* gene driven by a stress- and maturation-induced promoter (P_{SAKK}) showed a suppression of drought-induced leaf senescence, which resulted in outstanding drought tolerance due to enhanced oxidative tolerance (Rivero et al. 2007).

Zinc finger genes constitute a large and diverse gene family, of which an unusual zinc finger family characterized by three cysteines (Cys) followed by one histidine (His) residue ($Cx_{6–14}Cx_{4–5}Cx_3H$, where x represents any amino acid) was defined (Blackshear 2002). CCCH proteins contain one to six CCCH motifs. Tandem CCCH zinc finger (TZF) genes in particular belong to one of the smallest zinc finger families in mammals. *Tristetraprolin* (*TTP*) is a well characterized TZF gene in humans. It is characterized by two identical $Cx_8Cx_5Cx_3H$ motifs separated by 18 amino acids (Carrick et al. 2004; Pomeranz et al. 2011). *TTP* can directly bind to AU-rich elements (AREs) within the 3′-untranslated region of the mRNA encoding tumor necrosis factor-α (*TNF-α*) and granulocyte–macrophage colony-stimulating factor (*GM-CSF*) via its central TZF motif and recruit ARE-mRNA to P-bodies (PBs) for degradation (Carrick et al. 2004; Pomeranz et al. 2010). A genome-wide analysis of CCCH-type zinc finger genes identified 68 *AtC3Hs* and 67 *OsC3Hs* in *Arabidopsis* and rice, respectively. Based on the different amino acid spacing between the Cys and His residues in the zinc fingers and the number of zinc finger motifs, the *AtC3Hs* and *OsC3Hs* were classified into 11 and 8 subfamilies, respectively (Wang et al. 2008). Analysis of several subfamily members in both *Arabidopsis* and rice revealed that there is a plant-unique variant TZF motif $Cx_{7\text{-}8}Cx_5Cx_3H\text{-}x_{16}\text{-}Cx_5Cx_4Cx_3H$. In addition, a stretch of plant-unique conserved domains, containing an uncharacterized CHCH motif ($Cx_5Hx_4Cx_3H$) and two other motifs (SHDWTEC and ARRRDPR), were found upstream of the variant TZF motif. Previous detailed research speculated that genes in this specific subfamily may participate in a variety of stress responses (Pomeranz et al. 2010; Wang et al. 2008).

Molecular functions of *TZF* genes have been well studied in animals. They control a variety of cellular processes via the regulation of gene expression at transcriptional and post-transcriptional levels (Carrick et al. 2004; Stumpo et al. 2004). However, only a few plant CCCH proteins have been functionally characterized in model plants. For instance, *AtTZF1* is involved in both plant developmental and stress responses, and it acts as a positive regulator of abscisis acid (ABA)/sugar responses and a negative regulator of gibberellic acid (GA) responses (Lin et al. 2011; Pomeranz et al. 2010). The seed-specific *SOMNUS* (*AtTZF4*) gene is negatively involved in phytochrome-mediated seed germination by interacting with *PIL5* downstream to mediate ABA and GA metabolic gene expression (Kim et al. 2008). A number of other *TZF* genes in *Arabidopsis* (*AtTZF2*, *AtTZF3*, *AtTZF10*, and *AtTZF11*) regulate responses to drought and salt in plants (Lee et al. 2012a; Sun et al. 2007). *OsDOS*, characterized in rice, is involved in delaying leaf senescence by integrating developmental cues to the JA pathway (Kong et al. 2006). Although a number of CCCH proteins have been identified in plant genomes, their function remains unclear, especially in cotton. Here, we report that *GhTZF1* is involved in the drought stress response. Overexpression of *GhTZF1* in *Arabidopsis* enhanced drought tolerance. Moreover, stress-induced leaf senescence was also delayed in plants with *GhTZF1* ectopic overexpression by negatively regulating ROS production and accumulation.

Materials and methods

Plant materials, growth conditions, and stress treatments

Gossypium hirsutum cv. YZ1 seeds were cultivated in commercially sterilized soil (a complex of soil, peat, and composted pine bark) under natural conditions. Three-week-old seedlings at the stage of one fully expanded euphylla and one bud were used for gene expression analysis in response

to various abiotic stresses or MeJA treatment. The leaves were harvested at different time points after treatment (15 % PEG and 200 mmol L^{-1} NaCl at 1, 3, 6, 12, and 24 h; 0.5 mmol L^{-1} H_2O_2 at 0, 1, and 3 h; 100 μmol L^{-1} methyl jasmonate (MeJA) at 0, 3, and 12 h) for RNA isolation. Meanwhile, three-week-old seedlings grown in normal conditions were sampled as controls. For expression analysis, protoplast isolated from cotton embryonic callus were cultivated in KM8P medium and collected at 0, 3, 6, 9, 12, 24, and 48 h as previously described (Yang et al. 2008). Various tissues (root and stem from 3-week-old wild type seedlings and young leaves, mature leaves, senescent leaves, petals, anthers, and zygotic embryos from mature plants) were harvested and stored at −70 °C until analysis. *Arabidopsis thaliana* ecotype Columbia (Col-0) were grown under standard growth conditions (130 μmolm-2s-1, 22 °C, 16 h light/8 h dark cycle).

Cloning and plant transformation

The *GhTZF1* expression sequence was isolated from a cotton cell wall regeneration SSH library. *GhTZF1* was up-regulated at the early stage of cell wall regeneration (Yang et al. 2008). Using the cDNA of the cell wall regeneration 3 h sample as the template, the full-length sequence was amplified through 5′- and 3′-rapid amplification of cDNA end (5′-RACE and 3′-RACE) following the SMART RACE cDNA amplication kit user manual (Clontech, Mountain View, CA, USA). The gene-specific primers used are as follows: *GhTZF1*-5r-1: 5′-CATGGGCGAACCAACATAA TCCAAATC-3′; *GhTZF1*-5r-2: 5′-AAGCGGACC-CATTTCCTCTCGGACTC-3′; *GhTZF1*-3 s-1: 5′-AATGA ATGGTGCTTGTTCCTGGGGC-3′; and *GhTZF1*-3s-2: 5′-TTCCACACTCCGACCCGGTTTTTGC-3′.

For multiple alignment and phylogenic analysis, the amino acids of *GhTZF1*, 11 CCCH subfamily IX members, *AtC3H14*, *AtC3H15*, *OsDOS*, and *CsSEF1* were aligned using the clustalx program, and maximum parsimony analysis was performed using MEGA4.0 software with the neighbor-joining method used as the default (Tamura et al. 2007; Thompson et al. 1997).

To construct the overexpression vector, the open reading frame (ORF) of 1,089 bp was amplified using a pair of primers (*GhTZF1*-F: 5′-CAAAAATGATGATCGGAGA-3′ and *GhTZF1*-R: 5′-TCATTTCACCAACTCAGATAC-3′) and cloned into PK2GW7.0 (Ghent University). The expression vector was introduced into *Arabidopsis thaliana* (Col-0) by the *Agrobacterium tumefaciens* strain GV3101.

RT-PCR and qRT-PCR

Expression levels were assayed by quantitative real-time PCR (qRT-PCR) or RT-PCR. To determine the expression of *GhTZF1* in cotton and *Arabidopsis* plants, cotton RNA was isolated from the collected samples as previously described (Zhu et al. 2005). *Arabidopsis* total RNA was isolated using Trizol reagent (Invitrogen, USA). The first-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and qRT-PCR was carried out with the ABI Prism 7000 system (Applied Biosystems, Foster City, USA).

For cotton expression analysis, gene-specific primers (*GhTZF1*-RTS: 5′-TCGGTGGCCTTTGATTCTTCT-3′ and *GhTZF1*-RTA: 5′-AGGCAGTAGCACCACAAAATAG AG-3′) were used to analyze *GhTZF1* expression patterns in cotton. *GhUB7* (GenBank Accession Number: DQ116441) was used as an internal standard. Relative changes in gene expression levels were calculated with the $2^{-\Delta CT}$ method as described previously (Schmittgen and Livak 2008). The cycle number at which the transcripts were detectable ($C_{T,\ Target}$) was normalized to the cycle number of *GhUB7* gene detection ($C_{T,\ GhUB7}$), referred to as ΔCT. To investigate the expression of *GhTZF1* and *Arabidopsis* homologue genes in *Arabidopsis* under both normal and drought conditions, RT-PCR analysis was performed and gene-specific primers (*GhTZF1*-RTS: 5′-TCGGTGGC CTTTGATTCTTCT-3′ and *GhTZF1*-RTA: 5′-AGGCAG TAGCACCACAAAATAGAG-3; *AtTZF1*-RTS: 5′-CACA CTCTCCGTCACCGTATCTC-3′ and *AtTZF1*-RTA: 5′-GACGCAGAGACGACGAAAAAGGT-3′; *AtTZF2*-RTS: 5′-CGTCATACAACAATCAAATCGGAG-3′ and *AtTZF2*-RTA: 5′-TCACATAACCAAGTCAGAGACCCA CC-3′; *AtTZF3*-RTS: 5′-GAGCCCTGACAGAGTTGATT CTTTTG-3′ and *AtTZF3*-RTA: 5′-CTCAACGACACGCT CCATTACG-3′) were used. *AtACT2* (At3g18780) was used as an internal standard.

