Plant Cell Reports (2019) 38:869–882
https://doi.org/10.1007/s00299-019-02408-y

ORIGINAL ARTICLE

The trehalose-6-phosphate synthase TPS5 negatively regulates ABA signaling in *Arabidopsis thaliana*

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Received: 2 January 2019 / Accepted: 27 March 2019 / Published online: 8 April 2019
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Abstract

Key message **The TPS5 negatively regulates ABA signaling by mediating ROS level and NR activity during seed germination and stomatal closure in *Arabidopsis thaliana.***

Abstract Trehalose metabolism is important in plant growth and development and in abiotic stress response. Eleven *TPS* genes were identified in *Arabidopsis*, divided into Class I (*TPS1–TPS4*) and Class II (*TPS5–TPS11*). Although Class I has been shown to have TPS activity, the function of most members of Class II remains enigmatic. Here, we characterized the biological function of the trehalose-6-phosphate synthase TPS5 in ABA signaling in *Arabidopsis*. *TPS5* expression was induced by ABA and abiotic stress, and expression in epidermal and guard cells was dramatically increased after ABA treatment. Loss-of-function analysis revealed that *tps5* mutants (*tps5-1* and *tps5-cas9*) are more sensitive to ABA during seed germination and ABA-mediated stomatal closure. Furthermore, the H_2O_2 level increased in the *tps5-1* and *tps5-cas9* mutants, which was consistent with the changes in the expression of *RbohD* and *RbohF*, key genes responsible for H_2O_2 production. Further, *TPS5* knockout reduced the amounts of trehalose and other soluble carbohydrates as well as nitrate reductase (NR) activity. In vitro, trehalose and other soluble carbohydrates promoted NR activity, which was blocked by the tricarboxylic acid cycle inhibitor iodoacetic acid. Thus, this study identified that TPS5 functions as a negative regulator of ABA signaling and is involved in altering the trehalose content and NR activity.

Keywords ABA · TPS5 · NR activity · Stomatal closure · Seed germination

Abbreviations

ABA	Abscisic acid
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
NR	Nitrate reductase
ROS	Reactive oxygen species
T6P	Trehalose-6-phosphasynthetase

Introduction

Trehalose, a non-reducing disaccharide consisting of two glucose molecules linked by 1,1-glycosidic bonds, is synthesized in a two-step reaction from UDP-glucose (UDPG) and 6-phospho-glucose (G6P) by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) in plants (Eastmond and Graham 2003). Although trehalose is ubiquitous in bacteria, yeast, fungi, nematodes, and insects, serving as a reserve carbohydrate and stress protection (Crowe et al. 2003; Goddijn et al. 1997), its level in plants is extremely low and it was once thought to occur only in drought-tolerant plants (Joachim et al. 1995). Following whole-genome sequencing, 11 TPSs and 10 TPPs were identified in *Arabidopsis* (Genome 2000; Leyman et al. 2001). The TPS proteins, carrying a TPS- and a TPP-like domain,

Communicated by Prakash P. Kumar.

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00299-019-02408-y) contains supplementary material, which is available to authorized users.

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are further divided into Class I (TPS1–TPS4) and Class II (TPS5–TPS11) based on their homology with the yeast TPS (ScTps1) and TPP (ScTps2) proteins, respectively; the TPP proteins display significant similarity to the highly conserved phosphatase box elements of ScTPS2 (Leyman et al. 2001; Ramon et al. 2009).

Class I (e.g., TPS1), which has been shown to have TPS activity in vitro, is well characterized (Blázquez et al. 1998; Gomez et al. 2010a, b). Studies have shown that the *TPS1* gene is essential for normal growth, especially in flowers and buds, in embryogenesis and in ripening fruits (Eastmond et al. 2002; van Dijken et al. 2004; Wahl et al. 2013). Beyond being closely linked to growth and development in plants, *TPS1* is highly expressed in guard cells and the *tps1-12* mutant, which carries a weak allele of the *AtTPS1* gene, has a smaller stomatal aperture than wild-type plants (Gomez et al. 2010a, b). In addition, constitutive overexpression of TPS1 reduces the sensitivity of seed germination to abscisic acid (ABA) (Avonce et al. 2004; Van Houtte et al. 2013; Vandesteene et al. 2012). These results suggest that TPS1 is also involved in ABA signaling.

Interestingly, although most of the functions of Class II TPS remain unclear, many of the Class II genes appear to be extensively regulated at the transcriptional level by abiotic stress (Baena-Gonzalez et al. 2007; Bates et al. 2012; Scheible et al. 2004; Usadel et al. 2008; Wang et al. 2003); TPS6 was reported to regulate plant architecture, epidermal pavement cell shape, and trichome branching (Chary et al. 2008); TPS5 showed stomatal function effects without a change in stomatal density (Bates et al. 2012), and appears to be involved in thermotolerance, possibly by interaction with the transcriptional co-activator MBF1c (Suzuki et al. 2008); Ramon used a set of promoter-GUS protein reporters to demonstrate that Class II proteins have a significantly different tissue-specific expression and responsiveness to carbon availability and hormones (Ramon et al. 2009). Very recently, a rice TPS (OsTPS8) was reported conferring salt stress tolerance and controlling yield-related traits in rice (Vishal et al. 2019). In summary, evidence is increasing that Class II proteins play an important role in many key processes of plant growth and abiotic stress response.

In nature, plants face many challenges from the environment. Trehalose metabolism is considered an important pathway for plants to improve stress tolerance. Trehalose is reported to be an essential virulence factor for the rice blast fungus, *Magnaporthe oryzae* (Foster et al. 2014). T6P, the intermediate in trehalose biosynthesis, was found to play a possible role in ABA-mediated regulation of stomatal conductance, involving covalent modifications of nitrate reductase (NR) and the sucrose nonfermenting-1-related protein kinase2.6 (SnRK2.6) (Chen et al. 2016; Emanuelle et al. 2015; Figueroa and Lunn 2016).

TPS5 expression is closely related to ABA-induced and abiotic stress. *tps5* mutants (*tps5-1* and *tps5-cas9*) are more sensitive to ABA during seed germination and ABA-mediated stomatal closure. The obvious question that arises is how TPS5 could be involved in the regulation of ABA signaling.

As a further step towards the elucidation of TPS5 exact functions in ABA signaling, the level of H_2O_2 and ABA-responsive genes, such as RbohD and RbohF, were analyzed. In addition, the amounts of trehalose and other soluble carbohydrates as well as NR activity in both *tps5* mutants (*tps5-1* and *tps5-cas9*) and the wild type were determined. Finally, we determined whether trehalose metabolism is directly or indirectly involved in ABA signaling. Collectively, this study will elucidate the important role of TPS5 in ABA signaling and help develop strategies to improve stress tolerance in plants for better crop production.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* (L.) Heynh. 'Columbia' and *tps5* mutant (salk_144791) used in this study were purchased from the Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/). Plant growth conditions and *Agrobacterium tumefaciens*-mediated transformation were performed as described previously (Clough and Bent 1998). The primers for identifying *tps5-1* mutants were as follows: TPS5-T-F (5′-CACTCCTCAACGCTGATTTGA-3′); TPS5-T-R (5′-ATCTTGTATAAAGCTACGCGCC-3′); LBb1.3 (5′-ATTTTGCCGATTTCGAAC-3′).

Plasmid constructs

The *TPS5* cDNA was amplified by RT-PCR using the primer pair *TPS5*-F and *TPS5*-R and ligated to the pMD18-T vector. For subcellular localization analysis, the *TPS5* cDNA was amplified without its stop codon using the primer pair *TPS5*-SF and *TPS5*-SR, subsequently digested with *BsaI*, and then inserted into transient expression vector pEZS-N. The $TPS5_{pro}$::GUS for histochemical analysis was constructed as follows: a 1.6-kb fragment upstream of the ATG start codon of *TPS5* was amplified by PCR using the primers *TPS5*-GF and *TPS5*-GR and cloned into the pMD18-T vector, subsequently digested with Pst I and BamHI, respectively, then subcloned into the expression vector pBI101.2.

TPS5-Cas9 vectors for transgenic plants were also designed. The chimeric sgRNA for *TPS5* was constructed by cloning the annealed oligos *TPS5*-sgF and *TPS5*-sgR into pBlueScript SK-sgRNA, then both the sgRNA and hSpCas9

were subcloned into the expression vector pCAMBIA1300 (Lu et al. 2017).

