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RESEARCH PAPER

SpUSP, an annexin-interacting universal stress protein, enhances drought tolerance in tomato

Rachid Loukehaich*, Taotao Wang*, Bo Ouyang, Khurram Ziaf, Hanxia Li, Junhong Zhang, Yongen Lu and Zhibiao Ye†

Key Laboratory of Horticultural Plant Biology, Ministry of Education, and National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China

* These authors contributed equally to the article.
† To whom correspondence should be addressed: E-mail: zbye@mail.hzau.edu.cn

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Abstract

Universal stress protein (USP) appears to play an active role in the abiotic stress response, but their functions remain largely unknown in plants. A USP gene (*SpUSP*) was cloned from wild tomato (*Solanum pennellii*) and functionally characterized in cultivated tomato in the present study. The *SpUSP* transcript is abundantly accumulated in leaf stomata and its expression varied with the circadian rhythm. *SpUSP* was remarkably induced by dehydration, salt stress, oxidative stress, and the phytohormone abscisic acid (ABA) etc. This protein was predominantly localized in the nucleus and cell membrane. Overexpressing *SpUSP* increased drought tolerance of tomato in the seedling and adult stages. Under drought stress, the ABA content significantly increased in the *SpUSP*-overexpressing plants, which induced stomatal closure and reduced water loss, leading to the enhancement of drought tolerance. Based on the microarray data, a large number of chlorophyll *a*/*b*-binding proteins and photosystem-related genes were up-regulated in the *SpUSP*-overexpressing plants under drought conditions, which possibly enhanced the stomatal sensivitity to ABA and maintained the photosynthetic function. *SpUSP* overexpression also alleviated the oxidative damage accompanied by oxidative stress-responsive gene activation and osmolyte accumulation. Annexin (SGN-U314161) was found to interacte with *SpUSP* in the yeast two-hybrid method. This interaction was further confirmed by the bimolecular fluorescence complementation assay. The present study demonstrated that the annexin-interacting *SpUSP* plays important roles in the drought tolerance of tomato by influencing ABA-induced stomatal movement, increasing photosynthesis, and alleviating oxidative stress.

Key words: ABA, abiotic stress, annexin, *Solanum pennellii*, *SpUSP*, tomato.

Introduction

Sessile plants have to cope with various environmental stresses during their life cycle (Takashi and Kazuo, 2010). Among the different abiotic stresses, drought is a major agronomic threat to crop growth and yield, especially in arid and semi-arid areas (Adams *et al*., 2009). In drought conditions, 80–90% of water loss occurs via stomata in the leaf epidermis. Although various stimuli affect stomatal closure, the phytohormone abscisic acid (ABA)-induced stomatal closure is considered as a crucial mechanism for preventing water loss from plants (Lyudmila *et al*., 2011). ABA is a key regulator involved in diverse developmental processes and responses to abiotic stress (Cutler *et al*., 2010). Drought and salt stress conditions dramatically increase the ABA level which, in turn, induce the expression of many stress-related genes and activate signal transduction pathways that lead to stomatal movement (Tuteja, 2007; Zou *et al*., 2010).

Stomatal movement affects CO_2 assimilation and further affects photosynthesis. The light-harvesting chlorophyll *a*/*b*-binding proteins (LHCBs) fulfil a constitutive light-harvesting function for photosystem II (PSII) during photosynthesis (Roberto *et al*., 1997). Previous reports have shown that the members

of the LHCB family play an important role in plant photosynthesis and adaptation to environmental stresses (Ganeteg *et al*., 2004; Kovacs *et al*., 2006), as well as in guard cell signalling in response to ABA (Xu *et al*., 2012). The absence of the Lhcb1 and Lhcb2 proteins affects photosynthesis and results in the decrease of light absorption by the leaf (Andersson *et al*., 2003). The down-regulation of LHCB members also reduces plant tolerance to environmental stresses and affects seed production (Ganeteg *et al*., 2004; Kovacs *et al*., 2006).

Drought and salinity can lead to oxidative stress, thus, plants accumulate different kinds of osmoprotective solutes to reduce oxidative damage (Mahajan and Tuteja, 2005). These adaptive changes are achieved through a series of stress-dependent signal transduction pathways involving different types of genes (Chinnusamy *et al*., 2004). Some genes involved in osmoregulation have been cloned, such as *pyrroline-5-carboxylate synthetase* (*P5CS*) (Choudhary *et al*., 2005), *betaine aldehyde dehydrogenase* (Jia *et al*., 2002), and *trehalose-6-phosphate synthase* (Li *et al*., 2011). Transgenic plants overexpressing *P5CS* have significantly increased proline levels and tolerance to drought and salt stress (Kishor *et al*., 1995; Vendruscolo *et al*., 2007). The osmotic-stress-related genes are largely regulated by specific transcription factors, such as the members of the APETELA2, bZIP, and MYB families (Jung *et al*., 2010; Yang *et al*., 2009; Shin *et al*., 2011).

A type of protein called universal stress protein (USP) appears to play a positive role in the abiotic stress response. This protein features with the conserved USP domain in plants together with other functional domains (Kvint *et al*., 2003). USP was first discovered in bacteria whose expression is enhanced when the cell is exposed to stress agents (Nystrom and Neidhardt, 1992). The six USP genes of *Escherichia coli* have different functions linked to motility, adhesion, and oxidative stress resistance. Among these genes, *UspA* and *UspD* are required in the defence against superoxide-generating agents (Nachin *et al*., 2005).

USP homologues are ubiquitous in plants and encoded by gene families, while the functions of USPs remain largely unknown. The 44 putative USP genes in *Arabidopsis* are divided into two groups: ATP binding and non-ATP binding (Kerk *et al*., 2003). Two *Arabidopsis* USP genes, At3g62550 and At3g53990, that encode an ATP-binding motif have recently been up-regulated in a drought microarray dataset. (Isokpehi *et al*., 2011). *GUSP1* and *GUSP2* have been detected in water-stressed leaves of *Gossypium arboretum* (Maqbool *et al*., 2009). The present study is the first to report the cloning and functional characterizations of a *SpUSP* gene in tomato. The results suggest that *SpUSP* plays an important role in abiotic stress tolerance together with annexin via an ABA-dependent way.

Materials and methods

Isolation of SpUSP *cDNA and sequence analysis*

Total RNA was extracted from the leaves of the wild tomato species *Solanum pennellii* LA716 using Trizol reagent (Invitrogen, USA). Reverse transcription PCR (RT-PCR) was performed using a reverse transcription kit (Toyobo, Japan). The cDNA of *SpUSP* was amplified via a PTC-100 programmable thermal cycler (MJ Research, USA) using the primers USP1-F and USP1-R (see Supplementary Table S1 at *JXB* online). The amplified PCR fragment was cloned into pMD18-T (TaKaRa, Japan), transformed into DH5α *E. coli* cells, and sequenced. Sequence and phylogenetic analysis were conducted as previously described by Yang *et al*. (2011). The *cis*-acting regulatory elements in the promoter region were analysed using the PlantCARE (Lescot *et al*., 2002) and PLACE databases (Higo *et al*., 1999).

Plant growth and stress treatments

For gene expression profiling analysis, 2-month-old uniformly developed tomato plants (LA716, *S. pennellii*) were grown in a greenhouse under a 14/10 h light/dark regime at 25 °C and subjected to various stresses or hormone treatments. Salt stress was induced by watering the plants with 200 mM NaCl solution. Drought stress was simulated by placing detached leaves on a filter paper in 70% relative humidity at 25 °C. Cold or hot stress conditions were imposed by transferring the plants to a growth chamber and holding the plants at 4 °C or 40 °C, respectively. Wounding was performed by pinching the leaves with forceps. For hormone treatments and oxidative stress, 100 μM ABA, 100 μM ethylene (ETH), 100 μM gibberellic acid (GA_3), and 100 μM paraquat were directly sprayed onto tomato plants. After each treatment, leaves from different plants (three biological replicates) were collected and immediately frozen in liquid nitrogen and stored at –80 °C until RNA isolation.

