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ORIGINAL ARTICLE

Overexpression of the trehalose-6-phosphate phosphatase *OsTPP3* increases drought tolerance in rice

Dagang Jiang[1] · Weiting Chen[1] · Jieer Gao[1] · Fen Yang[1] · Chuxiong Zhuang[1]

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Abstract

Trehalose plays an important role in mediating stress responses in plants, and trehalose-6-phosphate synthases and trehalose-6-phosphate phosphatases are essential for trehalose biosynthesis. Here, we address the function of rice (*Oryza sativa*) *OsTPP3*. We analyzed the expression of *OsTPP3* in different tissues and stress conditions, and generated *OsTPP3*-overexpressing rice plants. These plants showed a higher tolerance to simulated drought conditions (10% PEG treatment) than wild-type (WT) plants. Reverse-transcription quantitative PCR analysis indicated that transcript levels of genes related to stress responses and abscisic acid biosynthesis were significantly higher in the *OsTPP3* overexpressors than in WT plants. These results highlight the importance of *OsTPP3* in conferring drought tolerance in rice.

Keywords Rice · Trehalose · *OsTPP3* · Drought tolerance · ABA

Introduction

The non-reducing disaccharide trehalose and its intermediate product, trehalose-6-phosphate (T6P), play important roles in the regulation of metabolism and stress (Paul et al. 2008). In trehalose biosynthesis, trehalose-6-phosphate synthases (TPSs) catalyze the synthesis of T6P, and trehalose-6-phosphate phosphatases (TPPs) dephosphorylate T6P to produce trehalose (Paul 2007; Paul et al. 2008). Genome analyses have identified variable numbers of *TPS* and *TPP* genes in different plant species. For example, rice (*Oryza sativa*) has 11 *TPS* genes clustered into two subfamilies (Zang et al. 2011). Winter wheat (*Triticum aestivum*) has 12 *TPS* genes (Xie et al. 2015), and different types of cotton have different numbers of these two genes, with 15, 14, and 24 *TPS* genes in *Gossypium raimondii* (group D), *G. arboreum* L. (group A), and *G. hirsutum* L. (group AD), respectively (Mu et al. 2016). This diversity and expansion of these two gene families likely reflects the many roles TPS and TPP proteins play in plant growth and development, as well as stress tolerance.

In growth and development, inflorescence branching is a major yield-related trait in grain crops and is controlled by the developmental fate of axillary shoot meristems (Ward and Leyser 2004). In maize, *RAMOSA3* (*RA3*) encodes a trehalose-6-phosphate phosphatase that is expressed in discrete domains subtending axillary inflorescence meristems; RA3 regulates inflorescence branching by modifying a sugar signal that moves into axillary meristems (Satoh-Nagasawa et al. 2006). Sucrose levels decrease and trehalose levels increase in salt-treated flowering maize, thereby providing a resource for studying primary metabolic pathways in C4 plants (Henry et al. 2015). In garden peas (*Pisum sativum* L.), T6P regulates bud dormancy, activation, and growth through sucrose (Fichtner et al. 2017). In *Arabidopsis thaliana*, loss of *TPS1* function causes delayed embryonic development and disrupts vegetative growth by affecting sugar metabolism and abscisic acid (ABA) biosynthesis (Gómez et al. 2010).

Genes associated with trehalose biosynthesis also function in responses to abiotic stress (Garg et al. 2002), and manipulation of these genes can affect key agronomic traits. For example, in maize (*Zea mays*), *TPP* overexpression significantly increases yield under drought and normal

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✉ Chuxiong Zhuang
zhuangcx@scau.edu.cn

[1] State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China

conditions (Nuccio et al. 2015). In rice, *OsTPP7* promotes seed germination under anaerobic conditions, which allows rice farmers to shift from transplanting seedlings to direct seeding, thus reducing labor and increasing yield (Kretzschmar et al. 2015). In rice, *OsTPP1* expression is regulated by stress and *OsTPP1*-overexpressing plants exhibit increased resistance to salt and low temperature, with increased expression of stress response genes (Ge et al. 2008). In rice, the mitogen-activated protein kinase (MAPK) OsMAPK3 phosphorylates the helix–loop–helix (HLH) transcription factor OsbHLH002 to increase *OsTPP1* expression, which in turn increases trehalose levels (Zhang et al. 2017). *OsTPP2* expression is regulated by stress factors including low temperature, drought, and salinity stress, as well as ABA treatment (Shima et al. 2007). Overexpression of *OsTPS1* increases tolerance to cold, salt, drought, and other abiotic stresses in transgenic rice (*OsTPS1*; Li et al. 2011a). Resistance to drought, salt, and low temperature can be improved through heterologous expression of *Escherichia coli* TPS and TPP in rice (Jang et al. 2003).