To explore the possible effects in *Arabidopsis* of *GhTZF1* overexpression on oxidative-related and stress-induced senescence triggering genes, *AtRBOHC* (At5g51060), *AtRBOHF* (At1g64060), *AtFSD1* (At4g25100), *AtCAT1* (At1g20630), *AtAPX1* (At1g07890), *AtGPX3* (At2g43350), *AtGPX4* (At2g48150), *AtGPX5* (At3g63080), *AtERD11* (At1g02930), *AtGSTU5* (At2g29450), *AtORE9* (At2g42620), *AtSAG21* (At4g02380), *AtSAG14* (At5g20230), *AtSAG15* (At5g51070), *AtELI3-2* (At4g37990), and *AtACS6* (At4g11280) were used for RT-PCR or qRT-PCR analysis in plants under both control and stress conditions. *AtACT2* (At3g18780) was used as an internal standard. The relative expression was determined using the $2^{-\Delta CT}$ method as described previously (Schmittgen and Livak 2008). The primers used are listed in Table 1.

Drought tolerance assay in transgenic *Arabidopsis*

Homozygous transgenic lines were used to analyze stress tolerance. To test the effects of drought on seed germination,

Table 1 The primers used in this study

Gene	Accession number	Forward primers (5′–3′)	Reverse primers (5′–3′)
AtRBOHC	At5g51060	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
AtRBOHF	At1g64060	GGTGTCATGAACGAAGTTGCA	AATGAGAGCAGAACGAGCATCA
AtFSD1	At4g25100	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
AtCAT1	At1g20630	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
AtAPX1	At1g07890	GGACGATGCCACAAGGATAGG	GACCAAAGGACGGAAAACAGG
AtGPX3	At2g43350	AGGAGGATTGTTTGGGGATGC	TTCAAGAGGTGATGTAGTTGGAGC
AtGPX4	At2g48150	GTAAACGGTCAAAACGCAGCA	CCATAACGATCAATCACTAAGCCAT
AtGPX5	At3g63080	AAGGTTTTGTGGTATTGGCGTT	ACTGGTGCAGCGTTTTGTCC
AtERD11	At1g02930	GCAGGAATCAAAGTTTTCGGTCAC	AAGGGGTTGCGAAGGATGAAAG
AtGSTU5	At2g29450	CTTTTGGGGATATGGGCGAG	CATTGTGGACAAGAACAGGGACT
AtORE9	At2g42620	GACACAATCGGTTTCGCACTG	CTCCTCTGGTTAACATCTCTATCCTG
AtSAG21	At4g02380	ATCGTATCTGCTTTCGTCTCTCG	TTCCACTCCCTTCTTCTTCATCAC
AtSAG14	At5g20230	GGAGGACTACGATGTTGGTGATGA	TGTGGCTAATGGGTTTCTCTTTCT
AtSAG15	At5g51070	ATGTCACCTCCATCGCCGCT	GAACCGTTCGAAAACCGCTG
AtELI3-2	At4g37990	GACTCATTCACTTCTTCCGTTGC	CCTCCTATCATACTTCCCATTACCA
AtACS6	At4g11280	CAAACCCGCTTGGTACGACG	CAAAAGTAGTAGCAGCATAAATCTCATC
AtACT2	At3g18780	TTCCTCATGCCATCCTCCGTCTT	CAGCGATACCTGAGAACATAGTGG
GhUB7	DQ116441	GAAGGCATTCCACCTGACCAAC	CTTGACCTTCTTCTTCTTGTGCTTG

seeds were sown on 1/2 MS medium in the presence of different concentrations of PEG to mimic an osmotic pressure of −0.5 and −0.7 MPa (Verslues et al. 2006). Seeds germinated on 1/2 MS medium (osmotic pressure of −0.25 MPa) were used as the control. The germinating rate was scored. The experiments were conducted in three biological replicates and each replicate represents 60 seeds for each line.

To explore the drought tolerance response of transgenic plants in soil, 5-week-old plants grown under normal water conditions were then deprived of water for 7 days. The relative water contents were then measured. The seedlings grown under normal water conditions were used as controls. The experiments were conducted in three biological replicates and each replicate represents at least 20 plants for each line.

Enzyme activities determinations

For enzyme assays, leaf samples were lyophilized using a Freeze-Dryer (Millrock LD85). The equal lyophilized leaf samples from three independent drought treatments were then extracted with 1.8 mL ice-cold 50 mM phosphate buffer (pH 7.8). The extracts were centrifuged at 4 °C for 20 min at 13,000 rpm min^{-1} and the resulting supernatants were collected and used for enzyme activities.

The total superoxide dismutase (SOD) activity was determined according to the method described by Giannopolitis and Ries (1977). To a 200 μL reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitro blue tetrazolium, 0.1 μM EDTA, 2 μM riboflavin, and 5 μL of enzyme extract was added. The tube was shaken and illuminated with 4,000 lux for 20 min and the absorbance read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate nitroblue tetrazolium chloride reduction. The enzyme activity was expressed as U mg^{-1} dry weight.

The activity of peroxidase (POD) was determined spectrophotometrically at 470 nm using guaiacol as a substrate (Scalet et al. 1995). Assays were performed in 0.2 M phosphate buffer (pH 6.0), 50 mM guaiacol, 2 % H_2O_2, and 2 μl of enzyme extract was added. Peroxidase activity is reported as U mg^{-1} min^{-1} dry weight, which corresponded to the change of absorbance, in 1 min, per milligram of dry weight.

Senescence testing using in vitro leaves

To examine the drought-induced leaf senescence, 5-week-old plants grown under normal water conditions were deprived of water for 7 days, re-watered, and then deprived of water for another 7 days. The plants grown under normal water conditions were used as controls.

To test dark-induced senescence, leaves (the eighth-tenth true rosette leaves) from 4-week-old plants were floated in water in the dark for 7 days, and the chlorophyll content was then used to measure the chlorophyll degradation.

To explore MeJA-induced senescence, leaves (the eighth-tenth true rosette leaves) from 40 days plants were incubated in water (mock) or a solution containing 45 μM MeJA in darkness for 4 days and the chlorophyll content was detected.

H_2O_2 treatment

To calculate H_2O_2-induced senescence, leaves (the tenth-eleventh true rosette leaves) from 4-week-old plants were incubated in water (mock) and a solution containing 10 mM H_2O_2 for 4 days.

To test the oxidative tolerance, seeds were allowed to germinate on 1/2 MS medium containing 0, 1, or 3 mM H_2O_2. The H_2O_2-induced damage was evaluated by measuring the fresh weight (FW). Experiments were conducted in triplicate for each line (30 seeds each).

Measurement of H_2O_2, chlorophyll and MDA content

The amount of H_2O_2 was quantified using a H_2O_2 quantification kit (Sangon Biotech, Shanghai, China) as previous described (Patterson et al. 1984). Briefly, 100 mg fresh leaves from wild type and *GhTZF1* transgenic plants were collected, ground into powder, added to 1.8 ml precooling acetone, shaken for 20 min, and then centrifuged at 4 °C (13,000 rpm min^{-1} for 15 min). The supernatant was then transferred to a new centrifuge tube for further testing. The amount of H_2O_2 was then measured. The H_2O_2 concentration was expressed as micromoles per gram of fresh leaves (μmol H_2O_2 g^{-1} FW). Data were analyzed and a Student's *t* test was used to determine significance.

Chlorophyll was extracted from leaf samples using 10 ml 80 % acetone for 16 h in the dark, and chlorophyll content was measured spectrophotometrically at 652 nm in accordance with a previous study (Arnon 1949).

Malondialdehyde (MDA) was measured as previously described (Fu and Huang 2001). The samples were homogenized in a 10 % (W/V) trichloroacetic acid (TCA) solution on ice. The homogenate was centrifuged for 10 min at 12,000 rpm. The supernatant was collected for a chromogenic reaction. An equivalent volume of 10 % (W/V) TCA containing 0.6 % (W/V) thiobarbituric acid (TBA) and the supernatant were mixed and maintained for 15 min in a boiling water bath and then immediately cooled on ice. The absorbance of the chromogenic reaction mixture was determined at 532 nm and was corrected for non-specific absorbance at 600 nm (the absorbance at 600 nm was subtracted from that at 532 nm), and the amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as μmol g^{-1} fresh weight as previously described.