Quantitative RT-PCR (qRT-PCR) analysis

Quantitative RT-PCR was performed on a LightCycler 480 system (Roche, Switzerland). The reaction mixture contained 5 μl of 2× SYBR Premix Ex Taq mix (TaKaRa, Japan), 0.5 μM each of forward and reverse primers, and 1 μl of 10-fold diluted first-strand cDNA. The PCR cycling regime comprised an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The primers used were QUSP1-F and QUSP1-R (see Supplementary Table S1 at *JXB* online). The tomato β-actin gene (GenBank accession no. BT013524) was used as an internal control. The threshold cycle value was given by the program automatically. The gene expression data were analysed using the $^{\Delta\Delta}$Ct method (Schmittgen and Livak, 2008).

Plasmid construction and production of SpUSP*-overexpressing plants*

SpUSP promoter::GUS was generated by fusing the promoter fragment of *SpUSP* (1.981 kb) in front of the GUS coding sequence in the PV3P vector. The promoter fragment of *SpUSP* was amplified using the primer pairs of *SpUSP* Pro F/R with an attB site (see Supplementary Table S1 at *JXB* online). The fragment was then integrated to the PV3P vector by BP and LR reactions (Invitrogen, USA), and then transformed into the cultivated tomato. The tissues of positive transgenic plants were treated with pre-chilled 90% acetone for 20 min, rinsed with chilled water, and incubated with chilled GUS stain at 37 °C overnight in the dark. An ethanol series was used to destain the tissues, and the expression patterns were analysed under a microscope.

To overexpress *SpUSP* in tomato, the 35S promoter was employed to drive the target gene. A 550 bp *Bam*HI–*Xho*I fragment containing the *SpUSP* cDNA was cloned into the plant binary vector pMV, replacing the GUS reporter gene. The construct was introduced into the *Agrobacterium tumefaciens* strain LBA4404. Genetic transformation was performed on a drought-sensitive tomato cultivar ZS6 (*S. lycopersicum*) as previously described by Ouyang *et al*. (2005). Kanamycin-resistant plants were further confirmed through PCR using 35S-F (a forward primer in the 35S promoter) and USP1S-R (a reverse primer specific for *SpUSP*) (see Supplementary Table S1 at *JXB* online). The kanamyicn spraying test was used in the genetic segregation analysis (Weide *et al*., 1989), and three single-copy homozygous T_3 lines (OE44, OE45, and OE69) were used for further study.

Stress tolerance assays in the transgenic tomato plants

To investigate the functions of *SpUSP* in stress tolerance, stress assays were conducted at the germination and adult-plant stages

including three transgenic lines (OE44, OE54, and OE69) and one Wt line. Approximately 30 seedlings at uniform developmental stages for each line were transferred to half-strength Murashige and Skoog (MS) solid medium supplemented with either 200 mM mannitol, 100 mM NaCl, or 3 μM ABA. After growing for 10 d, the length and weight of the seedlings were measured. In addition, adult OE and Wt plants were drought stressed in the identical 2.0 l preweighed pots. Water was withheld until the plants began to wilt, then the plants were reirrigated, and their ability to recover was investigated. All measurements were conducted with six replicates.

Meanwhile, some stress-related biochemical markers were examined under drought stress at 25% and well-watered conditions at 100% field capacity (FC). The drought-stress treatment was initiated at the four-leaf stage. After 9 d of treatment, the chlorophyll contents were determined at 6-d intervals according to Wellburn (1994). Two months later, the dry and fresh weights, soluble sugars, proline, and malondialdehyde (MDA) were determined using previously described methods (Dubois *et al*., 1956; Bates *et al*., 1973). All measurements were conducted with three replicates.

To investigate the oxidative tolerance of *SpUSP*, excised 5 h leaves from well-watered transgenic and Wt lines were stained using the diaminobenzidine (DAB) method according to Orozco-Cardenas and Ryan (1999). The oxidative tolerance of *SpUSP* was further studied by spraying 100 μM of paraquat (inducer of reactive oxygen species, ROS) onto well-watered plants for 24 h. The oxidative damage of the leaves was then determined using the above-mentioned DAB staining method.

Subcellular localization of SpUSP

To detect the subcellular localisation of *SpUSP*, a green fluorescent protein (GFP) reporter system was constructed. The GFP gene and full-length cDNA of *SpUSP* without stop codon were amplified using the high-fidelity *Taq* polymerase (KOD plus, Toyobo) with primers GFPsub-F/GFPsub-R (with *Kpn*I) and USPsub-F (with *Xba*I)/USPsub-R, respectively (see Supplementary Table S1 at *JXB* online). Overlapping PCR was conducted to assemble the resulting PCR products, and the chimeric fragment (*SpUSP::GFP*) yield was cloned into pMV through the restriction sites *Xba*I and *Kpn*I to replace the GUS reporter gene. The resulting GFP reporter vector was designated as *35S::SpUSP::GFP*. A GFP control vector (*35S::GFP*) was also constructed, and then transformed into the tobacco protoplast using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, USA). After incubation for 12–18 h at 25 ± 2 °C, the GFP signals were detected under a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Germany).

Yeast two-hybrid (Y2H) screening and bimolecular fluorescence complementation (BiFC)

Y2H screens were performed using BD Matchmaker Library Construction and Screening kits (Clontech, USA) according to the manufacturer's instructions. *SpUSP* was used as bait to screen the interacting proteins with the yeast library of tomato Ailsa Craig, and 40 randomly selected positive clones were sequenced and analysed. The interactions were further confirmed *in vivo* using the BiFC method as previously described by Walter *et al*. (2004). The cDNAs of *SpUSP* and *Annexin* (Unigene U314161), named *AnnSp2* based on the report of Lu *et al*. (2012), were amplified with the primers listed in Supplementary Table S1 at *JXB* online and cloned into the vectors pUC-SPYCE and pUC-SPYNE, respectively. Then, the BiFC constructs were bombarded into tobacco cells using a gene gun. Photographs were taken 12–18 h after transformation using a LSM510 confocal microscope.

ABA content and water loss assays

The ABA content was determined according to the method described by Seiler *et al*. (2011) with some modifications. After 7 d of exposure to drought and well-watered conditions, about 1 g of leaf sample was ground and extracted several times in 10 ml of extraction buffer (90% methanol+5% acetic acid+5% water), then the supernatant was collected by centrigugation and evaporated to dryness. The dried samples were redissolved in pure methanol and filtered. The filtrate was used for subsequent quantification through the chromatography using a Shimadzu UFLC syetem. Each sample analysis was repeated three times.

A water-loss assay was conducted as follows. Young fully expanded leaves of the transgenic and Wt lines were detached and placed on a filter paper under white florescent light and weighed periodically every 1 h for 5 h. The percentage of decreases in the fresh weight was expressed as the percentage water loss, and the stomatal conductance and transpiration rate were detected using the PP Systems-CIRAS-2 Portable Photosynthesis System according to the manufacturer's protocol. The stomatal aperture was measured as previously described by Zou *et al*. (2010).