Although many studies on TPS and TPP have been conducted to date, investigations of *OsTPP3* remain limited. This study addresses this gap by measuring *OsTPP3* expression in various tissues and in response to different abiotic stressors. Additionally, we generated transgenic lines overexpressing *OsTPP3* and show that these lines have increased tolerance to drought treatment. Our findings further indicate that the enhanced drought tolerance in transgenic rice resulted from changes in the expression of ABA biosynthetic and abiotic stress-related genes.

Materials and methods

Plant and other experimental materials

Rice (*Oryza sativa* L.) plants were grown in a paddy field at South China Agricultural University under natural conditions. Zhonghua 11 (ZH11) was used as the wild type. *E. coli* DH10B and *Agrobacterium tumefaciens* EHA105 were used for cloning and transformation experiments. pCAMBIA1380 was used as the binary vector for *Agrobacterium*-mediated transformation.

ABA treatments

To evaluate root length, seedling height, and germination rates, rice seeds were immersed in water and 3 or 6 μM ABA. Germination rates were determined 6 days after treatment. The root length and seedling height were directly measured 10 days after germination. Germination and seedling growth took place in a greenhouse with a 13/11-h day/night cycle (25/23 °C).

Stress treatments

Six-day-old seedlings were transferred into Kimura B nutrition solution. Two-week-old seedlings were subjected to abiotic stress treatments. For salt and simulated drought conditions, seedlings were treated with Kimura B solution supplemented with 10% PEG 6000 and were maintained in a greenhouse with a 13/11-h day/night cycle (25/23 °C). Seedlings were individually harvested at 0-, 2-, 4-, 6-, and 8-day intervals. For low- and high-temperature treatments, seedlings in Kimura B solution were subjected to 8/10 °C (day/night) or 42/37 °C (day/night) in the growth chamber (Canada Conviron PGV-36) with a 13/11-h day/night cycle. Seedlings were individually harvested at 0-, 3-, 12-, and 48-h intervals. The tissues were frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

To analyze drought tolerance of *OsTPP3*-overexpressing plants, WT and T_2 homozygous seeds were germinated and transferred to Kimura B solution. The 2-week-old seedlings were then transferred into new Kimura B nutrition solution with 10% PEG 6000. The plants were photographed before treatment and at 6 and 10 days after drought treatment followed by 4 days of rewatering recovery.

Vector construction and genetic transformation

For overexpression of *OsTPP*3 (Os07g0624600, LOC_Os07g43160), the open reading frame was amplified from cDNA using the primers OETPPF (5’-aaagcttttgcttcggcttccgctgctc-3′) and OETPPR (5′-aaggatcccatgatcagttaccatccatgc-3′). After subcloning and sequencing, the correct gene was inserted into pCAMBIA 1380-Ubi (driven by the maize *Ubiquitin* promoter). The transformation was performed as previously described (Li et al. 2011b; Zhou et al. 2014).

DNA extraction and PCR detection

The DNA extraction was conducted as described previously (Zhou et al. 2014). The primers used in detection of *hygromycin phosphotransferase* (*HPT*) are as follows (HPTF, 5′-gaagtgcttgacattggggagt-3′; HPTR, 5′-agatgttggcgacctcgtatt-3′). The total volume of PCR reaction was 25 μL with 1 U Taq DNA polymerase (Takara, Dalian), 0.3 μM HPTF and HPTR, and 50 ng of genomic DNA. The wild-type plants were set as the negative control, and verified transgenic rice was set as the positive control. The PCR product was sent to Thermo Fisher Scientific for sequencing.