Statistical analysis

Each graphical plot represents the results from three independent experiments, and the values are mean ± SD. Statistical significance was determined by Student's *t* tests, and *p* values <0.05 were considered statistically significant.

Results

Cloning and sequence analysis of *GhTZF1*

A 393 bp differentially expressed EST (GenBank accession number: EF403655) was identified from a cotton cell wall regeneration SSH library (Yang et al. 2008) with a putative full-length of 1,318 bp and cloned by 5′ and 3′ RACE. The ORF was 1,089 bp, encoding a polypeptide of 362 amino acids with a calculated molecular weight of 40.1 kDa and an isoelectric point of 8.38 (http://web.expasy.org/compute_pi/). A structural analysis revealed that the protein contained typical TZF motifs characterized by $Cx_7Cx_5Cx_3H$ and $Cx_5Cx_4Cx_3H$ motifs (where x represents any amino acid) separated by 16 amino acids and an upstream plant-unique TZF motif $Cx_5Hx_4Cx_3H$ (Fig. 1a). The protein was then designated *GhTZF1*. Phylogenetic analysis of amino acids among *GhTZF1,* 11 *Arabidopsis* CCCH-type zinc finger protein family subfamily IX members, and two *TZF* genes (*AtC3H14* (At1g66810) and *AtC3H15* (At1g68200)), which possess identical TZF domains to those of human *TTPs* (*hTTP*s), revealed that *GhTZF1* was a plant-unique TZF gene that clustered together with *Arabidopsis* subfamily IX member genes and was a homologue of *Arabidopsis AtTZF1* (Fig. 1b). Comparison of the *GhTZF1* zinc finger sequence with several plant-unique *TZF* genes from other species (*AtTZF1* (At2g25900), *AtTZF2* (At2g19810), *AtTZF3* (At4g29190), *OsDOS* (Q9FU27), and *CsSEF1* (CAI30889)) indicated a high conservation within the plant-unique TZF domain (Fig. 1c).

The *GhTZF1* gene is induced by various abiotic stresses

To obtain insights into the role of *GhTZF1*, the expression pattern of the *GhTZF1* gene was examined. Organ-specific expression analysis showed that *GhTZF1* was expressed in both vegetative and reproductive tissues, with a high expression in vegetative tissues (Fig. 2a). Interestingly, up-regulation of *GhTZF1* expression was observed in leaves in an age-dependent manner (Fig. 2a). To investigate the effect of *GhTZF1* expression on cell wall regeneration, the expression pattern of *GhTZF1* was examined. *GhTZF1* was highly expressed in the 3 h sample of cell wall regeneration (Fig. 2b). The effect of abiotic stresses on the expression of *GhTZF1* was also determined. The mRNA level of *GhTZF1* was increased within 24 h following PEG and NaCl treatment in leaves (Fig. 2c, d). The external application of H_2O_2 and MeJA was used to explore the effects of oxidative stress and growth hormones on the expression of *GhTZF1*. The expression level of *GhTZF1* was increased at 3 h after H_2O_2 treatment (Fig. 2e) and significantly up-regulated following exogenous MeJA treatment (Fig. 2f).

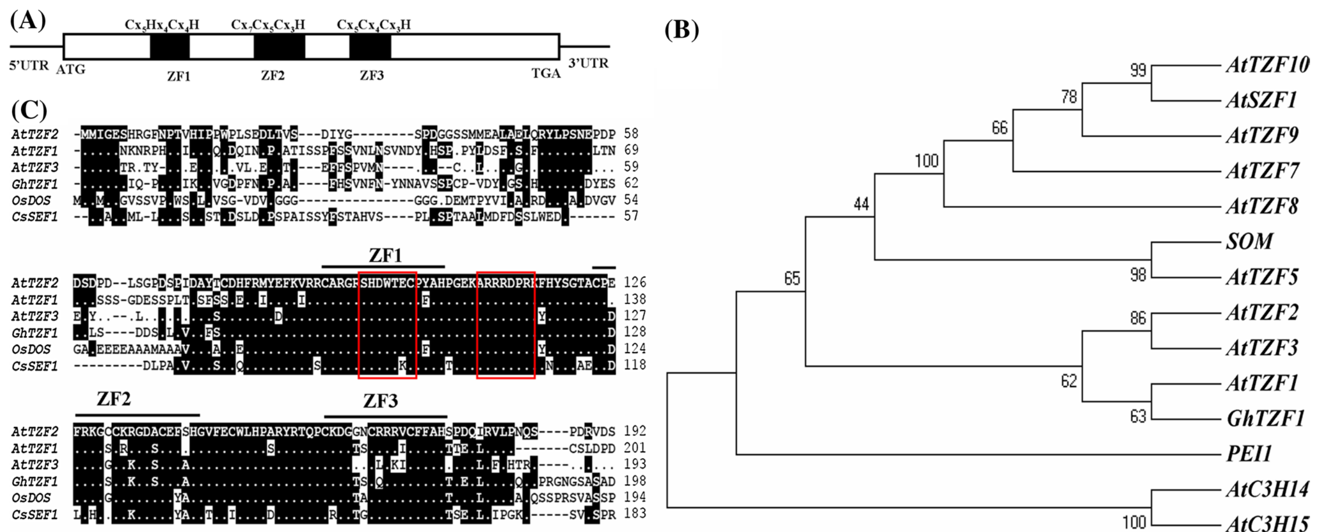


Fig. 1 Sequence analysis and structural features of *GhTZF1*. **a** The structure of the conserved TZF domains of *GhTZF1*. The primary structure possesses three tandem zinc fingers characterized by two classic motifs Cx7Cx5Cx3H-x16-Cx5Cx4Cx3H (*x* represents any amino acid) and one plant-unique motif Cx5Hx4Cx4H. **b** Phylogenic analysis of *GhTZF1* with *TZF* genes in the *Arabidopsis* subfamily IX and two *TZF* genes that possessed identical TZF motifs with h*TTP*. *AtTZF1* (At2g25900), *AtTZF2* (At2g19810), *AtTZF3* (At4g29190), *SOM* (At1g03790), *AtTZF5* (At5g44260), *PEI1* (At5g07500), *AtTZF7* (At2g41900), *AtTZF8* (At5g12850), *AtTZF9* (At5g58620), *AtTZF10* (At2g40140), *AtSZF1* (At3g55980), *AtC3H14* (At1g66810) and *AtC3H15* (At1g68200). *GhTZF1* is a plant-unique *TZF* gene clustered together with *Arabidopsis* subfamily IX members and is a homologue to *AtTZF1*. **c** Sequence alignment of *GhTZF1* and *TZF* genes in other species. *AtTZF1* (At2g25900), *AtTZF2* (At2g19810), *AtTZF3* (At4g29190), *OsDOS* (Q9FU27), and *CsSEF1* (CAI30889). Three TZF domains are marked by a *black bar* above the alignment. Two other plant-unique conserved domains are marked by a *red box*

Taken together, these data suggest that *GhTZF1* might play a role in the response to abiotic stresses.

Enhanced drought tolerance in *GhTZF1* overexpressed plants

In an effort to assess the in vivo function of *GhTZF1*, overexpression of *GhTZF1* was induced via a construct under the 35S promoter which was transferred to *Arabidopsis*. Several T3 generation transgenic plants were harvested and two representative lines (OX3 and OX4) were selected for phenotype and functional analysis (Fig. 3a). The homologous genes in *Arabidopsis* were investigated to make sure the referred phenotypes of transgenic plants in *Arabidopsis* were due to *GhTZF1* overexpression. There were no significant difference between wild type and transgenic lines (OX3 and OX4) on the expression of *AtTZF1* at drought condition, however, the expression of *AtTZF2* and *AtTZF3* was slightly reduced in transgenic lines both under the normal and drought conditions compared to wild type (Fig. 3a). Therefore, it was inferred that the phenotype of transgenic lines in present work was caused by *GhTZF1*. To gain insight into the effect of *GhTZF1* expression in the context of abiotic stress, the effects of PEG-simulated drought stress on seed germination was examined. Under a normal osmotic pressure condition (−0.25 MPa), all plant genotype seeds were germinated normally. We compared the germination rate of seeds grown in 1/2 MS medium with different osmotic pressures (−0.5 and −0.7 MPa) with seeds grown in 1/2 MS medium without PEG (−0.25 MPa) as a control. The seed germination rate of *GhTZF1*-overexpressing plants was comparable to that of wild type plants in 1/2 MS medium without PEG (Fig. 3b). However, the germination rate of seeds grown in 1/2 MS medium containing moderate PEG amounts (representing osmotic pressures of −0.5 and −0.7 MPa) was different, and indicated that *GhTZF1*-overexpressing seeds were more tolerant to PEG treatments than control seeds. The transgenic lines germinated and grew to a greater extent than did the wild type seeds (Fig. 3b). For example, on medium with an osmotic pressure of −0.5 MPa, the germination ratios of the transgenic lines were 94 and 79.2 % at 4 days of growth, respectively, while the germination ratio of the wild-type line was only 23.5 % (Fig. 3c).