Microarray hybridization analysis

When the OE *SpUSP* and Wt lines reached the six-leaf stage, drought stress was imposed by withholding water but the control plants were watered as usual. The total RNA extracted from the third leaf was sent for microarray hybridization using the tomato TOM2 oligo microarray, including 12 000 elements (CapitalBio Corp., China). The unigenes with changes higher or lower than 2-fold were considered as differentially expressed genes. These unigenes were converted to their corresponding probe ID and annotated using the online software Plant MetGenMAP (http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi) (Joung *et al*., 2009). Some unannotated unigenes were further analysed using the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Results

Structure of the SpUSP *gene*

Based on previous microarray results (Gong *et al*., 2010), a differential expression drought-responsive USP gene (SGN-U214690, http://solgenomics.net) was identified. The unigene information and full-length cDNA from KafTom (http://www.pgb.kazusa.or.jp/kaftom/) were successfully employed to design the gene amplification primer USP1-F/R (see Supplementary Table S1 at *JXB* online) and obtain the full-length cDNA of *SpUSP*. This cDNA was 572 bp in length and included a 438 bp open reading frame, a 91 bp 5'-untranslated region, and a 43 bp 3'-untranslated region. A BLAST homology search of the tomato genome database (http://solgenomics.net/) revealed that the *SpUSP* gene was located in chromosome 1. The genomic DNA of *SpUSP* was cloned using the same primers amplified for cDNA to investigate the gene structure. The sequence alignments between the genomic DNA and cDNA showed that the gene contained three exons separated by two introns (see Supplementary Fig. S1 at *JXB* online). *SpUSP* was predicted to encode a protein of 145 amino acids, with a molecular weight of 16.2 kDa and an isoelectric point of 5.9. A protein BLAST search of GenBank using SpUSP as a probe revealed the best homologue from grape (GenBank accession no. XP_002266746) with 48% amino acids identities, and 44% identities from *Arabidopsis* (GenBank accession no. NP_566108). The alignment of the 13 best hits (one from each species) showed that a conserved USP domain was present in all sequences (see Supplementary Fig. S2 at *JXB* online). The predicted secondary structure of *SpUSP* had conserved ATP-binding regions and other binding sites, and showed very similar distributions of α-helices and β-strands to that described for the crystal structure of MJ0577.

Expression pattern of SpUSP *in tomato*

Quantitative RT-PCR was employed to investigate the expression profile of *SpUSP* in tomato tissues. *SpUSP* was highly expressed in the leaf but barely in the root, although *SpUSP* expression was detected in all organs tested (Fig. 1A). Similar expression patterns were found in the wild relative *S. pennellii* LA716 and in cultivated tomato M82. A relatively higher expression level was detected in the stem of LA716 than M82 compared with other tissues. Noticeably, during the day/night cycles, the *SpUSP* transcripts exhibited maximum expression in the afternoon, some fluctuations at night, and minimumal expression in the morning (Fig. 1B).

Further investigation using the *SpUSP* promoter::GUS system showed that GUS fluorescence was mainly detected in leaves with very low expression in roots. These results agree with the expression pattern of *SpUSP* examined using qRT-PCR. High GUS fluorescence was also detected in the stomata and trichomes of the leaf epidermis (Fig. 1C). In addition, the subcellular localization of SpUSP protein was determined. The green fluorescence of *35S*::*SpUSP*::*GFP* was exclusively detected in the nucleus and cell membrane, whereas the cells transformed with the vector containing GFP alone displayed fluorescence throughout the cells (Fig. 1D). The promoter of *SpUSP* was cloned and analysed. Some *cis*-acting regulatory elements involved in the light response, circadian control, and the phytohormone response, such as ABA, MeJA, GA_3, and abiotic stress (e.g. MYB binding site and heat stress response), were found in the *SpUSP* promoter region (see Supplementary Fig. S3 at *JXB* online).

SpUSP *expression was induced by various stress conditions and hormones*

The expression patterns of *SpUSP* were investigated under different stress and hormone treatments using qRT-PCR assays. The *SpUSP* gene was significantly induced by some stress conditions (Fig. 2). Under drought stress, the *SpUSP* transcripts initially accumulated to 15-fold after 6 h and then decreased after 12 h. Under high salt stress (200 mM NaCl), *SpUSP* expression increased after 1, 6, and 10 h, but was unchanged after 3 h. Wounding resulted in a quick increase and then a decrease in the expression level of *SpUSP*. Interestingly, after 100 μM paraquat (oxidation-inducing agent) treatment, the *SpUSP* transcripts declined within 3 h and then increased after 12 h. Subjecting the tomato plants to cold stress (4 °C) resulted in the accumulation of *SpUSP* transcripts by 15-fold after 6 h. On the other hand, heat stress (40 °C) up-regulated the expression by approximately 4-fold after 6 h and stabilized it after 12 h. Regarding phytohormone treatments, the *SpUSP* transcripts rapidly increased up to 24-fold within 12 h with ABA (100 μM), gradually increased with ETH (100 μM), and had a limited effect with GA_3 (100 μM). Overall, the ABA and drought stresses exerted the strongest effects on all hormones or stressors.

OE of SpUSP *increased abiotic stress tolerance in tomato*

To evaluate the abiotic tolerance of *SpUSP*, three homozygous OE lines of *SpUSP* (OE44, OE54, and OE69) with a high transcript level were selected (Fig. 3A). Abiotic stress tolerance tests were performed with mannitol (200 mM) or NaCl (100 mM) in MS medium at the seedling stages. ZS6 was used as the control. Under mannitol stress, the seedling length decreased by 52% for Wt and only by 28–34% for the three *SpUSP* OE lines. The seedling weight decreased by 56% for Wt and only by 34–47% for the OE lines (Fig. 3B; see Supplementary Fig. S4 at *JXB* online). Under NaCl stress, the seedling length decreased by 32% for Wt and 15–20% for the OE lines, whereas seedling weight decreased by 53% for Wt and 30–41% for the OE lines (Figs 3C). Under ABA (3 μM) treatment, the seedling length and weight of the OE lines decreased by 30%, whereas those of the Wt line decreased by approximately 10% (Fig. 3D; see Supplementary Fig. S5 at *JXB* online). After the different treatments, significant differences were found in the seedling length and weight between the OE and Wt lines, except for a few cases.

The *SpUSP* OE and Wt lines were also challenged with drought stress by withholding watering in adult plants. After 3 weeks without watering, the three *SpUSP* OE lines (OE44, OE54, and OE69) showed only mild wilting, whereas severe wilting occurred in the Wt plants (Fig. 4A). After undergoing three cycles of drought and recovering, approximately 80% of the *SpUSP*-overexpressing plants survived, whereas all Wt plants died.

Elevated ABA contents increased stomatal closure in drought condition

The endogenous ABA content of 2-month-old *SpUSP*-overexpressing and Wt plants in well-watered or without watering conditions for 7 d were measured. No significant difference was found between the transgenic and Wt plants under the well-watered conditions. Under drought stress, the ABA content in the transgenic lines increased from 53% to 126%, whereas that in the Wt plants increased by only 20% (Fig. 5A, 5B). The expression of *NCED3*, a key gene in ABA biosynthesis, was also assessed under drought stress. Its expression was significantly higher in the transgenic lines than in the Wt plants (Fig. 5C).

In addition, a drought test was performed *in vitro* to investigate whether the elevated ABA content affected stomatal closure. The third leaf from the bottom was detached and stored for over 5 h at room temperature. The water loss rate was assayed in the Wt and three transgenic lines *in vitro* every hour for 5 h. The water loss rate of the transgenic plant leaves was slower than that of the Wt plant leaves (Fig. 6A). The transpiration rate and stomatal conductance of the transgenic plants were significantly reduced compared with those of the Wt (Fig. 6B, 6C). Furthermore, the stomatal apertures of OE44 and Wt were investigated. Under drought stress, the stomatal aperture of the OE44 lines was significantly smaller than that of the Wt line (Fig. 6D). These results further confirm the notion that *SpUSP* functions in drought stress by regulating stomatal movements to reduce water loss.