Stress induction and qRT-PCR analysis

The 20-day-old seedlings were subjected to various stress treatments. Drought stress was applied by removing the seedlings from the 0.5 × Murashige and Skoog (MS) medium plate and placing them on filter paper for a specified period of time; high-temperature stress was induced by transferring the seedlings from the 0.5 × MS medium into an incubator at 42 °C; 250 mM NaCl and 100 µM ABA treatments were performed as described previously (Ding et al. 2015). Total RNA was extracted with TRIzol reagent (Invitrogen) following the provider's instructions. Two micrograms of DNA-free RNA was used for reverse transcription according to the kit instructions (Invitrogen). Quantitative RT-PCR was performed on a Quant Studio 5 Real-Time PCR Instrument (96-well 0.2-ml block). The primers for RT-PCR and qRT-PCR are shown in Supplemental Table 1.

Histochemical GUS analysis

For GUS (β-glucuronidase) staining assays, transgenic plants were selected based on kanamycin resistance. The $TPS5_{pro}$ activity was analyzed via histochemical staining according to slightly modified published protocols (Li et al. 2015). Briefly, $TPS5_{pro}$::GUS transgenic plants were immersed in staining buffer containing 0.1 M phosphate-buffered sodium (pH 7.0), 10 mM EDTA, 2 mM potassium ferrocyanide, 0.1% (v/v) Triton X-100, 20% (v/v) methanol, 0.5 mM X-gluc, and incubated at 37 °C overnight. After staining, tissues were observed and photographed with an Olympus SZX12 microscope equipped with a camera.

Analysis of subcellular localization of TPS5

Subcellular localization of TPS5 was done according to slightly adjusted published protocols. The recombinant plasmids *35S::TPS5-EGFP* and *35S::BIP-RFP* (ER marker) were used to cotransfect *Arabidopsis* mesophyll protoplasts transiently. TPS5–EGFP fusion protein was excited at 488 nm using a laser, and the emitted fluorescence signal was collected with a band-pass filter at 490–540 nm (GFP panel). BIP-RFP marker was excited by laser emission at 561 nm, and the emitted fluorescence was collected with a band-pass filter at 592 nm (RFP panel) (Li et al. 2015).

Stomatal movement assay

Stomatal movement assays were performed as described previously (Ding et al. 2015) with slight modification. For ABA-induced stomatal closure experiments, 20-day-old rosette leaves were isolated and incubated in stomatal opening solution (OS solution) containing 10 mM KCl, 100 mM $CaCl_2$, and 10 mM 2-morpholinoethanesulphonic acid (MES)–KOH (pH 6.1), for 30 min in the light, and then 1 µM, 5 µM, and 10 µM ABA were added, respectively. The stomatal apertures were measured after 1 h of ABA treatment. Subsequently, the epidermis was gently torn off and mesophyll cells were carefully scraped off; the epidermal layer was mounted on glass slides and observed using an Olympus microscope.

Determination of soluble carbohydrates content

Soluble carbohydrates were detected by ion chromatography (ICS 5000, Thermo Fisher Scientific) by Shanghai Sanshu Biotechnology (www.sanshubio.com). Briefly, soluble carbohydrates were extracted with an 80% ethanol solution and shaken and mixed in a 70 °C water bath. After 2 h, the EP tube was taken out; after cooling, 700 µl of sterile water was added, and the solution was centrifuged at 1200 rps for 5 min to remove the supernatant. An aliquot of 700–1000 µl of chloroform was added to the supernatant, mixed well, and centrifuged three times. The supernatant was placed in a clean EP tube for testing. After addition of 700 µl of 80% alcohol solution to the weighed sample, it was extracted in a 70 °C water bath, and shaken 3–5 times during this period.

Construction of double mutants of *tps5* and *tps6*

First, we selected flower buds from *tps5* and *tps6* single mutants, labeled as parents, and carefully removed the petals and stamens. Then, the pollens of *tps5* and *tps6* mutants were applied to the stigma of the emasculated flowers reciprocally. Three days after pollination, the siliques were formed and elongated, which denoted successful hybridization. An F2 generation was obtained from the F1 hybrid, and F2 homozygotes were identified by their DNA and RNA levels.

DAB staining assay of rosette leaves

The accumulation of H_2O_2 was monitored by 3,3′-diaminobenzidine (DAB) staining (Thordal-Christensen et al. 1997). Twenty-day-old leaves grown under control conditions were treated with 100 µM ABA for 3 h and immersed in freshly prepared 1 mg/mL DAB solution, followed by vacuuming for 10 min and shaking at 180 rpm in the dark overnight. Stained leaves were fixed with a solution of 3:1:1 ethanol:acetic acid:glycerol, and then photographed (Ding et al. 2015).

Nitrate reductase (NR) activity assay

The nitrate reductase (NR) activity was measured using 20-day-old seedling tissue extracts of both *tps5* mutants (*tps5-1* and *tps5-cas9*) and the wild type. The NR Activity in plants was determined by Nitrate Reductase (NR) Assay Kit (Solarbio, Beijing, China). The absorbance of NR was measured at 520 nm ($n = 9$).

Results

tps5 mutants are more sensitive to ABA during seed germination

A *TPS5* T-DNA insertion allele (SALK_144791) was identified from ABRC, and named *tps5-1*. The site where T-DNA was inserted was determined to be 1472 bp downstream of the translational start site of *TPS5* (Fig. 1A). Subsequently, we created a *tps5-cas9* line using targeted genome editing of the *TPS5* by applying CRISPR/Cas9 technology and we found that *tps5-cas9* line lost four base pairs at 771–774 bp downstream of the translational start site of *TPS5* compared with wild-type(Fig. 1A). Semi-quantitative PCR analysis suggested that the *tps5-1* was an effective *TPS5* knockout mutant (Fig. 1B).

Under normal growth conditions, *tps5-1* and *tps5-cas9* did not show any morphological alterations. Then, the germination of *tps5-1* and *tps5-cas9* was compared with that of wild-type plants (Columbia). The germination rate (germination rate was defined by cotyledon greening) for the mutants was the same as that of the wild type on ABA-free medium. In the presence of ABA, *tps5-1* and *tps5-cas9* mutants showed lower cotyledon-greening percentages than wild-type plants (Fig. 2A). To quantify the effect of ABA on germination rate, we measured the cotyledon-greening percentages of *tps5* mutants and the wild type at different concentrations of ABA. When the concentration of exogenous ABA was 0.2 μM, the germination rates of *tps5-1*, *tps5-cas9* and wild type were 90.3%, 89.6% and 99%, respectively. At 0.6 μM ABA concentration, the germination rates of *tps5-1* and *tps5-cas9* were significantly reduced, only 35.7%, 26.3%, while the wild-type germination rate was 78.3% indicating that the mutation in *TPS5* led to ABA hypersensitivity during seed germination (Fig. 2B).

TPS5 is localized to the endoplasmic reticulum (ER) of *Arabidopsis* protoplasts

To determine the subcellular localization of the TPS5 protein, we constructed a vector for TPS5–GFP fusion proteins whose expression was regulated by cauliflower mosaic virus (CaMV) 35S promoter (*35S::TPS5-EGFP*). When transiently expressed in *Arabidopsis* mesophyll protoplasts, the appearance of the GFP signal in the cytoplasm was reticular, similar to the distribution and appearance of the endoplasmic reticulum (ER) (Fig. 3). *Arabidopsis* protoplasts were

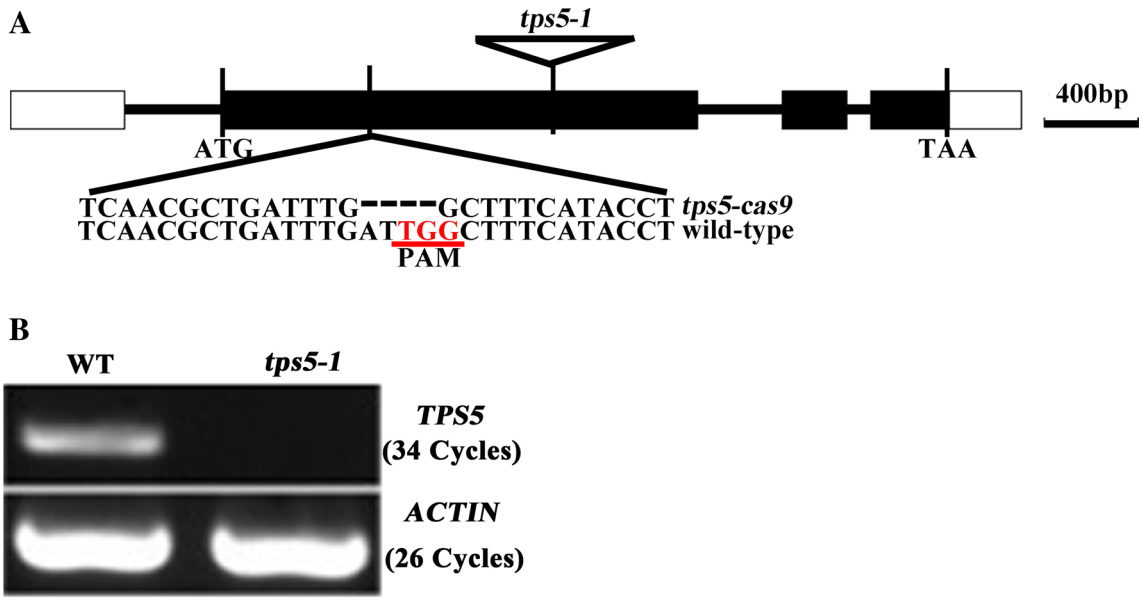

Fig. 1 Schematic structure of *TPS5* and the identification of *tps5* mutants. **A** T-DNA insertion site in the *tps5* mutant and Cas9 editing site in the *tps5-cas9* mutant. The ATG start codon and the TAA stop codon are indicated. The black straight lines show introns of *TPS5* and the black boxes show exons of TPS5 in the genome. The T-DNA insertion point is shown by the triangle above the gene diagram. **B** Identification of the *tps5-1* mutants based on the RNA level