To further decipher the drought tolerance *GhTZF1* overexpression confers, we conducted drought tolerance assays in soil. Water was withheld from 5-week-old wild type and homozygous transgenic plants for 7 days. After water deprivation, the leaves of wild type plants were significantly wilting, whereas the leaves of the transgenic lines were similar to the control leaves (Fig. 3d). There were no significant differences in the relative water content between wild type and transgenic lines under normal watering conditions. However, under drought conditions the relative water

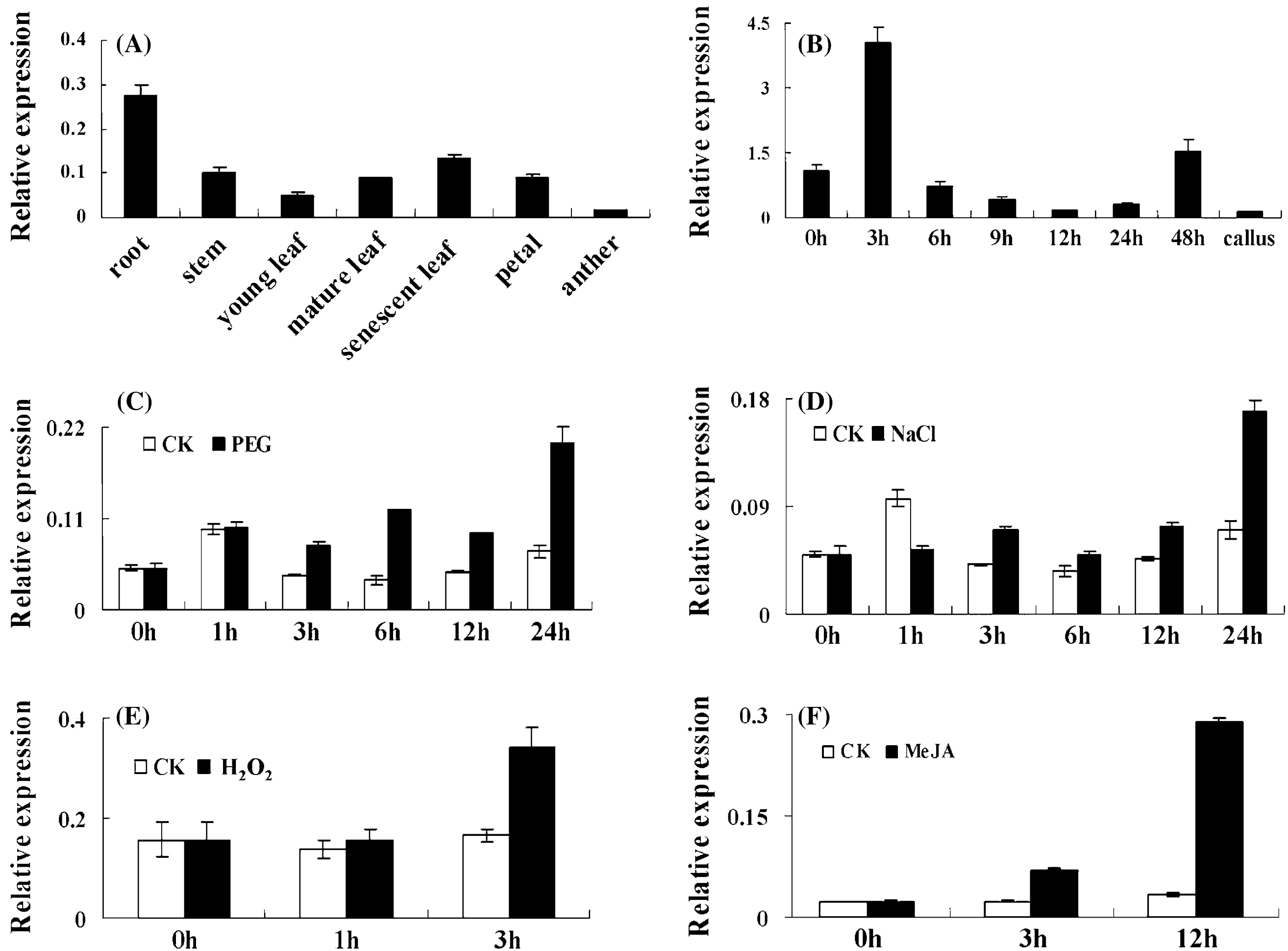
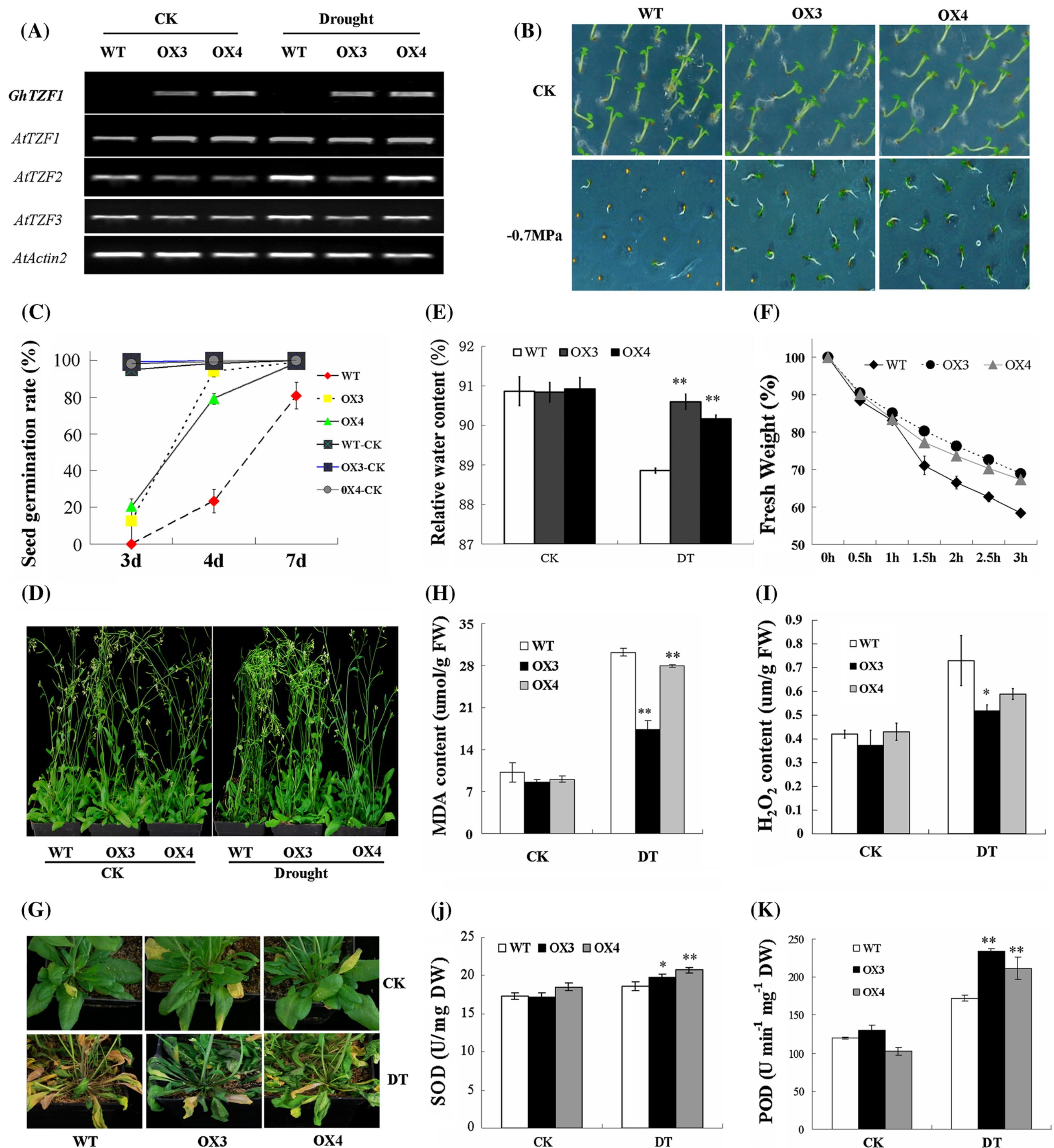


Fig. 2 Expression analysis of *GhTZF1*. **a** *GhTZF1* was expressed in various tissues. **b** The expression level of *GhTZF1* was increased at the early stage of cell wall regeneration. **c**, **d**, and **e** *GhTZF1* expression was induced by treatment with PEG, NaCl, and H_2O_2. **f** *GhTZF1* expression was induced by treatment with MeJA. The expression levels were normalized to *GhUB7* expression levels, and the relative expression was calculated with $2^{-\Delta CT}$, $\Delta CT = (C_{T,\ Target} - C_{T,\ GhUB7})_{Time\ x}$. *Error bars* represent ± SD of three biological replicates

content of the wild type line was decreased to 88.9 %, whereas the transgenic lines were just slightly decreased to 90.6 and 90.2 %, respectively, compared with the controls (Fig. 3e). Water loss assays showed that the water content of the transgenic lines decreased more slowly upon air drying compared with the wild type line (Fig. 3f), suggesting a role for *GhTZF1* in conferring drought resistance.