SpUSP *improves photosynthesis and alleviates oxidative stress during drought stress*

The chlorophyll content and biomass of the *SpUSP* OE lines grown under optimal (at 100% FC) and reduced watering regimes

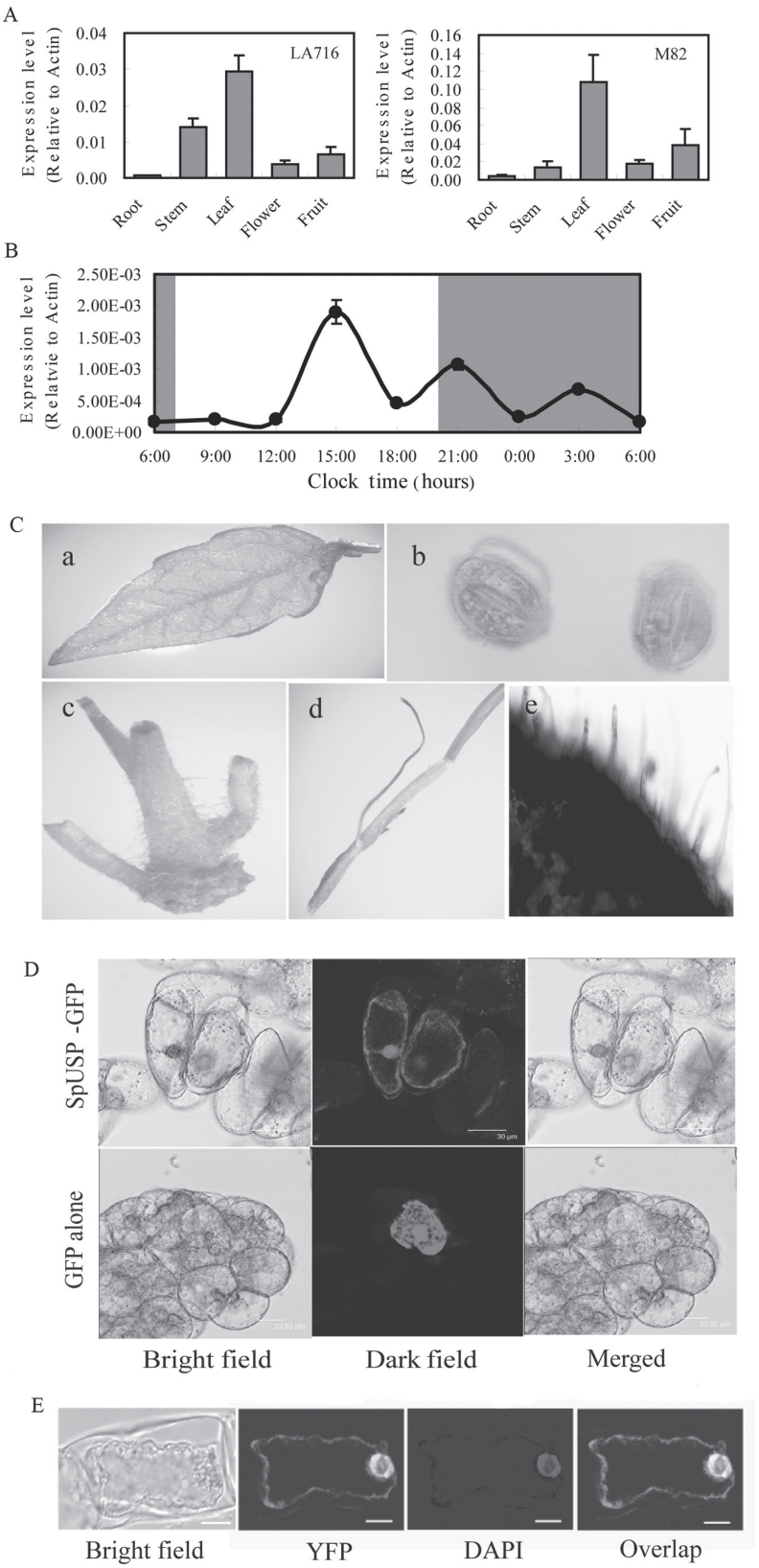

Fig. 1. Expression patterns of SpUSP. (A) Tissue profiling analysis of SpUSP in different organs of wild tomato LA716 (Solanum pennellii) and cultivated tomato M82 (S. lycopersicum) using qRT-PCR. (B) Expression pattern of SpUSP during a 24 h period. Leaf samples were collected every 3 h for 24 h starting from 06.00 h. (C) Expression patterns of SpUSP via GUS staining: (a) leaf, (b) stoma, (c) stem, (d) root, and (e) trichome. (D) Subcellular localization of SpUSP. The photographs were taken under bright light, in the dark field for the GFP-derived green fluorescence and merged respectively. (E) Interaction of SpUSP with annexin via BiFC. The photographs were taken under bright light, in the dark field for YFP-derived green fluorescence, staining with DAPI and overlap, respectively. Scale bars=10 μm.

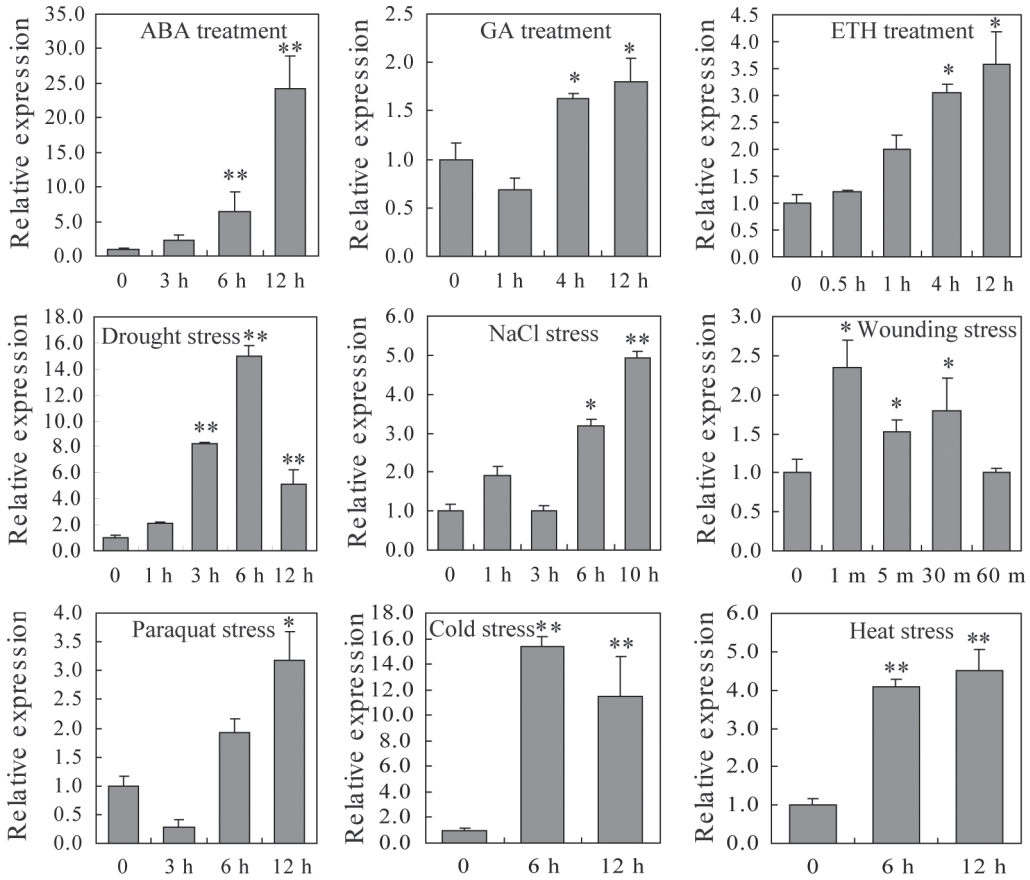

Fig. 2. Expression levels of *SpUSP* in tomato leaves under phytohormones and different stress conditions. The leaves of 2-month-old plants were used for RNA extraction in LA716 (*Solanum pennellii*) after treatment with 100 μM ABA, 100 μM GA, and 100 μM ETH, drought, 200 mM NaCl, wounding, 100 μM paraquat, 4 °C cold, 40 °C heat, respectively. All samples were collected at the indicated time points ('h' and 'm' refer to hours and minutes after treatment, respectively) from three biological replicates. Single (*P <0.01) and double (**P <0.01) asterisks denote statistically significant differences between the stress treatment and the 0 h control. The β-actin gene (BT013524) was used as an internal control in the qRT-PCR.