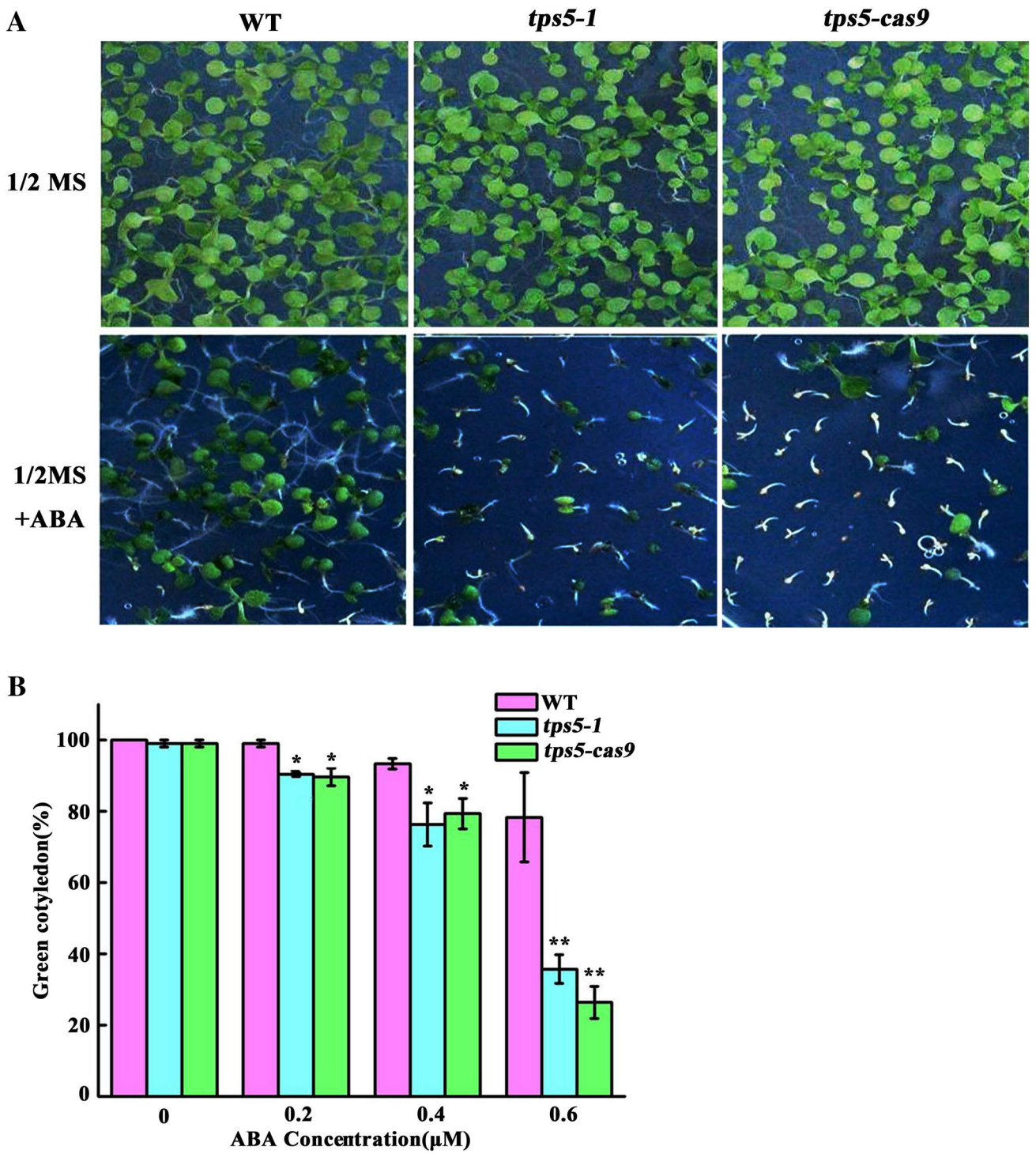

Fig. 2 ABA sensitivity of *tps5-1* and *tps5-cas9* during seed germination. **A** Seed germination of wild type and *tps5* mutants in response to ABA. Germination rates were defined by cotyledon greening. Wild type, *tps5-1*, and *tps5-cas9* seedlings grown on 0.5 × Murashige and Skoog medium with or without 0.5 μM ABA for 7 days were compared. **B** The percentage of cotyledon greening in the wild type and in *tps5* mutants in response to different ABA concentrations. Values represent mean ± SD ($n = 3$) from three biological replicates. Statistical significance of the difference between the wild type and *tps5* mutants was determined using a *t* test (*$P < 0.05$; **$P < 0.01$)

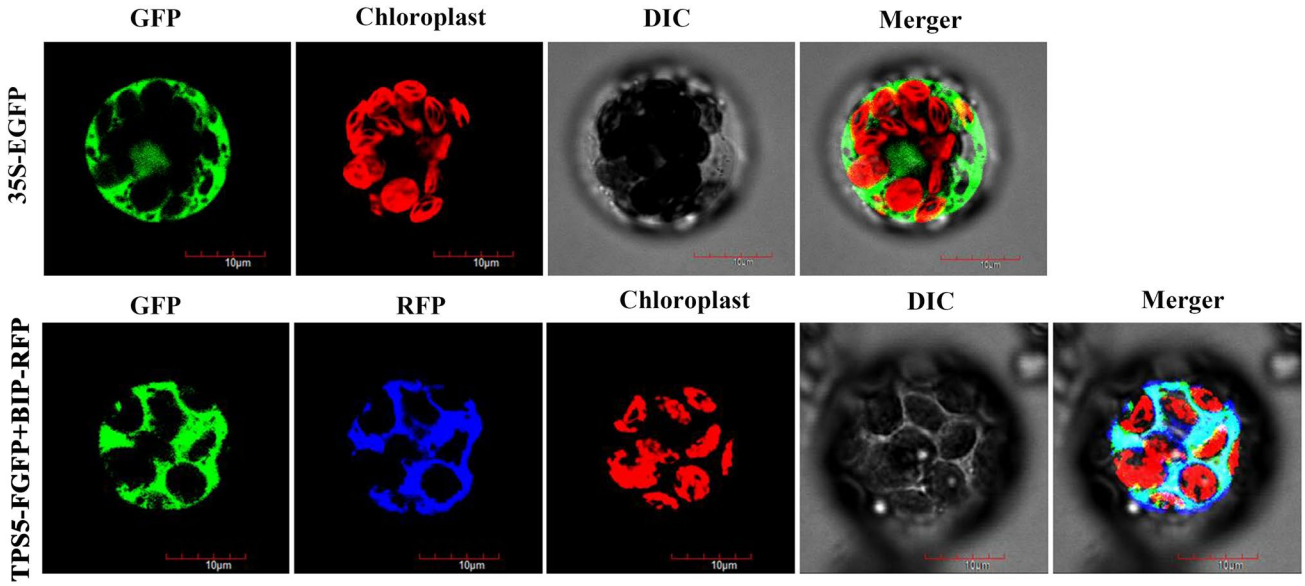

Fig. 3 Subcellular localization of TPS5 in protoplasts from *Arabidopsis* mesophyll cells. The recombinant plasmids *35S::TPS5-EGFP* and *35S::BiP-RFP* (ER marker) were used to cotransfect *Arabidopsis* mesophyll protoplasts transiently. TPS5–EGFP fusion protein was laser-excited at 488 nm, and the emitted fluorescence signaling was collected with a band-pass filter at 490–540 nm (GFP panel). BiP-RFP marker was laser-excited at 561 nm, and the emitted fluorescence was collected with a band-pass filter at 592 nm (RFP panel). The GFP and RFP fluorescence signals were mostly overlapping (overlay panel). The DIC panel shows the bright-field fluorescence microscopic image of the protoplast. Bar, 10 μm

then co-transformed with *35S:TPS5-GFP* and *35S: Bip-RFP* plasmids (a fluorescence marker for ER localization) (Lee et al. 2011). The co-localization of both TPS5-GFP and BiP-RFP was clearly detected (Fig. 3), leading to the conclusion that TPS5 was localized to the ER.