Moreover, under normal conditions, the processes of leaf senescence in all plant genotypes were not discernibly different. In contrast, under drought conditions, leaf senescence was accelerated in the wild type line, but was notably delayed in the transgenic lines when water was withdrawn for twice (Fig. 3g). The content of MDA, which is an indicator of lipid peroxidation caused by oxidative damage, was significantly increased in all groups during drought conditions compared with normal conditions. However, the increase in the amount of MDA generated under drought conditions in the transgenic lines was significantly less than the increase in the amount of MDA in the wild type line (Fig. 3h), indicating that the severe oxidative damage generated by drought stress was attenuated in the transgenic plants. Therefore, we hypothesized that the amount of H_2O_2 accumulation might be related to *GhTZF1* function. There was no significant difference in H_2O_2 content between the transgenic and wild type lines under normal conditions. Drought treatment induced a rapid accumulation of H_2O_2 in all plant genotypes; however, the amount of H_2O_2 accumulated in the transgenic lines was significantly lower than the amount of H_2O_2 accumulated in the wild type line (Fig. 3i).

To explore the molecular mechanisms of drought tolerance and delayed leaf senescence that overexpression of *GhTZF1* confers, two ROS scavenging enzymes activities were detected under both normal conditions and drought conditions. No obvious difference was observed in the activity of SOD between the wild type and transgenic



plants under normal conditions. The activity of SOD was increased under drought conditions in all plants. However, in comparison to the increasing of 7.8 % in the wild type, the activity of SOD increased to a high degree in transgenic lines, increasing to 115.1 % and 112.1 % to normal conditions respectively (Fig. 3j). Similar results were observed in the activity of POD (Fig. 3k). It was indicated that the activities of ROS scavenging enzymes were enhanced in transgenic lines and it was consistent with the lower H_2O_2 level in transgenic lines. The expression of a specific set of ROS homeostasis-related genes and stress-induced senescence triggering genes was also analyzed. qRT-PCR analysis determined that the expression levels of *AtRBOHC* and *AtRBOHF,* which are involved in ROS generation, were down-regulated in the transgenic lines compared with the wild type line. The expression of

◀ **Fig. 3** Overexpression of *GhTZF1* confers drought resistance and delayed drought-induced leaf senescence. **a** RT-PCR analysis of expression levels of *GhTZF1* and *Arabidopsis* homologue genes in wild type and transgenic lines under both normal and drought conditions. Two representative overexpression lines (OX3 and OX4) were selected for further experiments. **b** The transgenic lines germinated faster than the WT line in the presence of PEG (−0.7 MPa). 60 seeds for each line and the picture were taken after 7 days. **c** The germinating rate of seed germinated in the presence of PEG (−0.5 MPa). **d** Image of 5-week-old *Arabidophsis* plants deprived of water for 7 days. Leaf wilting was observed in WT plants. **e** The relative water content was reduced in WT plants compared with transgenic plants under drought conditions. **f** Water loss was reduced in transgenic plants compared with WT plants during air-drying conditions. **g** Drought-induced leaf senescence was delayed in the transgenic lines. **h** The amount of MDA was diminished in the transgenic lines compared with the WT line under drought conditions. **i** The amount of H_2O_2 was reduced in the transgenic lines compared with the WT line under drought conditions. **j** The activity of SOD in wild type and transgenic lines under normal and drought conditions. **k** The activity of POD in wild type and transgenic lines under normal and drought conditions. WT: wild type; OX3 and OX4: *GhTZF1*-overexpressing transgenic lines. CK: normal condition; DT: drought condition. *Asterisks* indicate statistically significant differences between wild type and transgenic lines (OX3 and OX4), as determined by Student's *t* test ($*p < 0.05$; $**p < 0.01$). *Error bars* represent ± SD of three biological replicates

AtFSD1, which is associated with ROS generation/removal, was also down-regulated in the transgenic lines compared with the wild type line. The expression levels of *AtAPX1*, *AtCAT1*, *AtGPX3*, *AtGPX4*, *AtGPX5*, and *AtERD11*, which are involved in modulating cellular redox status, were up-regulated in all lines under drought conditions compared with lines under normal conditions; however, the drought-induced up-regulation in expression levels of these genes was reduced in transgenic plants compared with the wild type plants (Fig. 4). This attenuation of increased expression may be because of reduced oxidative stress encountered in the transgenic plants. Moreover, the expression of *AtSAG14*, *AtSAG21*, *AtORE9*, and *AtACS6*, which are induced by oxidative stress, was dramatically up-regulated in wild type plants under water deficit conditions, whereas the drought-induced increase in *AtSAG14*, *AtSAG21*, *AtORE9*, and *AtACS6* mRNA expression was significantly attenuated in the transgenic lines (Fig. 4), indicating that the drought tolerance and delayed leaf senescence conferred by *GhTZF1* overexpression may be related to ROS homeostasis.

Delayed dark- and MeJA-induced leaf senescence in transgenic *Arabidopsis*

To confirm *GhTZF1*-overexpressing plants delay leaf senescence, a dark-induced leaf senescence test was conducted using the above two *GhTZF1*-overexpressing plants (OX3 and OX4) and wild type plants. Detached leaves of wild type and transgenic plants were floated in water in the dark, and chlorophyll degradation was visually detected by observing leaf yellowing. After 7 days of dark treatment, the wild type leaves turned yellow, while the leaves from transgenic lines were minimally influenced and retained green coloring (Fig. 5a). By measuring the amount of chlorophyll in the leaves, it was determined that the amount of total chlorophyll in wild type leaves was decreased to approximately 31.8 % compared with the amount found in the untreated control. However, the amount of total chlorophyll in the two *GhTZF1*-overexpressing plants (OX3 and OX4) was decreased to 81.8 and 87 %, respectively, compared with the amount found in the untreated control (Fig. 5b). Taken together, these data suggest that dark-induced leaf senescence was delayed in the transgenic lines.

MeJA is known to promote the process of leaf senescence by generating H_2O_2 (Hung et al. 2006). To confirm whether the JA response and JA-mediated redox status were affected in transgenic lines, detached age-matched leaves were incubated in the presence of 45 uM MeJA for 4 days in the dark or in water (mock treatment) and chlorophyll loss was visually scored. Under MeJA treatment for 4 days, wild type leaves showed an evident leaf yellowing, while the transgenic lines displayed delayed leaf senescence (Fig. 5c). The total amount of chlorophyll in wild type leaves decreased to 37.2 % of that of the mock-treated leaves, while the amount of chlorophyll in the transgenic lines decreased to only 61.5 and 61 %, respectively (Fig. 5d). Therefore, our results indicate that JA-induced leaf senescence was delayed in the transgenic lines. The amount of H_2O_2 was significantly increased in wild type leaves when subjected to MeJA treatment compared with mock treated leaves. However, the amount of H_2O_2 was significantly lower in transgenic lines than in wild type lines under MeJA treatment, suggesting MeJA-induced H_2O_2 production was attenuated in transgenic lines (Fig. 5e).