RNA extraction, cDNA synthesis, RT-PCR, and RT-qPCR

RNA was isolated using the RNA extraction kit with TRIzol reagent (Invitrogen, USA) and quantified with a DU730 spectrophotometer (Beckman Coulter, Germany).

cDNA was synthesized with a 5 × iScript RT supermix kit (Takara, Dalian) using about 2 µg of total RNA. RT-qPCR was performed with SYBR premix Ex Taq II (Takara, Dalian) in a total volume of 20 µL on the Bio-Rad CFX 96 following the manufacturer's protocol. Data were normalized to the internal rice *UBIQUITIN* (*UBI*) gene, and relative quantification was used for data analysis. RT-PCR was performed with the Takara Ex Taq Hot Start Version kit following the manufacturer's manual. The primers used are as follows: (TPPF, 5′-caaggagatcgtcgtgttcctcg-3′; TPPR, 5′-atgcacctcccgctcacgatcg-3′; UBIF, 5′-aaccagctgaggcccaaga-3′; UBIR, 5′-acgattgatttaaccagtccatga-3′).

Primers for *OsNCED1*, *OsNCED2*, *OsNCED3*, and *OsNCED4* were described by Zhu et al. (2009), and primers for *OsRAB21*, *OsDREB2a*, *OsMYB2*, *OsPP2C49*, *OsPP2C48*, *OsPP2C6*, *OsRAB21*, *OsbZIP23*, *OsProt*, and *OsRab1* were described by Hong et al. (2016).

Statistical analysis

The statistical significance of each parameter was determined using *t* tests. $P < 0.001$, $P < 0.01$ and $P < 0.05$ were considered significant differences and labeled ***, **, and *, respectively.

Results

OsTPP3 expression was induced in response to abiotic stress

A query of the public database (https://www.ncbi.nlm.nih.gov) showed that the *OsTPP3* gene is 1518 bp in length and encodes a 366-amino-acid protein. To study the expression of this gene, we used reverse-transcription quantitative PCR (RT-qPCR) to analyze the transcript levels of *OsTPP3* in different tissues. We found that *OsTPP3* is expressed in the roots, stems, stem nodes, seedlings, young panicles, leaves, and immature seeds, with expression levels in stem nodes being the highest (Fig. 1a).

Several studies have shown that expression of the *TPP* gene family is affected by stress conditions. Therefore, we measured the transcript level of *OsTPP3* in high-temperature, low-temperature, and drought stress conditions. The high- and low-temperature treatments induced *OsTPP3* expression, with low temperature producing a large response at 3 h after treatment. Under high temperature, *OsTPP3* expression increased at 3 and 12 h and decreased relative to its previous levels at 12 h (Fig. 1b). During the 10% PEG treatment simulating drought, the expression levels of *OsTPP3* were also increased at 2, 4, 6, and 8 days (Fig. 1c). These results indicate that *OsTPP3* expression responds to various abiotic stresses.

Overexpression of *OsTPP3* does not affect normal plant development

To investigate the function of OsTPP3 in rice, we generated 10 transgenic rice overexpressor lines containing *OsTPP3* driven by the *Ubiquitin* promoter. Wild-type (WT) and transgenic plants did not show any obvious phenotypic differences when grown in normal conditions (Fig. 2a). In addition, PCR detection of the hygromycin phosphotransferase (HPT) selectable marker gene in transgenic lines revealed a 500-bp band present in overexpression lines (OE), but not in the WT, indicating that the construct was successfully inserted into the rice genome (Fig. 2b).

To verify the expression levels of *OsTPP3* in the transgenic lines, RT-PCR was performed, which revealed that expression levels of *OsTPP3* in the transgenic lines OE1.1