TPS5 expression is ABA- and stress-related

To investigate the expression of *TPS5*, 20-day-old seedlings of the wild type were treated with exogenous ABA and subjected to various abiotic stresses, including drought, salt, and heat. Quantitative RT-PCR analysis showed that ABA induced the expression of *TPS5*, which was increased approximately twofold at 20 min after 100 μM ABA treatment, then gradually decreased for 4 h and maintained at a level close to the control, after which a significant rise occurred to an approximately fourfold increase at 24 h (Fig. 4A). High salt and temperature stresses also significantly induced the expression of *TPS5*, which increased about six- to tenfold, respectively, after the treatment was applied (Fig. 4A). By contrast, *TPS5* expression was mildly responsive to drought stress, changing only about one- to twofold relative to control plants (Fig. 4A). To analyze the *TPS5* expression pattern in more detail, a 1.5-kb upstream DNA fragment of *TPS5* was fused with a GUS gene, and the construct was introduced into *Arabidopsis thaliana* to generate transgenic plants. Activity of GUS during seed germination was only detected in the root (Fig. 4B, a–c). However, in the presence of ABA, GUS was expressed more strongly in the hypocotyls and cotyledons (Fig. 4B, f–h), supporting the role of TPS5 during seed germination under ABA treatment. At the rosette stage, in addition to the strong expression of GUS in the root, there is also a weak expression in the leaf veins (Fig. 4B, d). Similarly, under ABA treatment, the intensity of GUS activity increased significantly (Fig. 4B, i), and was consistent with the qRT-PCR analysis. Interestingly, the activity of GUS in epidermal cells, guard cells and vascular bundle was also significantly enhanced by ABA treatment (Fig. 4B, j, Supplemental Fig. 4), suggesting that TPS5 is involved in regulating ABA-mediated stomatal closure and the response to abiotic stress.

TPS5 is a negative regulator of ABA-mediated stomatal closure

Since *TPS5* is expressed in stomata and *TPS5* expression was induced by ABA and abiotic stress, we further analyzed whether *TPS5* regulates ABA-dependent stomatal closure. Stomatal closure or opening in *tps5-1* and *tps5-cas9* plants was similar to that of the wild type in both light and dark conditions. However, a significant difference was observed under ABA treatment. The stomatal apertures in detached leaves of the *tps5-1* and *tps5-cas9* mutants decreased more rapidly than those of the wild type after 1–2.5 h of ABA treatment (Fig. 5A, B). Then, the water loss in leaves of *tps5-1*, *tps5-cas9*, and the wild type was analyzed, showing that leaf water loss of

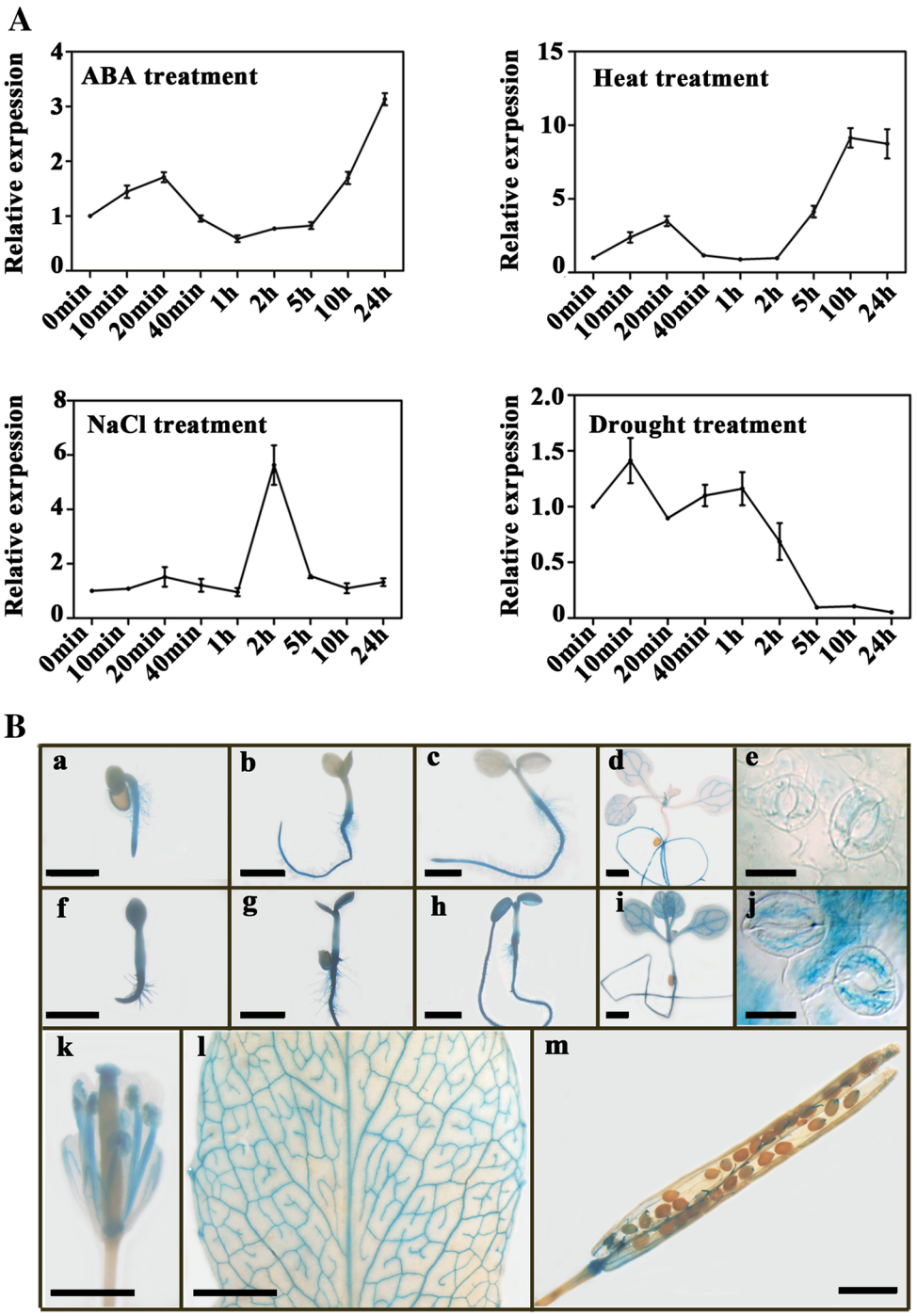

Fig. 4 Expression patterns of *TPS5*. **A** Relative expression of *TPS5* in response to treatments with 100 μM ABA, 42 °C, 250 mM NaCl, or drought. Total RNA was isolated from 3-week-old seedlings after the stress treatments. 18S rRNA transcript levels were used as an internal control for data normalization. Values represent mean ± SD ($n = 3$). **B** Histochemical analysis of *TPS5* promoter activity in different tissues. (a)–(d) and (f)–(i) GUS activity in seedlings, grown in Murashige and Skoog medium without (a–d) or with (f–i) 1 mM ABA. Bars, 2 mm. (e, j) GUS activity in guard cells with or without 0.5 μM ABA treatment for 3 h. Bars, 10 μm. (k–m) GUS activity in opened flower, rosette leaf, and silique. Bars, 2 mm. All GUS staining patterns were obtained by observing at least ten independent T2 transgenic lines

tps5 mutant was significantly lower than that of the wild type (Fig. 5C). Increased H_2O_2 levels have been reported to function as an early signaling in response to ABA signaling (Zhang et al. 2001). Therefore, ABA-mediated accumulation of ROS (reactive oxygen species) was also compared in the wild type, *tps5-1*, and *tps5-cas9*. Leaves of 20-day-old plants were treated with 0 or 100 μM ABA for 3 h, after which the leaves were incubated in DAB for 12 h. The results showed that in *tps5-1*, *tps5-cas9*, and the wild type, H_2O_2 levels increased in response to ABA, while the increase in *tps5-1* and *tps5-cas9* was significantly larger than in the wild type (Fig. 5D). Then, the transcript levels of the key genes responsible for the production of H_2O_2, *RbohD* and *RbohF* were examined in the wild type and in the *tps5* mutants. The expression of *RbohD* and *RbohF* in mutants was higher than that in the wild type (Fig. 5E), which was consistent with the changes in the H_2O_2 level. Collectively, these data indicate that *TPS5* negatively regulates ABA-mediated stomatal closure.