GhTZF1 regulates the response to H_2O_2

Gene expression analysis indicated that the expression of many genes involved in cell redox homeostasis decreased in *GhTZF1*-overexpressing lines under drought conditions. In addition, inhibiting H_2O_2 accumulation was also observed in the *GhTZF1*-overexpressing lines. To dissect the role of *GhTZF1* in the H_2O_2 response, the in vitro chlorotic leaf response to H_2O_2 was evaluated. Detached leaves of wild type and *GhTZF1*-overexpressing plants (OX3 and OX4) were incubated in H_2O_2 for 4 days. Chlorotic leaf lesions were present in all plant genotypes under H_2O_2 treatment. The number of chlorotic leaf lesions was severely attenuated in transgenic lines compared with the number of lesions in the wild type line. Control plants, which were

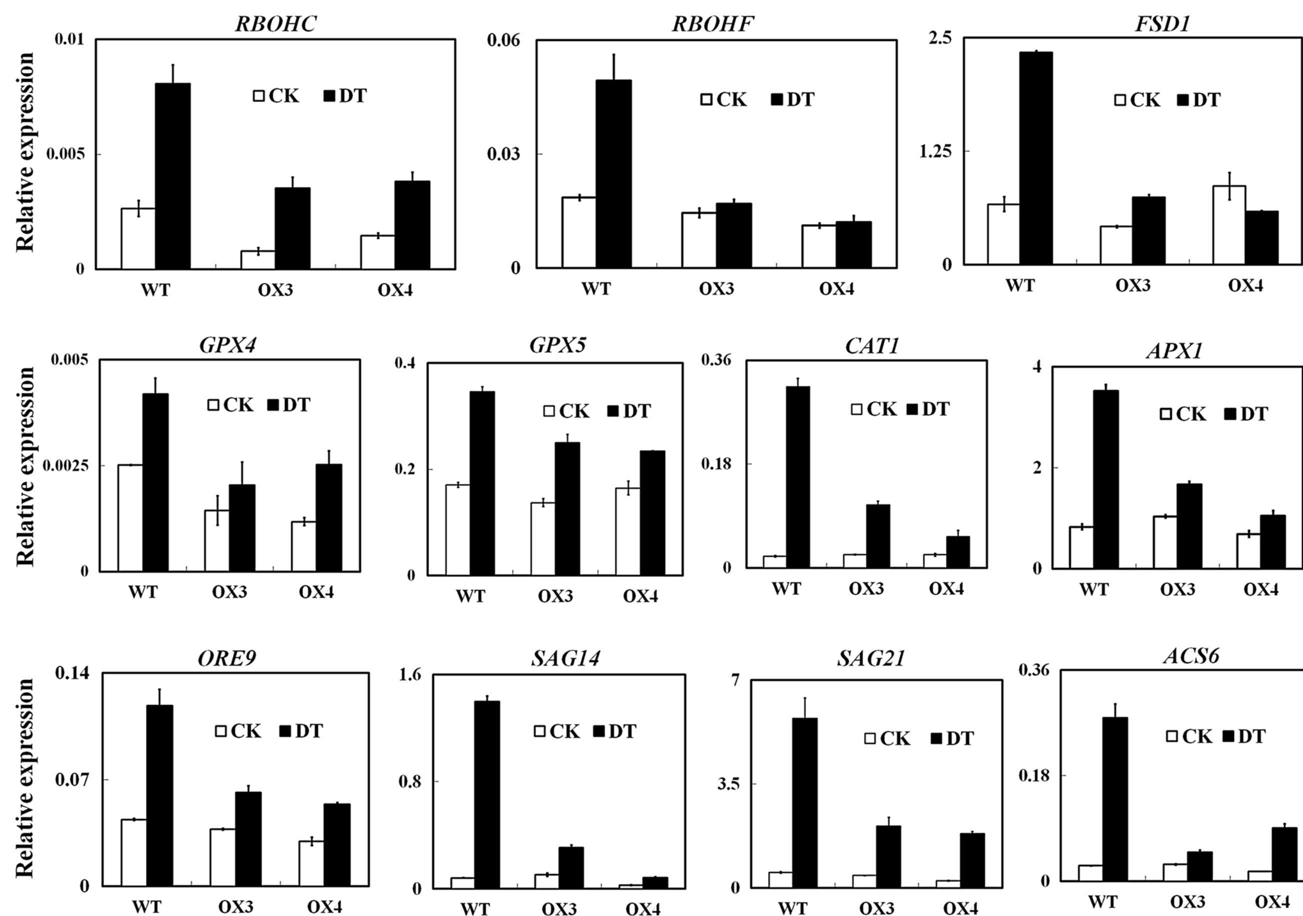


Fig. 4 The expression levels of ROS homeostasis-related genes and senescence-associated genes in *GhTZF1* transgenic lines under drought conditions. *AtRBOHC* (At5g51060), *AtRBOHF* (At1g64060), *AtFSD1* (At4g25100), *AtGPX4* (At2g48150), *AtGPX5* (At3g63080), *AtCAT1* (At1g20630), *AtAPX1* (At1g07890), *AtORE9* (At2g42620), *AtSAG14* (At5g20230), *AtSAG21* (At4g02380), and *AtACS6* (At4g11280). The expression levels were normalized to *AtActin2* expression levels and the relative expression were calculated with $2^{-\Delta CT}$, $\Delta CT = (C_{T,\ Target} - C_{T,\ GhUB7})_{Time\ x}$. *Error bars* represent ± SD of three biological replicates

incubated in water, exhibited no significant visible changes in the number of chlorotic leaf lesions (Fig. 6a). There were no significant changes in the amount of chlorophyll in all plant genotypes under the mock treated condition. In contrast, the amount of chlorophyll in the wild type line was significantly lower than the amount of chlorophyll in the transgenic lines in response to H_2O_2 (Fig. 6b). Supporting this observation, the expression of some oxidative stress-related *SAG*s was changed during H_2O_2 treatment. The expression of *ELI 3-2*, *SAG15*, and *ACS6* was up-regulated when exposed to H_2O_2 compared with expression of mock treated lines. However, these oxidative stress-related *SAG*s were up-regulated to a lesser degree in the transgenic lines compared with the wild type line, indicating that the overexpression of *GhTZF1* alleviated the H_2O_2-induced response (Fig. 6c). Furthermore, RT-PCR analysis indicated that the expression levels of genes encoding antioxidant-related genes, such as, *AtGPX3*, *AtGPX4*, *AtGPX5*, *AtERD11*, and *AtGSTU5*, were increased in transgenic lines compared with the wild type line when subjected with H_2O_2 treatment (Fig. 6c). The observed changes in gene expression were in agreement with the enhanced H_2O_2 tolerance. These observations indicated that *GhTZF1* might also regulate these antioxidant gene expressions in response to H_2O_2 treatment.

To further confirm the sensitivity of *GhTZF1* to H_2O_2, seeds of wild type and two overexpressing transgenic lines, OX3 and OX4, were germinated on 1/2 MS medium supplemented with 0-3 mM H_2O_2. There was no significant difference in seed germination rate among wild type and *GhTZF1*-overexpressing plants (OX3 and OX4) on 1/2 MS medium. However, the growth of wild type plants was retarded in 1 mM H_2O_2-supplemented medium, while *GhTZF1*-overexpressing transgenic plants grew well. In 3 mM H_2O_2-supplemented medium, the growth of both wild type and *GhTZF1*-overexpressing plants was inhibited, but *GhTZF1*-overexpressing plants grew better than wild type plants (Fig. 6d). The relative suppression in the