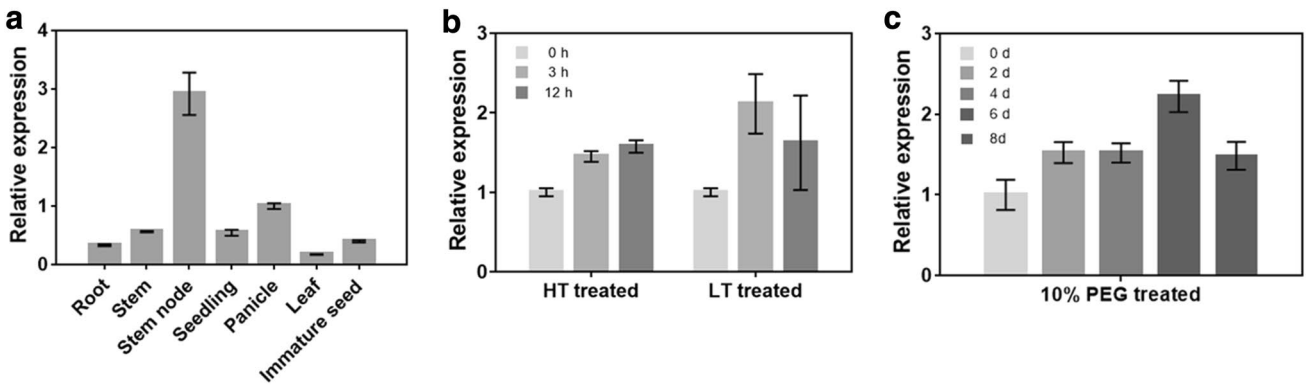

Fig. 1 Transcript analysis of *OsTPP3* in different tissues and abiotic stresses. Relative expression of *OsTPP3* was analyzed by RT-qPCR. **a** Expression pattern in different tissues. **b** Expression pattern of *OsTPP3* in 2-week-old rice seedlings exposed to low temperature (8/10 °C) or high temperature (42/37 °C). Samples were collected at 0, 3, and 12 h. **c** Expression pattern of *OsTPP3* in 2-week-old rice seedlings exposed to 10% PEG 6000 treatment. Samples were collected at 0, 2, 4, 6, and 8 days. RT-qPCR data were normalized using the rice *UBI* gene and are shown relative to 0 h. Error bars represent the mean ± SD of three biological replicates

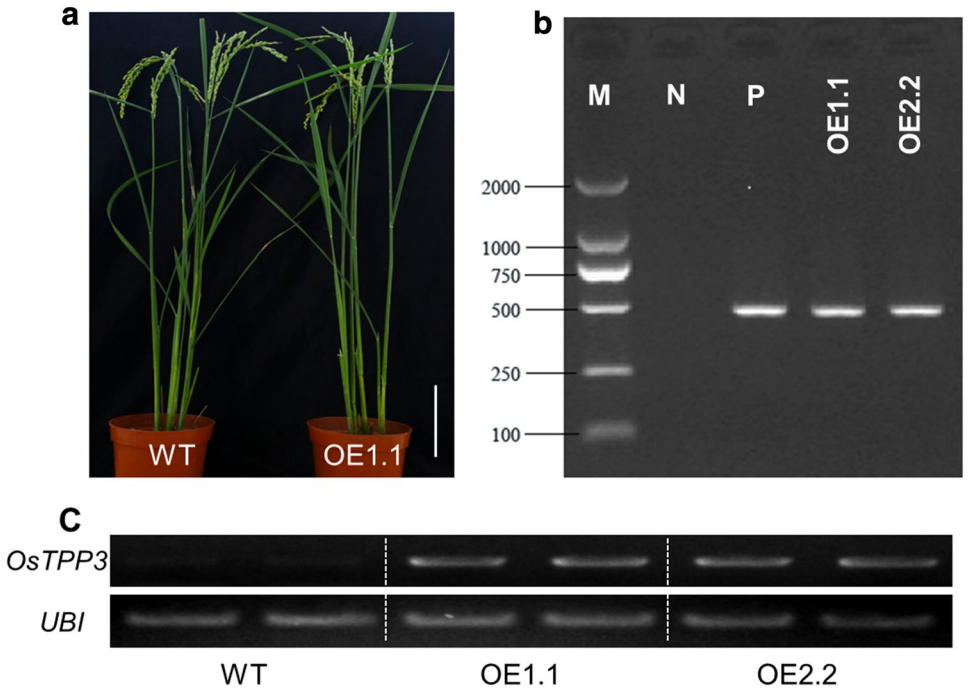

Fig. 2 Phenotypic comparison of ZH11 and *OsTPP3*-overexpressing rice plants. **a** Appearance of WT and *OsTPP3* overexpressor plants at ripening stage. **b** Detection of the *HPT* selectable marker. **c** *OsTPP3* expression measured by RT-PCR. *UBIQUITIN* was used as a loading control. WT ZH11, OE1.1 and OE2.2 *OsTPPT3* overexpression lines, M molecular marker DL2000, N negative control, *P* positive control