(at 25% FC) were measured. Under the optimal watering regime, the biomass and chlorophyll contents did not differ between the transgenic and Wt plants. However, reduced watering for one month severely affected the biomass and chlorophyll contents of the Wt plants. The biomass of Wt plants was decreased by approximately 60%, whereas that of the transgenic plants was decreased by only about 25% (Fig. 4B). The decrease in biomass was significantly different between the OE lines and ZS6. Under drought stress, the chlorophyll content of the Wt plants significantly decreased with prolonged drought duration, whereas that of the transgenic plants remained at a high level (Fig. 4C).

Small biomolecules, including proline, soluble sugars, and MDA, are important indicators of plant oxidative stresses. Under drought stress, the proline concentrations in the transgenic lines OE44 and OE54 were 3–4-fold higher than those in the control, whereas no significant difference was observed under well-watered conditions (Fig. 4D). The expression level of *P5CS*, a key gene in proline biosynthesis, was significantly higher in the *SpUSP*-OE plants than the Wt (Fig. 4E). Similarly, the concentration of soluble sugars increased by up to several fold in the transgenic plants, especially for the OE44 and OE69 lines (Fig. 4F). By contrast, MDA increased significantly in the drought-stressed Wt compared with the *SpUSP*-OE plants (Fig. 4G).

Under oxidative stress, ROS can cause cell damage. To determine whether the ectopically expressed *SpUSP* affected ROS production, leaves of the transgenic and Wt lines were detached. After 5 h of drought *in vitro*, the Wt leaves appeared to wilt more than the transgenic ones. DAB staining showed the Wt plants accumulated more hydrogen peroxide than the transgenic ones under drought stress (see Supplementary Fig. S6A at *JXB* online). When paraquat was spread directly onto the leaves to induce ROS production, although the leaves showed deeper brown spots than those under drought stress in both transgenic and Wt plants, the leaves of the transgenic plants accumulated much less hydrogen peroxide compared with the Wt plants (see Supplementary Fig. S6B at *JXB* online). Hence, *SpUSP* can alleviate excess ROS-induced oxidative damage and thus improve oxidative stress tolerance.

SpUSP interacted with annexin in vivo

The *SpUSP* full-length cDNA was used as the bait in the Y2H to explore its interacting proteins. After screening, 20 sequences were

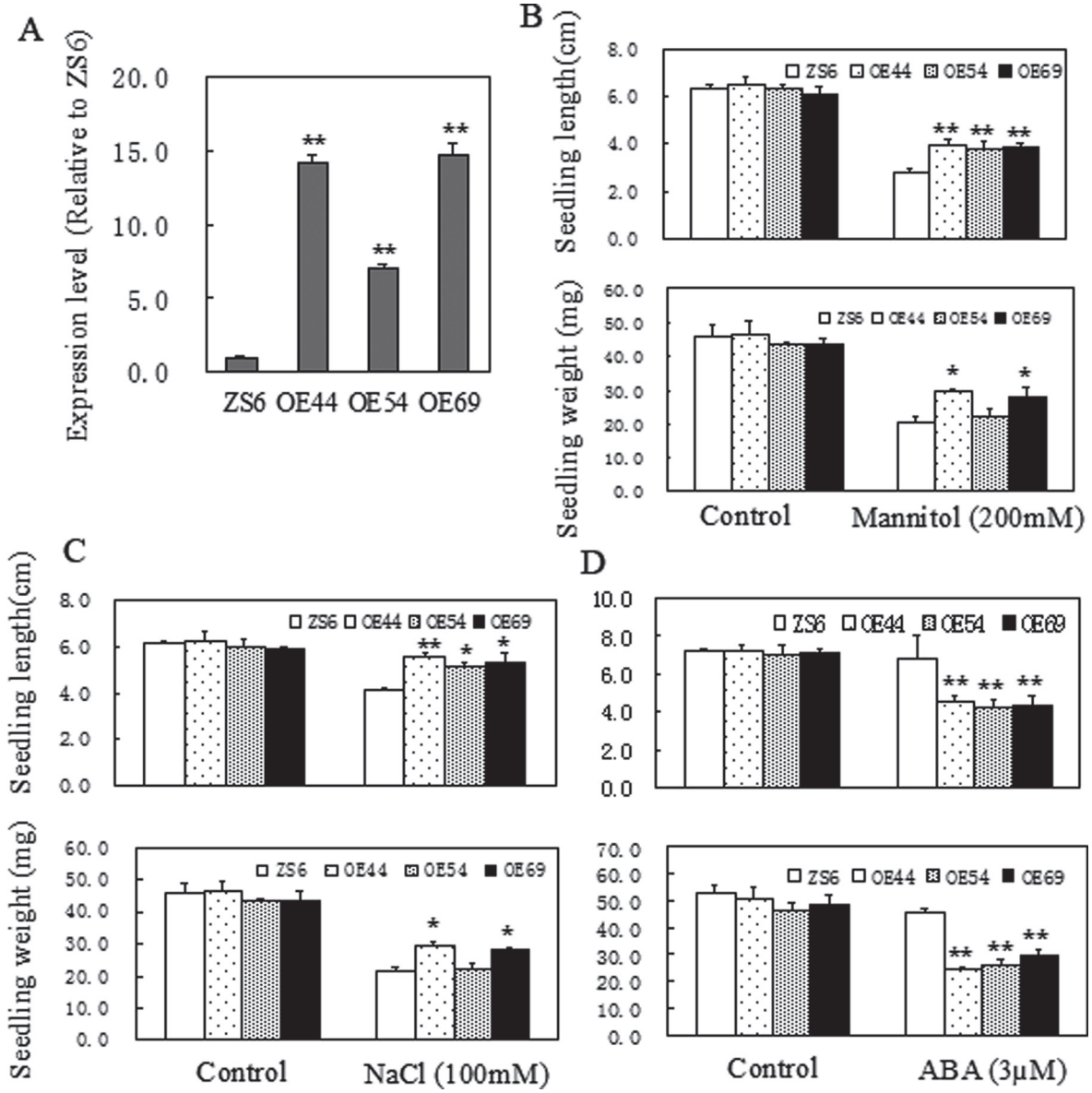

Fig. 3. Growth performances of OE *SpUSP* and wild-type seedlings treated with mannitol, salt, and ABA stresses. (A) Analysis of *SpUSP* transcriptional expression via qRT-PCR in overexpressing (OE44, OE54, and OE69) and wild-type (ZS6) lines. Seedling lengths and weights of transgenic and wild-type lines after treatment with 200 mM mannitol (B), 100 mM NaCl (C), and 3 μM ABA (D), and without stress as a control. The seedlings were grown in half-strength MS medium. The data shown are the mean ±SE (n= 6). Single (*P <0.05) and double (**P <0.01) asterisks denote statistically significant differences between transgenic and wild-type lines.

obtained from 40 positive clones encoded with 11 distinct entities. The database search revealed that seven of them were related to abiotic stress (see Supplementary Table S2 at *JXB* online). Based on the role of *Arabidopsis AnnAt1* in drought tolerance (Konopka-Postupolska *et al*., 2009), the annexin gene *AnnSp2* was selected as the top candidate for interaction with *SpUSP*. *AnnSp2* has more than 98% similarity with the annexin p35 gene (Lim *et al*., 1998) in tomato, and has the highest similarity of 71% with *AnnAt2* in *Arabidopsis*. To confirm the *SpUSP–AnnSp2* interaction, BiFC was performed using tobacco cells. The cells co-transfected with *SpUSP–YFPC* and *AnnSp2–YFPN* displayed strong yellow fluorescence that was mainly accumulated in the nucleus and plasmalemma (Fig. 1E). Hence, the interaction between *SpUSP* and *AnnSp2* occurred in both the nucleus and cell membrane, in agreement with the result of *SpUSP* subcellular localization.