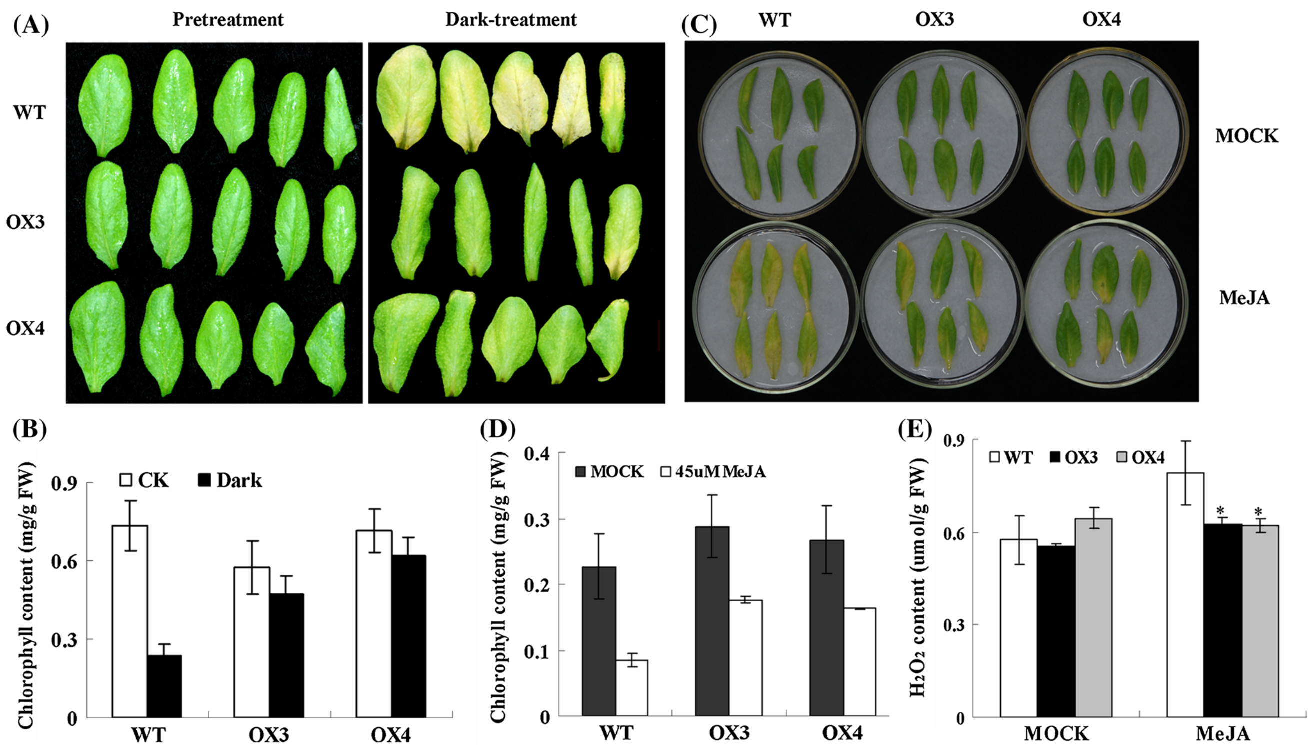


Fig. 5 Delayed dark-induced and MeJA-induced leaf senescence in *GhTZF1*-overexpressing plants. **a** Image of detached leaves (the eighth-tenth true rosette leaves) from 4-week-old plants incubated in the dark for 7 days and leaf yellowing was observed in WT plants. **b** The total amount of chlorophyll in WT plants was decreased compared with transgenic plants after dark treatment. **c** Image of detached leaves (the eighth-tenth true rosette leaves) from 40 days plants incubated in 45 uM MeJA for 4 days. **d** The total amount of chlorophyll was decreased in WT plants compared with *GhTZF1* transgenic plants when cultured in 45 µM MeJA solution for 4 days. **e** The H_2O_2 content of detached leaves incubated in 45 µM MeJA for 4 days. WT: wild type; OX3 and OX4: *GhTZF1*-overexpressing transgenic lines. *Asterisks* indicate statistically significant differences between wild type and transgenic lines (OX3 and OX4), as determined by Student's *t* test (*$p < 0.05$). *Error bars* represent ± SD of three biological replicates, $n \geq 5$

fresh weight (FW) of *GhTZF1*-overexpressing plants was less than the suppression in wild type plants at 14 days after germination. With treatment of 1 mM H_2O_2, the FW of *GhTZF1*-overexpressing lines (OX3 and OX4) was 1.25- and 1.2-fold that of the wild type line FW, respectively. With treatment of 3 mM H_2O_2, the FW of *GhTZF1*-overexpressing lines (OX3 and OX4) was 1.22- and 1.11-fold that of the wild type line FW, respectively (Fig. 6d). These results indicate that *GhTZF1*-overexpressing plants were more tolerant to H_2O_2 at the whole-plant level as well as in detached leaves.

Discussion

GhTZF1 is a link in the relationship of drought resistance and delayed leaf senescence

TZF genes are IX subfamily member of CCCH-type zinc finger protein gene in *Arabidopsis*. Expression profile and functional analysis of these members indicated that they were involved in many developmental and abiotic stresses responses (Wang et al. 2008). Overexpression of *AtTZF1* enhanced drought and cold tolerance (Lin et al. 2011). *AtTZF2* and *AtTZF3* were positively involved in drought and oxidative stress responses (Huang et al. 2011; Lee et al. 2012a). *OsTZF1*, a CCCH-tandem zinc finger protein in rice, conferred delayed leaf senescence and salt tolerance (Jan et al. 2013). Genetic studies have revealed that extended longevity is frequently associated with an increased resistance to stress (Kurepa et al. 1998). Chlorophyll retention or 'stay-green' is regarded as a key indicator of stress adaptation (Woo et al. 2004). The expression of most *SAG*s was induced by both senescence and stresses (Weaver et al. 1998). Previous research has elucidated that delayed leaf senescence confers extreme drought resistance (Rivero et al. 2007; Valente et al. 2009). Transgenic *PSAG12-IPT* plants with delayed leaf senescence had improved drought tolerance, which was due to the accumulation of several metabolites involved in the stress response pathways (Merewitz et al. 2012). Loss of *ZmACS6* expression delayed leaf senescence and enhanced drought

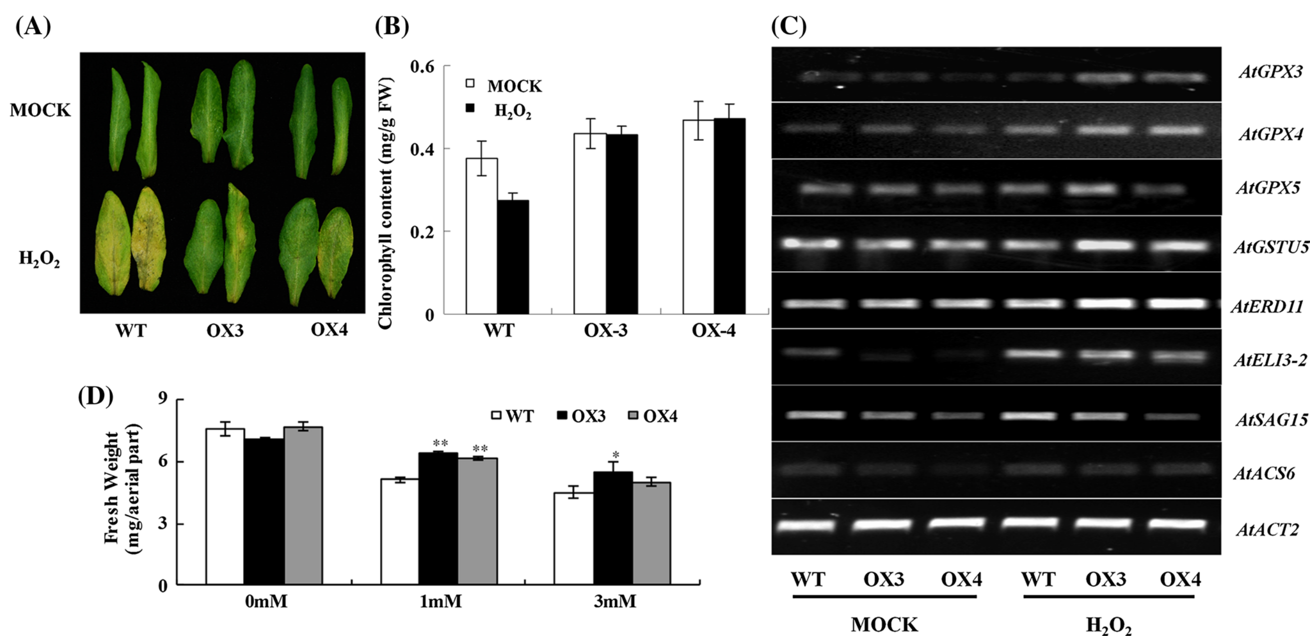


Fig. 6 *GhTZF1*-overexpressing plants showed diminished responsiveness to H_2O_2-induced oxidative stress. **a** Image of detached leaves (the eighth-tenth true rosette leaves) from 4-week-old plants incubated in 10 mM H_2O_2 solution for 4 days. **b** The chlorophyll contents of wild type and transgenic lines when treated with 10 mM H_2O_2 for 4 days. **c** The expression levels of genes influenced by H_2O_2 in *GhTZF1*-overexpressing plants. *AtGPX3* (At2g43350), *AtGPX4* (At2g48150), *AtGPX5* (At3g63080), *AtGSTU5* (At2g29450), *AtERD11* (At1g02930), *AtELI3-2* (At4g37990), *AtSAG15* (At5g51070), and *AtACS6* (At4g11280). D, The effect of H_2O_2 on growth in wild type and *GhTZF1*-overexpressing plants. WT: wild type; OX3 and OX4: *GhTZF1*-overexpressing transgenic lines. *Asterisks* indicate statistically significant differences between wild type and transgenic lines (OX3 and OX4), as determined by Student's *t* test (*$p < 0.05$; **$p < 0.01$). *Error bars* represent ± SD of three biological replicates, $n \geq 10$

tolerance (Young et al. 2004). Also, accelerated leaf senescence performed drought hypersensitivity, for example, overexpression of the active form of *NTL4* promoted ROS production and accelerated leaf senescence, meanwhile, plants with *NTL4* overexpression were hypersensitive to drought because of NTL4 binding directly to the promoters of genes encoding ROS biosynthetic enzymes, leading to ROS accumulation, in contrast, delayed leaf senescence and enhanced drought resistance were exhibited in *NTL4*-deficient *ntl4* mutants (Lee et al. 2012b).