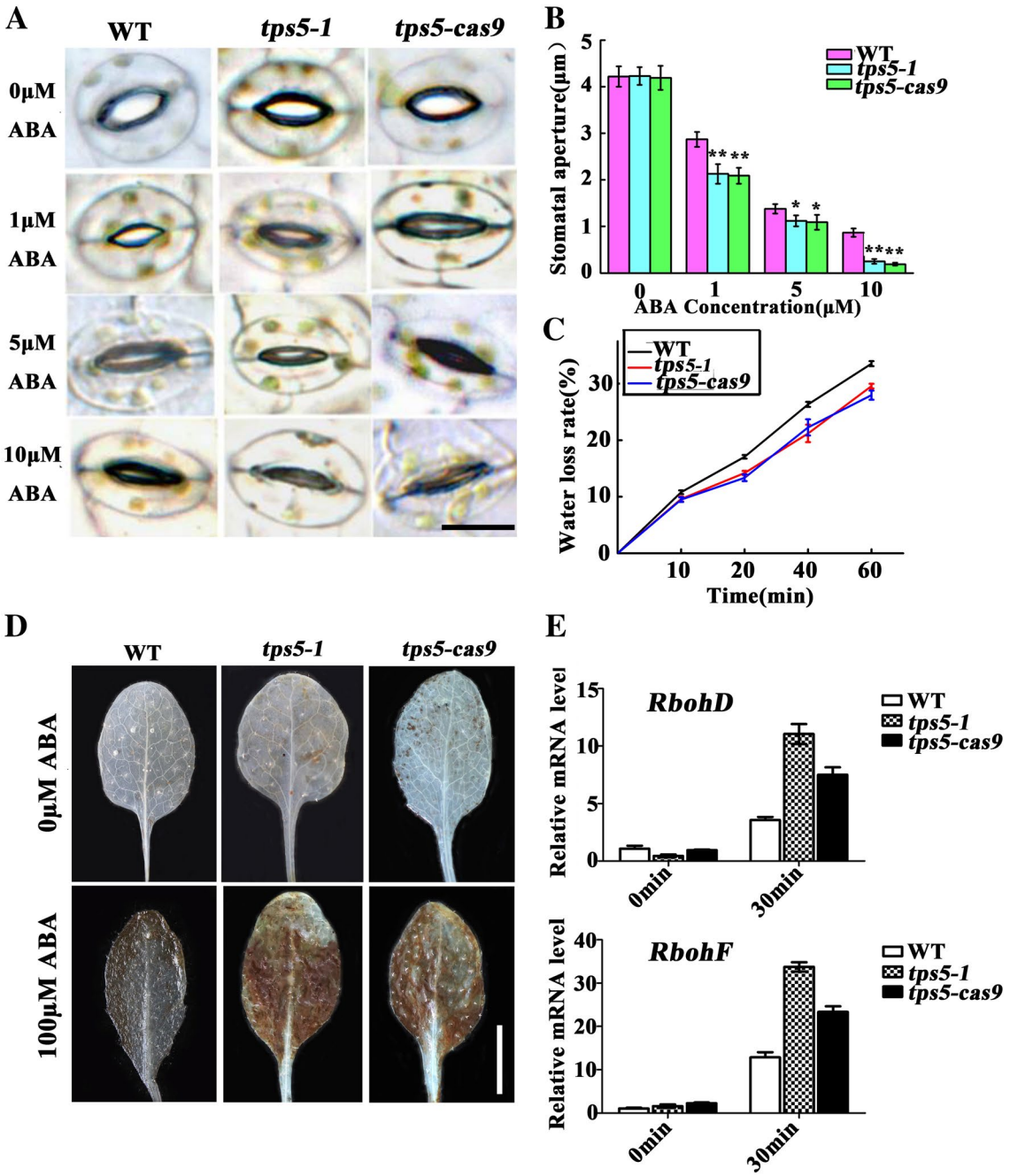

Fig. 5 Phenotypic analysis of the ABA-related stress response of *tps5-1* and *tps5-cas9* mutant plants. **A**, **B** Comparison of ABA-induced stomatal closure in the wild type, *tps5-1*, and *tps5-cas9* mutants. Leaves from 4-week-old plants were incubated in stomatal OS. ABA (0, 1, 5, and 10 μM) was added to the samples and stomatal closure was observed at 2.5 h of ABA treatment. The data were obtained from approximately 100 stomatal. **C** Water loss rate of wild-type, *tps5-1* and *tps5-cas9* mutants. **D** DAB (3,3′-diaminobenzidine) staining indicates different levels of ABA-induced H_2O_2 production in leaves of the wild type, *tps5-1*, and *tps5-cas9* mutants. The presence of H_2O_2 in the leaves is visualized as a brown color. Representative photographs are shown. **E** Relative expression of RbohD and RbohF in wild type, *tps5-1*, and *tps5-cas9* mutants. The 18S rRNA transcript levels were used as an internal control for data normalization. Error bars indicate SD ($n=3$). Values represent mean ± SD ($n>100$) from three to five biological replicates. The statistical significance of the difference between the wild type and *tps5-1* or *tps5-cas9* mutant lines was determined by a *t* test (*$P<0.05$; **$P<0.01$)

TPS5 knockout reduces the amounts of trehalose and other soluble carbohydrates

Accumulating evidence shows that trehalose metabolism plays a particularly important role in the regulation of stomatal closure (Figueroa et al. 2016; Gomez et al. 2010a, b;

Van Houtte et al. 2013; Vandesteene et al. 2012). Therefore, the negative regulation of ABA signaling by TPS5 might be associated with changes in T6P levels, resulting in downstream effects such as the accumulation of trehalose and other soluble carbohydrates. Nevertheless, trehalose has also been reported to have a signaling or regulatory function (Fernandez et al. 2012; Rodriguez-Salazar et al. 2009). To verify this possibility, the amounts of T6P, trehalose, and other soluble carbohydrates in plants were measured. Unfortunately, due to the extremely low T6P content in plants, the levels of T6P have not been detected in both mutants and wild-type plants. However, under normal conditions, the accumulation of trehalose, glucose and fructose in the *tps5* mutants was lower than that in the wild type, while there was no difference in sucrose levels (Fig. 6A). When treated with ABA for 12 h in the light, the trehalose, glucose and fructose contents in both wild type and mutants were significantly increased, but the increase in the wild