Overall gene expression changes in SpUSP-*overexpressing plants*

The gene expression profile of the *SpUSP*-overexpressing lines was compared with that of the Wt under both normal and drought-stress conditions using the tomato TOM2 microarray (12 000 probes). Under normal conditions, 259 genes were detected to have more than a 2-fold change in the *SpUSP*-overexpressing line OE44 (123 genes were up-regulated and 136 were down-regulated; see Supplementary Table S3 at *JXB* online)

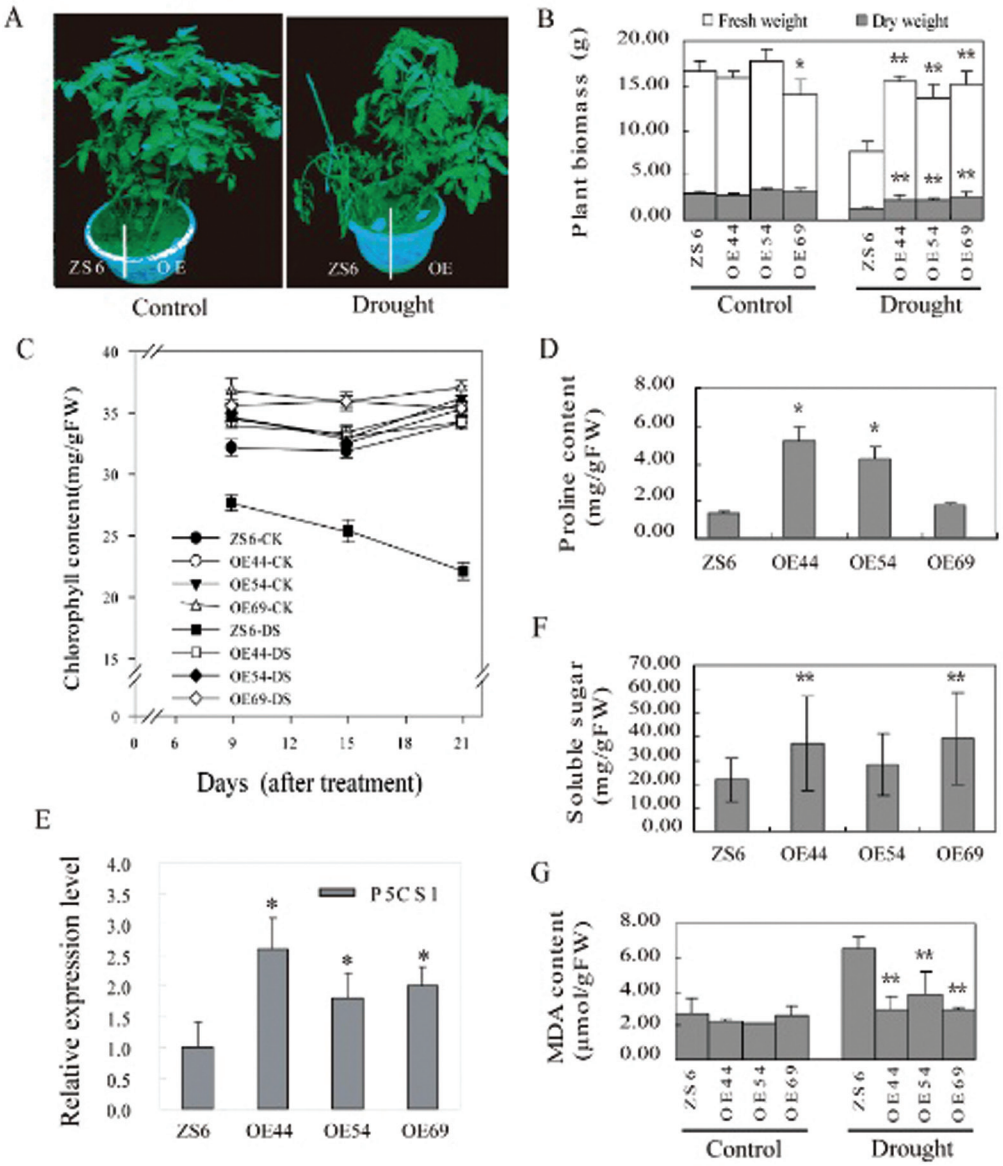

Fig. 4. *SpUSP* overexpression enhances drought tolerance in tomato. (A) Drought tolerance tests for *SpSUP*-overexpressing (OE44, OE54, and OE69) and wild-type ZS6 plants grown in the same pot. The phenotypes under well-watered ('Control') and drought-stress conditions ('Drought') are shown.. (B) Effects of drought on the fresh and dry weights of the OE and wild-type lines. (C) Chlorophyll contents in plant leaves under drought stress ('DS') or normal condition ('CK'). (D) Proline accumulation in plant leaves under drought stress. (E) Relative expression levels of P5CS1 in the OE and wild-type lines under drought stress via qRT-PCR. (F) Soluble sugar content in plant leaves under drought stress. (G) MDA content in plant leaves under stress ('Drought') or normal condition ('Control'). The data shown are the mean ±SE (n=3). Single (*P <0.05) and double (**P <0.01) asterisks denote statistically significant differences between the transgenic and wild-type lines under drought stress.

compared with the Wt. However, under the drought stress condition, 856 genes were detected to have more than a 2-fold change (479 genes were up-regulated and 377 were down-regulated; see Supplementary Table S4 at *JXB* online).

Many of the differentially expressed genes were functionally unknown. However, some photosystem-related genes were up-regulated (see Supplementary Table S5 at *JXB* online), involved in the entire photosystem process, including the main photosynthetic apparatus components (27 chlorophyll *a*/*b*-binding proteins and 11 photosystems I and II reaction centre subunits or proteins). The expression levels of seven ATP synthases and a cytochrome P450 gene were also changed. Few changes were observed in the microarray data of the well-watered tomato.

Several abiotic stress-responsive genes were also changed based on the microarray results, such as L-ascorbate oxidase (SGN-U219080), osmotin-like protein (SGN-U213934 and SGN-U212927), two NADP-malic enzymes (SGN-U232389 and SGN-U213228), calcium-dependent protein kinase 4 (SGN-U224039), aquaporin (SGN-U221263), MYB (SGN-U226315), ERD1-like (SGN-U216637), HSP (SGN-U220044), serine/threonine protein kinase (SGN-U225125), and bZIP transcription factor (SGN-U213708).