In the current study, analysis of the expression of *GhTZF1* indicated that *GhTZF1* may be involved in the response to drought stress. Following a PEG culture, which mimicked drought conditions, and water stress treatment, *GhTZF1* was shown to enhance drought tolerance (Fig. 3). Moreover, the expression level of *GhTZF1* was altered following leaf development (Fig. 2a), and the PEG-induced accumulation of *GhTZF1* mRNA was detected in leaves (Fig. 2c), and lower transpiration was also observed in *GhTZF1* transgenic plants than that in control plants under drought conditions (Fig. 3f). Furthermore, drought-induced leaf senescence was delayed in transgenic plants (Fig. 3g). Meanwhile, *GhTZF1*-induced delayed leaf senescence was further confirmed by the results of the dark- and MeJA-induced leaf senescence tests (Fig. 5). The expression levels of *SAGs*, such as *ORE9*, *SAG14*, *SAG21*, and *ACS6*, were attenuated in transgenic lines compared with the wild type line under drought conditions (Fig. 4). These results suggest that the conferred drought resistance might be coupled with leaf physiology and that the *GhTZF1* gene might link drought resistance with delayed leaf senescence in plants under drought stress.

GhTZF1 confers drought resistance and delayed leaf senescence by inhibiting ROS generation and accumulation

ROS production and accumulation has been shown to be associated with drought and leaf senescence (Khanna-Chopra 2012). ROS levels were rigorously regulated and retained in young and/or unstressed plants, and ROS homeostasis was disrupted, leading to oxidative stress, in senescent and stressed plants (Chen et al. 2012). Dark-induced leaf senescence was involved in an increase of H_2O_2, which was accompanied by an imbalance in the antioxidative system (Pastori and Río 1994). Methyl jasmonate promoted leaf senescence by inducing the production of ROS (Hung et al. 2006). JA-defective *Arabidopsis* mutants showed alleviated lipid peroxidation and a disturbance in redox homeostasis under drought conditions (Brossa et al. 2011). It has been previously reported that delayed leaf senescence is

closely correlated with tolerance to oxidative stress (Rivero et al. 2007; Woo et al. 2004). Delayed leaf senescence mutants *ore1*, *ore3*, and *ore9* were more tolerant of oxidative stress, and the mechanism in the *ore* mutants responsible for this response was suggested to be the altered oxidative stress response instead of the modulation of activity of antioxidant enzymes (Woo et al. 2004). Repressing the expression of the senescence-related ACC synthase (*ACS*) gene enhanced abiotic stress tolerance and diminished ethylene biosynthesis as a result of decreased ROS accumulation (Wi et al. 2010). Acclimation to drought stress generates oxidative stress tolerance in drought-resistant cultivar (Khanna-Chopra and Selote 2007). NADPH oxidases such as the Rbohs (respiratory burst oxidase homologs) are an important ROS-generating system in plants producing O_2^-, which is usually rapidly dismutated to hydrogen peroxide (Jaspers and Kangasjärvi 2010). The Fe superoxide dismutase gene (*FSD1*), which encodes a superoxide dismutase, is involved in reducing O_2^- to H_2O_2 (Attia et al. 2011).

In the present study, the amount of H_2O_2 under drought conditions was significantly decreased in the transgenic lines than in the wild type line, whereas there was no significant difference between wild type and transgenic lines under normal conditions (Fig. 3i). These results suggest a role of *GhTZF1* in regulating ROS levels to maintain redox homeostasis under drought conditions. Antioxidant enzymes are the most important components in the scavenging systems of ROS to maintain ROS homeostasis within cellular, of the antioxidant enzymes, SOD and POD play key roles in cellular ROS detoxification (Gao et al. 2010; Meloni et al. 2003). In the present study, the activities of SOD and POD were obviously increased to a higher degree in transgenic lines as compared with wild type under drought conditions (Fig. 3j, k), indicating *GhTZF1*-overexpressing transgenic lines had a higher capacity for scavenging ROS. In the transcription level, the expression levels of *RBOHC*, *RBOHF* were significantly decreased in the transgenic plants compared with the wild type plants under drought conditions, indicating that ROS generation might be inhibited in transgenic plants under drought conditions (Fig. 4). Concomitant with reduced ROS accumulation, the induction degree of some ROS homeostasis-related genes was compromised in drought treated transgenic lines. It was proposed that *GhTZF1* reduced the cellular ROS level, thereby minimizing the stimulatory effect on ROS-homeostasis related genes. Similar response has been reported before, overexpression of *OsTZF1* in rice reduced ROS accumulation and attenuated the induction ratio of significant numbers of stress-related genes at salt-treated conditions (Jan et al. 2013). In addition, there is the possibility that *GhTZF1* regulates drought tolerance and senescence through other target genes. Moreover, the expression levels of the oxidative-related *SAG*s, such as *ORE9*, *SAG14*, *SAG21*, and *ACS6*, were diminished in the transgenic lines compared with the wild type line under drought conditions (Fig. 4), indicating delayed drought-induced leaf senescence was related with diminished ROS accumulation. Meanwhile, the amount of MDA, a product of senescence-associated lipid peroxidation, which is generated by excessive accumulation of ROS, was significantly reduced in the transgenic lines compared with the wild type line (Fig. 3h). Therefore, we propose that *GhTZF1* inhibits the generation and accumulation of ROS to maintain the cell redox status under drought conditions. Supporting this hypothesis, dark- and MeJA-induced leaf senescence was dampened in *GhTZF1* transgenic lines. The amount of H_2O_2 in detached leaves was lower in the transgenic lines compared with the wild type line when subjected to MeJA (Fig. 5), indicating that *GhTZF1* plays a role in mediating ROS homeostasis. Consistent with the oxidative stress tolerance generated in transgenic lines, overexpression of *GhTZF1* enhanced H_2O_2-induced oxidative stress tolerance in detached leaves as well as in whole plants (Fig. 6a). The mRNA levels of senescence-associated genes (*SAGs*), such as *SAG25*, *ERD1*, and *ACS6*, which were induced when exposed to oxidative stress, were diminished in the transgenic lines compared with the wild type line when exposed to H_2O_2 (Fig. 6c). In addition, The expression of ROS detoxification related genes, such as *GPX3*, *GPX4*, *GPX5*, *GSTU5*, and *ERD11*, was higher in the transgenic lines than in the wild type line (Fig. 6c), suggesting that *GhTZF1* might regulate ROS detoxification to modulate H_2O_2 response. It is likely that *GhTZF1* conferred drought tolerance and delayed drought-induced leaf senescence by inhibiting cellular ROS generation and accumulation, which alleviated the oxidative damage caused by the drought stress.

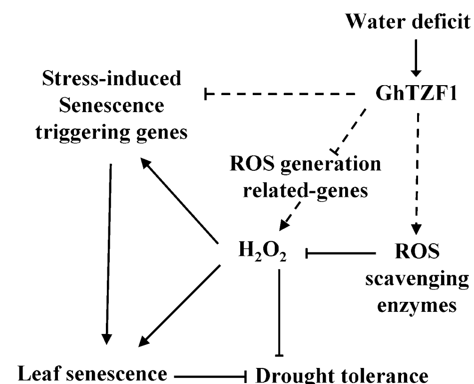


Fig. 7 A model in *Arabidopsis* of how *GhTZF1* modulates leaf senescence and drought stress through alterations in the generation and accumulation of H_2O_2. *Arrows* indicate positive regulation. *T-bars* indicate negative regulation. *Dotted lines* indicate hypothetical regulation

Results from the current work suggest that the role of *GhTZF1* in *Arabidopsis* might be linked to reactive oxygen species regulation, and thus the regulation of drought response and leaf senescence. *GhTZF1* might regulate the activities of antioxidant enzymes and the expression of ROS generation-related genes to modulate reactive oxygen species levels and regulated cellular redox homeostasis, which in turn influence the expression of ROS-homeostasis related genes. In addition, reduced ROS accumulation in turn repressed the expression of stress-induced senescence triggering genes, which delayed the stress-induced leaf senescence and enhanced drought tolerance (Fig. 7). Further work is necessary to address the intricacies of the underlying regulatory mechanisms in greater detail.

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