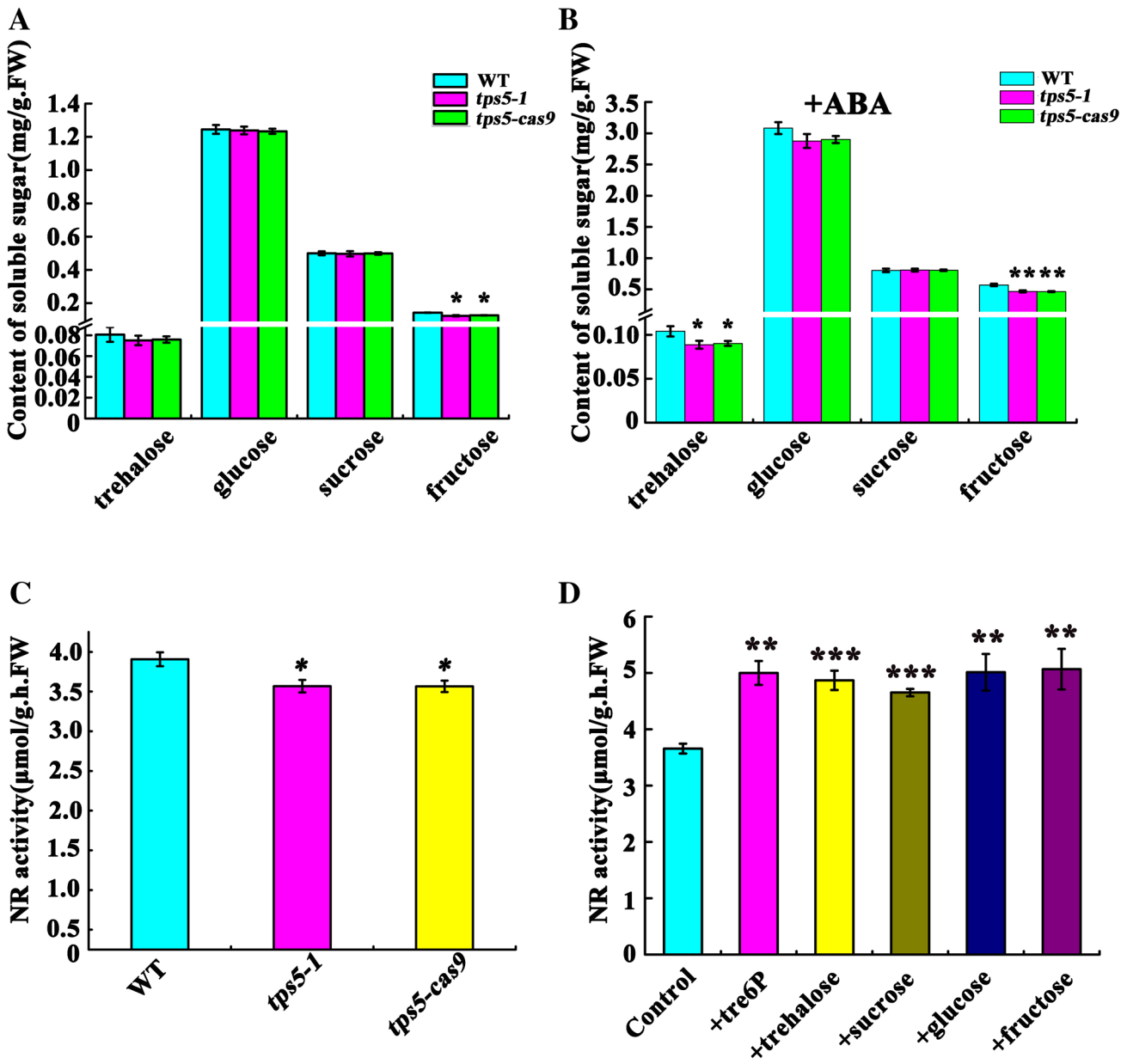

Fig. 6 Analysis of the content of soluble sugar and nitrate reductase (NR) activity. **A** The content of soluble sugars in the wild type (WT), *tps5-1*, and *tps5-cas9* under controlled conditions. **B** The content of soluble sugar in WT, *tps5-1*, and *tps5-cas9* under ABA stress. **C** NR activity in WT, *tps5-1*, and *tps5-cas9* under controlled conditions. **D** NR activity in WT, when T6P or soluble sugar is added. Values represent mean ± SD ($n = 9$) from three biological replicates. Statistical significance of the difference between the wild type and *tps5* mutants was determined by a *t* test (*$P < 0.05$; **$P < 0.01$)

type was more pronounced (Fig. 6B). Although inconsistent with the result that there was no catalytic activity of Class II TPS proteins (Harthill et al. 2006; Ramon et al. 2009), these results apparently implicate a possible role for TPS5 in regulating carbohydrate metabolism and in ABA signaling. Possibly, TPS5 may be involved in the regulation of trehalose metabolism through interactions with other proteins or complex formation (Ramon et al. 2009; Vishal et al. 2019).

TPS5 knockout decreases NR activity, while trehalose and other soluble carbohydrates promote NR activity in vitro

The findings that nitric oxide (NO)-mediated ABA signaling is impaired in *nia1* and *nia2* mutants lacking nitrate reductase (NR) activity (Chen et al. 2016), whereas *TPS* induction leads to activation of NR (Figueroa and Lunn 2016), suggests a possible role for the trehalose metabolism pathway in regulating ABA signaling involving changes in the activity of NR (Figueroa and Lunn 2016). In this study, NR activity was measured using 20-day-old seedling tissue extracts of both *tps5* mutants (*tps5-1* and *tps5-cas9*) and the wild type. The results showed that the NR activity in *tps5-1* and *tps5-cas9* was lower than that in the wild type (Fig. 6C). Then, NR activity was measured using 3-week-old seedling tissue extracts with and without T6P in vitro. NR activity in wild-type seedling extracts enhanced by T6P was observed when exogenous 1 μM T6P was added (Fig. 6D). The same effect as with T6P was observed when glucose, sucrose, trehalose and fructose were added (Fig. 6D), indicating that the change in NR activity is most correlated with the trehalose metabolic pathway. Thus, further studies are needed to understand why adding T6P and soluble sugars to seedling extracts can lead to activation of NR. NR is widely present in plants and is a key enzyme for the conversion of plant nitrate nitrogen to ammonia nitrogen. Its catalytic effect requires the presence of NADPH and H^+. Therefore, it is speculated that an increase in NR activity is associated with the production of NADH and H^+ by the glycolysis pathway. To further verify this possibility, we added iodoacetic acid, an inhibitor of the tricarboxylic acid cycle, to the extract and found that the addition of T6P, trehalose, glucose and sucrose no longer led to the activation of NR (Supplemental Fig. 1). This unexpected finding led to the proposal that in regulating ABA signaling, TPS5 is most likely involved in trehalose metabolism and is associated with changes in the activity of NR.

Discussion

Role of TPS5 in ABA signaling

ABA is an important regulator of stomatal closure, and several studies have found evidence of a link between trehalose metabolism and ABA signaling (Gomez et al. 2010a, b; Van Houtte et al. 2013; Vandesteene et al. 2012). The *Arabidopsis TPS* family has 11 *TPS* genes and is subdivided into 2 distinct clades, known as Class I (*TPS1–TPS4*) and Class II (*TPS5–TPS11*) (Leyman et al. 2001). In addition to the better-established roles of the gene products of Class I, the function of most of the Class II proteins remains enigmatic (Figueroa et al. 2016). In this study, we show that the *TPS5* gene, which encodes a trehalose-6-phosphate synthase and has been implicated in thermotolerance (Suzuki et al. 2008), plays an important role in ABA signaling.

ABA response assays indicated that during seed germination *tps5-1* and *tps5-cas9* mutants are more sensitive to ABA than the wild type (Fig. 2). However, if after germination the seedlings were shifted to ABA-containing medium, the differences in the effects between *tps5-1*, *tps5-cas9* and wild type were less apparent when the transfer occurred after more than 7 days, meaning that the role of *TPS5* in the ABA signaling pathway may be particularly important during seed germination. *AtTPS5* is temporally induced by salt, drought and heat treatment. However, the *tps5* mutants did not show any changes in phenotype when treated with these stresses. We supposed that it is caused by functional redundancy of the *TPS* genes.

After 1–2.5 h of ABA treatment, the *tps5-1* and *tps5-cas9* mutants show enhanced stomatal closure (Fig. 5A, B). These findings are supported by the enhanced expression of *tps5* in response to ABA and abiotic stresses (Fig. 4A). Under ABA stress, stronger GUS activity was observed in the hypocotyls, cotyledons and guard cells of the $TPS5_{pro}$::GUS transgenic plants (Fig. 4B). Furthermore, the expression of *RbohD* and *RbohF* in response to ABA in *tps5-1* and *tps5-cas9* mutants increased more significantly than in the wild type (Fig. 5E). These data were consistent with the findings obtained for changes in H_2O_2 content (Fig. 5D), which is a key signal for regulating stomatal closure. In addition, the accumulation of trehalose, fructose and glucose in the wild type under ABA treatment was also more than that in *tps5* mutants (Fig. 6A), which is consistent with the result that the *tps5* gene could link sucrose signaling to guard-cell movements (Bates et al. 2012). Collectively, these results suggest that TPS5 is a negative regulator of ABA signaling in seed germination and stomatal closure.

The obvious question that arises is how TPS5 could be involved in the regulation of ABA signaling. NO has been reported to suppress ABA signaling in guard cells by

S-nitrosylation of SnRK2.6 (Wang et al. 2015a, b), which plays a key role in ABA downstream signaling and belongs to the same SNF1/AMPK/SnRK superfamily of protein kinases as SnRK1. Lunn et al. speculated that T6P could participate in the ABA-mediated regulation of stomatal closure, involving covalent modifications of NR and SnRK2.6, with NO as an intermediary signal (Lunn et al. 2014). Although speculatively, this hypothesis could be tested by investigating the NR activity in *tps5* mutants and the wild type and the effect of exogenous T6P on NR activity. In this study, we demonstrated that the NR activity in the *tps5-1* and *tps5-cas9* mutants was lower than that in the wild type (Fig. 6C), and that exogenous T6P enhanced the NR activity significantly (Fig. 6D), which is consistent with the observation of activation of NR by Tre6P by Figueroa et al. (2016), and also explains the suppression of SnRK2.6 activity after the induction of TPS (Zhang et al. 2009). However, further proof is needed to understand how rising T6P levels lead to the activation of NR (Figueroa and Lunn 2016). It is striking that exogenous trehalose, glucose, sucrose, and fructose also increase the NR activity significantly, and that this enhancement is blocked by iodoacetic acid. Iodoacetic acid is an inhibitor of the tricarboxylic acid cycle and inhibits the production of NADPH and H^+. Recently, similar results were reported in rice that the levels of trehalose and other soluble sugars were also changed in *tps8* mutant. Besides, the ABA sensitivity was altered when *TPS8* was mutated (Vishal et al. 2019). From these findings, a model emerged that changes in trehalose content driven by the knockout of *TPS5* trigger related changes in glucose and fructose, which cause reductions of NADH and H^+ and decrease the activity of NR. Another possibility is that the trehalose and fructose in the guard cells are rapidly reduced in the *tps5* mutant, resulting in a decrease in malate synthesis that contributes to enhancing stomatal closure. Thus, experiments to measure T6P and related metabolites (e.g., trehalose, glucose,) in guard cells should provide new insight into how trehalose metabolism affects stomatal opening and closing.