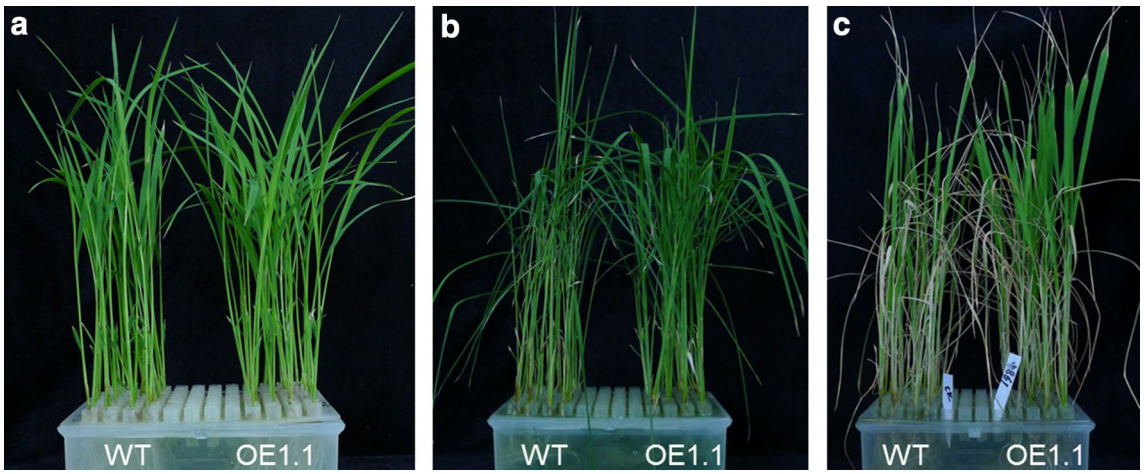

Fig. 3 Drought tolerance of WT and *OsTPP3*-overexpressing plants. Seeds were germinated in water and transferred to Kimura B nutrition solution. Two-week-old seedlings were treated in Kimura B nutrition solution with 10% PEG 6000. Plants before treatment (**a**), 6 days after drought treatment (**b**), and 10 days after drought treatment followed by 4 days of rewatering recovery (**c**) are shown. WT, wild type; OE1.1, *OsTPP3* overexpression line. Three independent experiments were performed

and OE2.2 were considerably higher than in WT (Fig. 2c), indicating that the incorporated *OsTPP3* gene was successfully overexpressed.

OsTPP3-overexpressing rice plants show increased drought tolerance

To investigate drought tolerance in *OsTPP3*-overexpressing plants, we used osmotic stress to simulate drought stress. To this end, we treated WT and transgenic plants with 10% PEG 15 days after germination. Before treatment, no significant phenotypic differences between the overexpressing plants and the WT were detected (Fig. 3a). At 6 days of PEG treatment, the leaves of the WT were all curled (Fig. 3b). Although older leaves in the overexpressor lines were curled, the young leaves appeared normal (Fig. 3b). When the drought treatment was extended to 10 days, followed by a 4-day recovery period in fresh water, a few new leaves emerged from the WT and exhibited normal green color, whereas most leaves from the transgenic plants recovered to normal, with only the tips of some old leaves remaining withered (Fig. 3c). These results suggest that *OsTPP3*

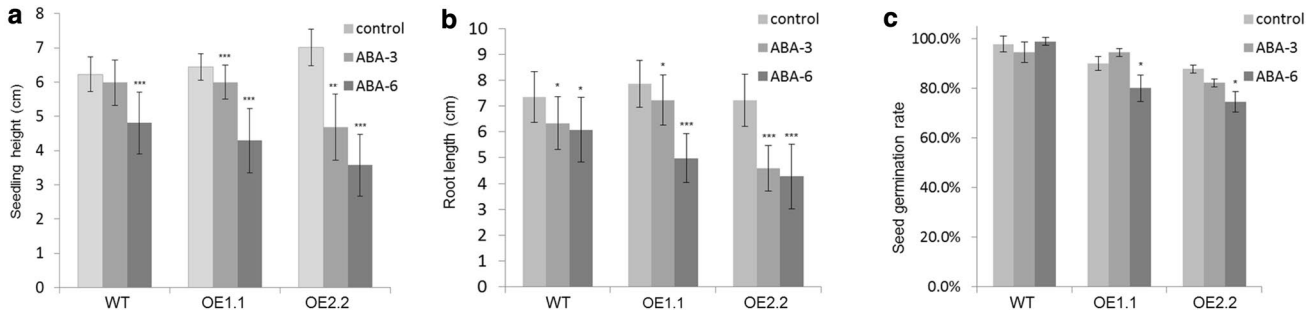

Fig. 4 Effect of ABA on root length, seedling height, and germination rate. The seedling height (**a**) and root length (**b**) were measured on 10-day-old plants after ABA treatment ($n=40$). **c** Germination rates after immersion in water solution for 6 days ($n>200$). The seeds and seedlings were cultured in hydroponic solution with 0, 3, or 6 μM ABA. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (*t test*)