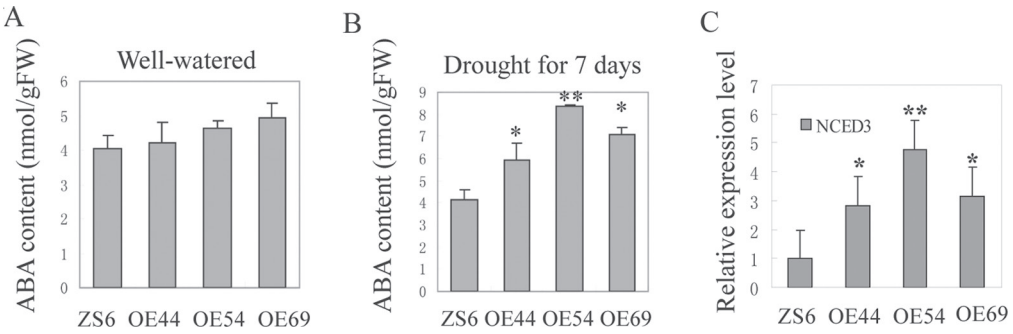

Fig. 5. Endogenous ABA content in *SpUSP*-overexpressing and wild-type 2 month-old plants measured by HLPC. (A) ABA content in well-watered condition. (B) ABA content after withholding water for 7 d. (C) Relative expression level of *NCED3* in OE and wild-type (ZS6) lines under drought stress via qRT-PCR. Variance analysis was performed to determine significant differences (*P <0.05 and **P <0.01) between the ZS6 and OE lines.

Discussion

USP, first identified in *E. coli* (Nystrom and Neidhardt, 1992), reportedly plays an important role in stress adaptation (Nachin *et al*., 2005). The present study explored the function of the *SpUSP* gene in the abiotic stress of tomato. *SpUSP* has low sequence similarity with the USPs of other species, the highest being only 48% with a USP of grape (GenBank accession no. XP_002266746), thus implying it is a novel USP gene. In plants, only a few homologues of the USP family have been isolated (Chou *et al*., 2007; Maqbool *et al*., 2009), and their functions remain unclear. The present study demonstrates that *SpUSP* plays a critical role in abiotic stress, particularly in drought tolerance in tomato, as supported by data from overexpressing lines.

SpUSP *expression is regulated by various stress and photoperiodic conditions*

SpUSP expression is regulated in response to abiotic stress conditions and several hormones, specifically for ABA. ABA mediates the core signalling network in the plant abiotic-stress response (Cutler *et al*., 2010). The ABA-responsive element (ABRE) widely exists in the promoters of ABA-induced genes. These promoters function in ABA-dependent gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). An ABRE element was also found in the *SpUSP* promoter region, and ABA treatment *in vitro* induced high expression levels of *SpUSP*. This result indicates that *SpUSP* is probably ABA dependent. Other abiotic stress-response elements such as the heat shock element and recognition sites for MYB were also found in the *SpUSP* promoter region. These *cis*-elements are the core elements for stress response (Urao *et al*., 1993). Surprisingly, *SpUSP* expression changed with the circadian rhythm and coincided with stomatal movement in a 1 d cycle. A *cis*-acting regulatory element involved in the circadian control (circadian CAACAGCATC) was found in its promoter region. Several other genes involved in stomatal movement have been affected by photoperiodic rhythms such as *AnnAt1* and *AnnAt4* (Huh *et al*., 2010), *PHOT1* and *PHOT2* (Kinoshita *et al*., 2001), and *AtMYB60* (Cominelli *et al*., 2005).

SpUSP-*mediated ABA signals enhance drought resistance by reducing the stomatal opening*

Plants can reduce water loss through stomatal closure and transpiration inhibition. Stomatal movement is generally regulated via the ABA signalling pathway (Jung *et al*., 2008; Zou *et al*., 2010). ABA treatment stimulates stomatal closure in leaves and enhances resistance to drought in myb96-overexpressing *Arabidopsis* (Seo *et al*., 2009). A positive correlation is found between the ABA level and enhanced abiotic stress tolerance (Lee *et al*., 2006). In the present study, the increased ABA content and transcripts of *SpUSP* in the stomata lead to less stomatal aperture, suggesting that *SpUSP* has a function in regulating stomatal opening and thus improves drought resistance by ABA. The transcript accumulations of some genes in guard cells also mediate abiotic stress responses by ABA (Zhang *et al*., 2007; Jung *et al*., 2008; Seo *et al*., 2009). Recently LHCB members have been identified as new players in ABA signalling in stomatal movement in *Arabidopsis*. The down-regulation of any member of the LHCB family reduces the responsiveness of stomatal movement to ABA thereby resulting in decrease in drought tolerance in *Arabidopsis thaliana* (Xu *et al*., 2012). The up-regulation of many LHCB members in *SpUSP*-OE lines (see Supplementary Table S5 at *JXB* online) probably contributes to enhanced stomatal sensitivity to ABA.

Interestingly, exogenous ABA application reduced the seedling size of the *SpUSP* overexpressing lines compared with the Wt, indicating that *SpUSP* may be related to ABA signalling in seedling growth regulation. The seed germination of the *SpUSP* overexpression lines was also significantly inhibited in ABA-containing media. The sensitivity of seed germination and growth to ABA has also been reported in *Arabidopsis* showing enhanced drought tolerance due to the OE of *MYB15* and *MYB96* (Ding *et al*., 2009; Seo *et al*., 2009).

Abiotic stress tolerance of SpUSP *is involved in the improvement of photosynthesis*

Under drought stress, stomatal closure reduces CO_2 availability, energy balance has been recognized as a key component of cell function under a limited supply of CO_2 (Lawlor and Tezara, 2009; Pfannschmidt *et al*., 2009). LHCB is the main photosynthetic