Studies have shown that TPSs possess multiple SnRK1 phosphorylation sites, and TPS5 can bind directly or indirectly to 14-3-3 proteins (Avonce et al. 2004; Harthill et al. 2006). These correlations between TPS5 and SnRK1 lead to the hypothesis that TPS5 can interact directly with SnRK2 s to modulate ABA signaling. Analysis of transcriptional levels showed that the expression of TPS5 was very similar to that of SnRK2.6 in the wild type under ABA and heat treatment (Supplemental Fig. 2), but no direct interaction between Class II TPSs and SnRK2 s has been reported so far (Emanuelle et al. 2015; Figueroa et al. 2016).

Although detailed dynamic changes in the levels of activated NR, T6P, and S-nitrosylated SnRK2.6 in guard cells are not yet known, available evidence and the well-established ABA signaling pathway (Chen et al. 2016; Ding et al. 2015; Figueroa and Lunn 2016; Wang et al. 2015a, b) suggest the following hypothetic model for a function of TPS5 as a negative regulator of ABA signaling in regulating stomatal closure and seed germination (Fig. 7). We hypothesize that ABA treatment leads to increased expression of TPS5 in seed and guard cells (Fig. 4b), causing the synthesis of T6P and triggering-related changes in trehalose, glucose, and fructose, and causing an increase in the activity of NR (Fig. 6D), resulting in the accumulation of NO (Chen et al. 2016). When NO accumulates to high levels, the SnRK2s are S-nitrosylated by NO at cysteine 137, a residue adjacent to the kinase catalytic site (Wang et al. 2015a, b), and their activation is inhibited. Since the expression pattern of TPS5 was similar to that of SnRK2.6 under ABA and abiotic stress (Supplemental Fig. 2), and TPSs possess multiple SnRKs phosphorylation sites that bind directly or indirectly to 14-3-3 proteins (Harthill et al. 2006). Another scenario could be reached, namely that ABA treatment causes very fast and strong activation of SnRK2 s in seed and guard cells, which phosphorylates many downstream effector proteins and leads to stomatal closure and the inhibition of seed germination (Wang et al. 2015a, b). The activated SnRK2 s

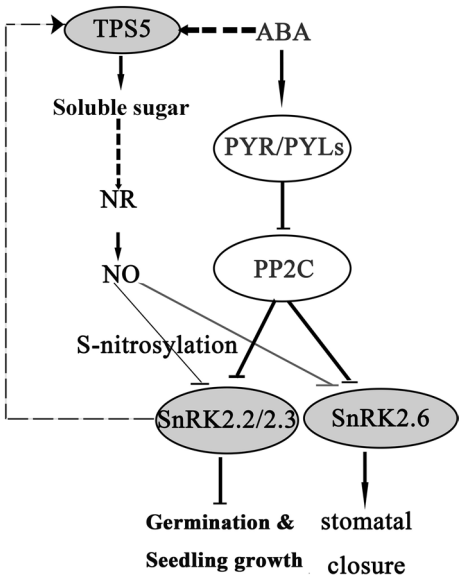

Fig. 7 Proposed model for the function of TPS5 in ABA-induced stomatal closure, germination and seedling growth. ABA treatment leads to increased expression of TPS5 in seed and guard cells, causing the synthesis of T6P and triggering-related changes in trehalose, glucose, and fructose, and causing an increase in the activity of NR, resulting in the accumulation of NO. When NO accumulates to high levels, the SnRK2s are S-nitrosylated, and their activation is inhibited. The expression pattern of *TPS5* was similar to that of SnRK2.6 under ABA and abiotic stress, another scenario could be reached, namely that ABA treatment causes very fast and strong activation of SnRK2s in seed and guard cells, which phosphorylates many downstream effector proteins and leads to stomatal closure and the inhibition of seed germination

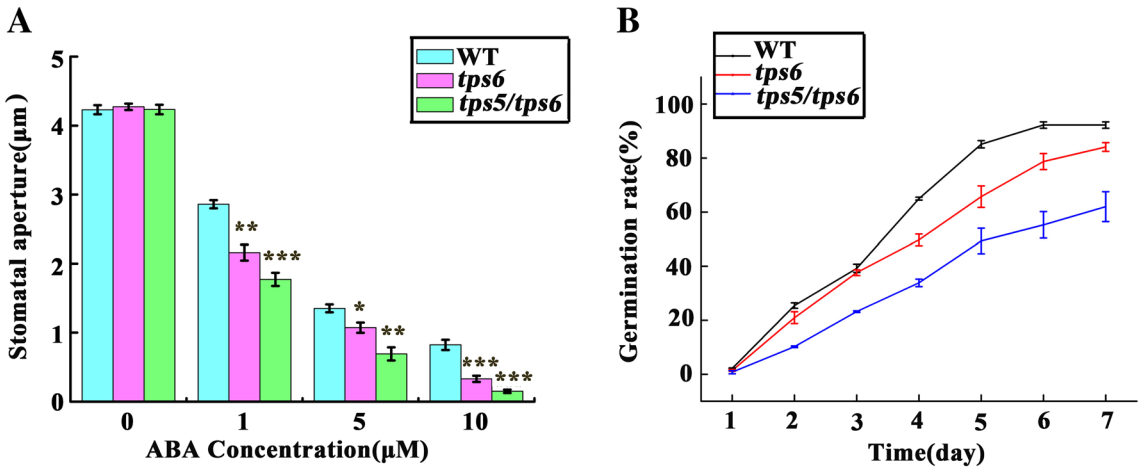

Fig. 8 Phenotypic analysis of *tps6 tps5/tps6* mutants plants in ABA stress response. **A** Comparison of ABA-induced stomatal closure in the wild type, *tps5*, and *tps5/tps6* mutants. Leaves from 4-week-old plants were incubated in stomatal OS. ABA (0, 1, 5, and 10 μM) was added to the samples and stomatal closure was observed after 2.5 h of ABA treatment. The data were obtained from more than 100 stomata. Values represent mean ± SD ($n > 100$) from three to five biological replicates. Statistical significance of the difference between the wild type and *tps6* or *tps5/tps6* mutant lines was determined with a *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). **B** Seed germination of wild-type *tps6* and *tps5/tps6* mutants in response to ABA. Germination rates were defined by cotyledon greening. In wild-type, *tps6,* and *tps5/tps6* seeds on Murashige and Skoog medium with 0.5 μM ABA are compared for 7 days

also promote the expression of *tps5* and increase the level of trehalose to activate NR, which in turn leads to the accumulation of NO and inhibits the activity of SnRK2.6. This inhibition serves as negative feedback on ABA signaling. Furthermore, when treated with ABA, not only H_2O_2 production was increased in *tps5* mutants, but the expression of RbohD and RbohF was also promoted (Fig. 5D, E). These results demonstrate unambiguously that the negative regulation of ABA-mediated stomatal closure by TPS5 is required for phosphorylation of the NADPH oxidases RbohD and RbohF.

Seed germination and stomatal closure analysis in mutants TPS6–TPS11 provide insight into the role of Class II TPS in regulating ABA signaling

For further insight into the role of *TPS5* in regulating ABA signaling, a step forward in the analysis is to elucidate the role of other members of the Class II TPS (TPS5–TPS11) family under ABA treatment. The *tps6* mutant is more sensitive to ABA than the wild type during seed germination (Fig. 8), while mutants *tps7–11* showed no significant differences. A similar result was observed in ABA-mediated stomatal closure (Fig. 8). Next, a *tps5/tps6* double mutant was constructed, and it exhibited higher sensitivity to ABA than the *tps5* and *tps6* single mutants during seed germination and ABA-mediated stomatal closure (Fig. 8). Furthermore, histochemical staining analysis of *TPS6* showed that GUS activity was also increased in epidermal and guard cells after ABA treatment (data not shown), which was very similar to the effect of *TPS5*. In summary, accumulating data suggest important roles for the Class II proteins in regulating ABA signaling and abiotic stress response. Although a detailed analysis of the expression patterns of the entire set of Class II *TPS* genes showed that they differ remarkably in their tissue-specific expression and responsiveness to hormones (Ramon et al. 2009), the *TPS5* and *TPS6* genes are likely to play similar roles in ABA signaling. In support of these results, a phylogenetic analysis of the Class II *TPS* family showed that *Arabidopsis TPS5* and *TPS6* were more closely related to each other than to *TPS7–11* (Supplemental Fig. 3).

In *Arabidopsis thaliana*, trehalose metabolism plays an important role in regulating stomatal conductance. However, it is unknown at present how trehalose metabolism participates in ABA signaling (Figueroa and Lunn 2016). Although many of the molecular details remain to be elucidated, our results reveal an important role for TPS5 in coordinating trehalose and NR activity in the regulation of ABA signaling in plants. The genetic mechanism defined in this study may, therefore, assist in understanding the possible mechanism by which metabolic and genetic regulation is integrated in ABA signaling.