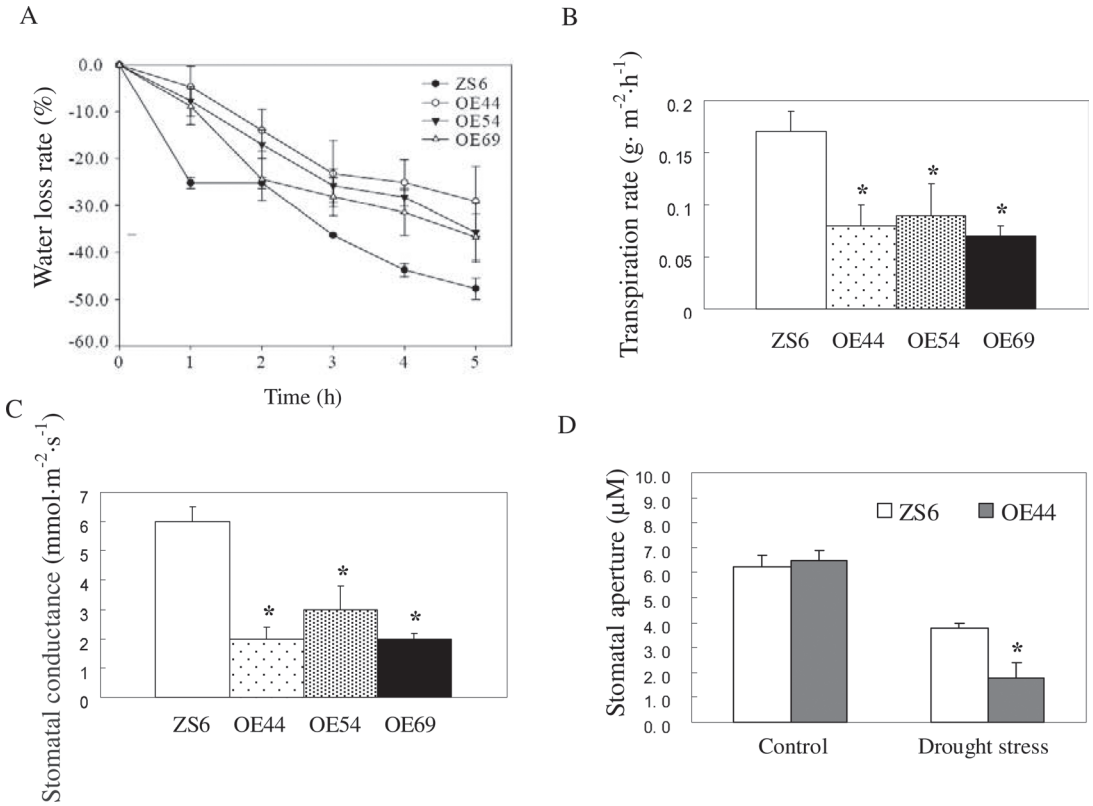

Fig. 6. Water loss test for the detached leaves excised from the wild type and transgenic plants. Variance analysis was performed to determine significant differences (*P <0.05) between the detached leaves of the ZS6 and OE lines under drought stress. (A) Water loss test of the detached leaves for 5 h. The detached leaves were collected from mature plants from at the same position and placed on a Wattman paper at room temperature. The mean weight was measured every hour. (B) Transpiration rate and (C) stomatal conductance of the detached leaves. The two parameters were detected after the detached leaves were placed on a filter paper and exposed under white florescent light for 5 h. (D) Stomatal aperture of transgenic and wild-type plants under drought stress. Leaves were detached from 2-month-old transgenic (OE44) and wild-type (ZS6) plants and subjected to drought stress for 5 h *in vitro*. The lower surfaces of the leaves with or without stress were examined under a microscope.

component that can mediate the distribution of excitation energy between photosystems I and II to balance photosynthesis (Asada, 2006). In the present microarray data, many chlorophyll *a*/*b*-binding proteins were up-regulated under drought stress in the *SpUSP*-OE lines. LHCB regulation is considered to be one of the most important mechanisms of plants in modulating chloroplast functions (Pruneda-Paz and Kay, 2010; Thines and Harmon, 2010). These up-regulated LHCBs possibly keep the PSII antenna complex intact and ensure its functional involvement in photosynthesis.

Several ATP synthase subunits that can enhance ATP production and provide chemical energy for plant growth were up-regulated. The importance of ATP synthase in photosynthetic regulation is well recognized (Wu *et al*., 2007). *SpUSP* is predicted to contain ATP binding residues similar to the bacterial MJ0577-type proteins (Zarembinski *et al*., 1998), suggesting *SpUSP* is an ATP-mediated molecular switch. UspA protein is described as an autophosphorylating serine and threonine phosphoprotein that uses either GTP or ATP as phosphate donors in *E. coli* (Freestone *et al*., 1997). Phopshorylation may be related to the function of the STK-N domain predicted in *SpUSP*. Thus, the regulation of LHCB and ATP may be involved in the improvement of photosynthesis in the *SpUSP* OE lines.

Overexpressing SpUSP *alleviates oxidative stress in tomato*

Drought is a kind of oxidative stress in plants. Some osmoprotectants including proline, soluble sugars, and oxidative-stress biohazards can be used as biochemical markers to indicate the oxidative condition. Proline accumulates in plants as an osmoprotectant under a wide range of biotic and abiotic stresses (Ashraf and Harris, 2004; Verbruggen and Hermans, 2008). In the present study, the amount of free proline in transgenic lines was higher than that in non-transgenic plants under drought stress (Fig. 4D). The high proline content may be involved in the enhanced expression of the *P5CS1* gene. In drought stress, the elevated expression of *P5CS1* improves drought tolerance in tobacco and *Arabidopsis* (Huh *et al*., 2010; Ziaf *et al*., 2011). In addition, *SpUSP* was also significantly induced by paraquat, an inducer of ROS. DAB staining results under drought and paraquat stress conditions suggest that *SpUSP* plays an important role in alleviating oxidative stress.

Oxidation-related genes have been extensively studied under drought stress at the molecular level (Allen and Tresini, 2000). In

the present microarray results, some genes involved in alleviating oxidative stress were changed, such as osmotin-like proteins (SGN-U213934 and SGN-U212927) that reportedly participate in defence response in potato (Castillo *et al*., 2005). Two NADP-malic enzymes (SGN-U232389 and SGN-U213228) whose homologues from rice confer salt tolerance in transgenic *Arabidopsis* (Cheng and Long, 2007) were changed. Some other changed genes such as ERD1-like (SGN-U216637), HSP (SGN-U220044), serine/threonine protein kinase (SGN-U225125), and bZIP transcription factor (SGN-U213708) are all involved in the alleviation of oxidative stress (Ziaf *et al*., 2011; Jiang *et al*., 2009; Mao *et al*., 2010; Orellana *et al*., 2010).

SpUSP *functions together with annexin in drought-stress signalling*

Annexins have been considered as targets of Ca^{2+} signals in eukaryotic cells and deemed to play an important role in plant stress responses (Mortimer *et al*., 2008; Laohavisit *et al*., 2009). Increased calcium influx and cytoplasmic Ca^{2+} are important in guard cell ABA signal transduction (Mcainsh *et al*., 1990; Roelfsema and Hedrich, 2010). The overexpression of annexin (*AnnAt1*) helps eliminate ROS and improve drought tolerance in *Arabidopsis* (Konopka-Postupolska *et al*., 2009). In the present study, *SpUSP* was also induced by ABA and paraquat (inducer of ROS), and the OE of *SpUSP* showed stronger antioxidative ability and drought tolerance. These functions of *SpUSP* agree well with those attributed to annexin. Thus, the interaction between the two proteins is suggested to be indeed dependable, and the stomatal closure induced by *SpUSP* probably involves Ca^{2+} signalling, although further confirmation is needed.

How does SpUSP *work in drought-tolerance improvement?*

The mechanism of the *SpUSP:AnnSp2* complex in alleviating drought stress may be highly complex. When the tomato plants were exposed to the abiotic stresses of drought and salt, the ABA content significantly increased in *SpUSP* OE plants. Drought tolerance mediated by elevated ABA may be attributed to the following aspects. First, OE *SpUSP* increases the expression of LHCB under drought stress, which probably enhances stomatal sensitivity to elevated ABA. The stomatal aperture is then reduced, which is likely to be the major factors contributing to the drought tolerance of *SpUSP*. Second, under less stomatal aperture condition, the higher expression of LHCBs and photosystem-related genes keeps the PSII antenna complex intact and maintains normal photosynthesis in *SpUSP* OE plants. Finally, *SpUSP* complex proteins activate some stress-responsive genes, which lead to the accumulation of some osmoprotective solutes to alleviate oxidative stress by eliminating ROS production. Altogether, these various mechanisms result in enhanced drought tolerance in tomato *SpUSP* OE lines.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1 List of primers used in this study.

Supplementary Table S2 *SpUSP*-interacting proteins identified by yeast two-hybrid screening.

Supplementary Table S3. A complete list of genes with expression levels that changed more than 2-fold under a well-watered condition in transgenic plants overexpressing *SpUSP*, as compared with the non-transformed wild-type control.

Supplementary Table S4. A complete list of genes with expression levels that changed more than 2-fold under drought stress in transgenic plants overexpressing *SpUSP*, as compared with the non-transformed wild-type control.

Supplementary Table S5. A list of genes that underwent changes in association with photosynthesis under drought stress in *SpUSP*-overexpressing plants compared with the non-transformed wild-type control.

Supplementary Fig. S1. *S*tructure of the *SpUSP* gene.

Supplementary Fig. S2. Multiple sequence alignment and phylogenetic tree analysis of USPs and USP-like proteins from different species.

Supplementary Fig. S3. *Cis*-acting element analysis of *SpUSP* promoter.

Supplementary Fig. S4. *SpUSP* overexpression improves the growth performance of seedlings under osmotic stress.

Supplementary Fig. S5. *SpUSP* overexpression affects the growth performance of seedlings under ABA treatment.

Supplementary Fig. S6. Oxidative stress assay on the leaves of transgenic and wild-type plants

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