Author contribution statement DL, LC, LT conceived and designed the experiments. LT, ZX, CL, XH, YH, SW performed the experiments. DL, LT, ZX, LC analyzed the data and wrote the paper.

Acknowledgements We thank Jiankang Zhu for kindly providing CRISPR/cas9 vectors. This work was supported by grants of NSFC (31570316; 31371244), HNNSF (2016JJ2089), Project of Hunan Provincial Education Department (17C0974, 13C638), China Postdoctoral Science Foundation (2013M542118).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The *Arabidopsis* trehalose-6-P synthase *AtTPS1* gene is a regulator of glucose, abscisic acid, and stress signaling. Plant Physiol 136:3649–3659

Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature 448:938–942

Bates GW, Rosenthal DM, Sun J, Chattopadhyay M, Peffer E, Yang J, Ort DR, Jones AM (2012) A comparative study of the *Arabidopsis thaliana* guard-cell transcriptome and its modulation by sucrose. PLoS ONE 7:e49641

Blázquez MA, Santos E, Flores CL, Martínezzapater JM, Salinas J, Gancedo C (1998) Isolation and molecular characterization of the *Arabidopsis TPS1* gene, encoding trehalose-6-phosphate synthase. Plant J 13:685–689

Chary SN, Hicks GR, Choi YG, Carter D, Raikhel NV (2008) Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in *Arabidopsis*. Plant Physiol 146:97–107

Chen ZH, Wang Y, Wang JW, Babla M, Zhao C, Garcia-Mata C, Sani E, Differ C, Mak M, Hills A, Amtmann A, Blatt MR (2016) Nitrate reductase mutation alters potassium nutrition as well as nitric oxide-mediated control of guard cell ion channels in *Arabidopsis*. New Phytol 209:1456–1469

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Crowe JH, Carpenter JF, Crowe LM (2003) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60:73–103

Ding S, Zhang B, Qin F (2015) *Arabidopsis* RZFP34/CHYR1, a ubiquitin E3 ligase, regulates stomatal movement and drought tolerance via SnRK2.6-mediated phosphorylation. Plant Cell 27:3228–3244

Eastmond PJ, Graham IA (2003) Trehalose metabolism: a regulatory role for trehalose-6-phosphate? Curr Opin Plant Biol 6:231–235

Eastmond PJ, Van Dijken AJ, Spielman M, Kerr A, Tissier AF, Dickinson HG, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant J 29:225–235

Emanuelle S, Hossain MI, Moller IE, Pedersen HL, van de Meene AM, Doblin MS, Koay A, Oakhill JS, Scott JW, Willats WG, Kemp BE, Bacic A, Gooley PR, Stapleton DI (2015) SnRK1 from *Arabidopsis thaliana* is an atypical AMPK. Plant J 82:183–192

Fernandez O, Vandesteene L, Feil R, Baillieul F, Lunn JE, Clément C (2012) Trehalose metabolism is activated upon chilling in grapevine and might participate in Burkholderia phytofirmans induced chilling tolerance. Planta 236:355–369

Figueroa CM, Lunn JE (2016) A tale of two sugars: trehalose 6-phosphate and sucrose. Plant Physiol 172:7–27

Figueroa CM, Feil R, Ishihara H, Watanabe M, Kolling K, Krause U, Hohne M, Encke B, Plaxton WC, Zeeman SC, Li Z, Schulze WX, Hoefgen R, Stitt M, Lunn JE (2016) Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon availability. Plant J 85:410–423

Foster AJ, Jenkinson JM, Talbot NJ (2014) Trehalose synthesis and metabolism are required at different stages of plant infection by Magnaporthe grisea. EMBO J 22:225–235

Genome IA (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815

Goddijn O, Verwoerd TC, Voogd E, Krutwagen R, Graff PD, Poels J, Dun KV, Ponstein AS, Damm B, Pen J (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol 113:181–190

Gomez LD, Baud SA, Li Y, Graham IA (2010a) Delayed embryo development in the *Arabidopsis* trehalose-6-phosphate synthase 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J 46:69–84

Gomez LD, Gilday A, Feil R, Lunn JE, Graham IA (2010b) AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. Plant J 64:1–13

Harthill JE, Meek SE, Morrice N, Peggie MW, Borch J, Wong BH, Mackintosh C (2006) Phosphorylation and 14-3-3 binding of *Arabidopsis* trehalose-phosphate synthase 5 in response to 2-deoxyglucose. Plant J 47:211–223

Joachim M, Thomas B, Andres W (1995) Effects of validamycin A, a potent trehalase inhibitor, and phytohormones on trehalose metabolism in roots and root nodules of soybean and cowpea. Planta 197:362–368

Lee J, Lee H, Kim J, Lee S, Kim DH, Kim S, Hwang I (2011) Both the hydrophobicity and a positively charged region flanking the C-terminal region of the transmembrane domain of signal-anchored proteins play critical roles in determining their targeting specificity to the endoplasmic reticulum or endosymbiotic organelles in *Arabidopsis* cells. Plant Cell 23:1588–1607

Leyman B, Dijck PV, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends Plant Sci 6:510–513

Li J, Huang Y, Tan H, Yang X, Tian L, Luan S, Chen L, Li D (2015) An endoplasmic reticulum magnesium transporter is essential for pollen development in *Arabidopsis*. Plant Sci 231:212–220

Lu C, Yu F, Tian L, Huang X, Tan H, Xie Z, Hao X, Li D, Luan S, Chen L (2017) RPS9 M, a mitochondrial ribosomal protein, is essential for central cell maturation and endosperm development in *Arabidopsis*. Front Plant Sci 8:2171

Lunn JE, Delorge I, Figueroa CM, Van Dijck P, Stitt M (2014) Trehalose metabolism in plants. Plant J 79:544–567

Ramon M, De Smet I, Vandesteene L, Naudts M, Leyman B, Van Dijck P, Rolland F, Beeckman T, Thevelein JM (2009) Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from *Arabidopsis thaliana*. Plant, Cell Environ 32:1015–1032

Rodriguez-Salazar J, Suarez R, Caballero-Mellado J, Iturriaga G (2009) Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance and biomass in maize plants. FEMS Microbiol Lett 296:52–59

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiol 136:2483–2499

Suzuki N, Bajad S, Shuman J, Shulaev V, Mittler R (2008) The transcriptional co-activator MBF1c is a key regulator of thermotolerance in *Arabidopsis thaliana*. J Biol Chem 283:9269–9275

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. Plant J 11:1187–1194

Usadel B, Blasing OE, Gibon Y, Retzlaff K, Hohne M, Gunther M, Stitt M (2008) Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in *Arabidopsis* rosettes. Plant Physiol 146:1834–1861

van Dijken AJ, Schluepmann H, Smeekens SC (2004) *Arabidopsis* trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant Physiol 135:969–977

Van Houtte H, Vandesteene L, Lopez-Galvis L, Lemmens L, Kissel E, Carpentier S, Feil R, Avonce N, Beeckman T, Lunn JE, Van Dijck P (2013) Overexpression of the trehalase gene *AtTRE1* leads to increased drought stress tolerance in *Arabidopsis* and is involved in abscisic acid-induced stomatal closure. Plant Physiol 161:1158–1171

Vandesteene L, Lopez-Galvis L, Vanneste K, Feil R, Maere S, Lammens W, Rolland F, Lunn JE, Avonce N, Beeckman T, Van Dijck P (2012) Expansive evolution of the trehalose-6-phosphate phosphatase gene family in *Arabidopsis*. Plant Physiol 160:884–896

Vishal B, Krishnamurthy P, Ramamoorthy R, Kumar PP (2019) OsTPS8 controls yield-related traits and confers salt stress tolerance in rice by enhancing suberin deposition. New Phytol 221:1369–1386

Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, Feil R, Lunn JE, Stitt M, Schmid M (2013) Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana*. Science 339:704–707

Wang R, Okamoto M, Xing X, Crawford NM (2003) Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol 132:556–567

Wang P, Du Y, Hou YJ, Zhao Y, Hsu CC, Yuan F, Zhu X, Tao WA, Song CP, Zhu JK (2015a) Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. Proc Natl Acad Sci USA 112:613–618

Wang P, Zhu JK, Lang Z (2015b) Nitric oxide suppresses the inhibitory effect of abscisic acid on seed germination by S-nitrosylation of SnRK2 proteins. Plant Signal Behav 10:e1031939

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. Plant Physiol 126:1438–1448

Zhang Y, Primavesi LF, Jhurreea D, Andralojc PJ, Mitchell RA, Powers SJ, Schluepmann H, Delatte T, Wingler A, Paul MJ (2009) Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. Plant Physiol 149:1860–1871

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