overexpression enhances drought tolerance and recovery compared to WT plants.

OsTPP3-overexpressing rice lines show increased sensitivity to ABA

The plant hormone ABA is involved in the response to drought. To test the sensitivity of *OsTPP3*-overexpressing plants to ABA, we treated germinated seeds with different concentrations of ABA and measured plant height, root length, and seed germination, all of which are affected by ABA. WT plants did not show any significant differences in height between untreated and 3 μM ABA-treated plants, whereas the overexpressor lines exhibited significant differences between untreated and 3 μM ABA-treated plants (Fig. 4a).

Furthermore, root lengths were more strongly affected in the *OsTPP3* overexpressor lines compared to WT. With 6 μM ABA treatment, the root length of the WT seedlings measured 6.08 ± 1.25 cm, which was significantly shorter than that of untreated plants, at 7.35 ± 0.98 cm ($P=0.015$). Difference between 6 μM ABA-treated *OsTPP3* overexpressor line OE1.1 and untreated plants was highly significant ($P=3.29\times10^{-8}$) (Fig. 4b). Root lengths of the 6 μM ABA-treated *OsTPP3*-overexpressing line OE1.1 were 4.98 ± 0.95 cm, whereas in untreated plants, root length measured 7.86 ± 0.91 cm (Fig. 4b). A significant difference was also observed in another overexpressor line, OE2.2 ($P=3.11\times10^{-8}$) (Fig. 4b). Root lengths of 6 μM ABA-treated OE2.2 were 4.27 ± 1.26 cm, whereas in untreated plants, root length measured 7.22 ± 1.01 cm (Fig. 4b).

In addition, we measured the effects of ABA on seed germination. When we treated seeds with 6 μM ABA, we observed no change in germination rates in WT plants, whereas seed germination in the two *OsTPP3*-overexpressing lines was significantly reduced (Fig. 4c). These results indicate that *OsTPP3* overexpression increased sensitivity to ABA compared to WT plants.

ABA biosynthesis-related genes are upregulated in *OsTPP3*-overexpressing plants

To explore the reasons for the increased drought tolerance observed in *OsTPP3*-overexpressing plants, we measured the expression levels of several ABA biosynthesis-related genes using RT-qPCR. Expression levels of rice 9-*cis*-epoxycarotenoid dioxygenase (*OsNCED*) genes *OsNCED1*, *OsNCED2*, *OsNCED3*, and *OsNCED4* in the two overexpressor lines were higher than in WT plants (Fig. 5). Of these genes, *OsNCED2* expression was the most affected, increasing 30-fold compared to WT plants (Fig. 5b). *OsNCED3* expression also increased 10-fold compared to WT levels (Fig. 5c). Expression of *OsNCED1* and *OsNCED4* was also significantly upregulated in the overexpressor lines relative to WT but to a lesser extent (Fig. 5a, d). These findings suggest that the increased expression of ABA biosynthesis-related genes may alter the ABA content in *OsTPP3*-overexpressing plants, which might explain the increased drought resistance in these plants.

Changes in expression levels of stress-responsive genes in transgenic plants

To examine whether *OsTPP3* overexpression affects expression of drought-tolerance-related genes, 10 genes known to be involved in the drought response were analyzed by RT-qPCR. Expression levels of these 10 genes (*OsRAB21*, *OsDREB2a*, *OsMYB2*, *OsPP2C49*, *OsPP2C48*, *OsPP2C6*, *OsRAB21*, *OsbZIP23*, *OsProt*, and *OsRab1*) all significantly increased (Fig. 6). In the two *OsTPP3*-overexpressing lines, *OsPP2C6* expression increased by 6.85- and 8.29-fold, respectively; *OsProt* expression increased by 6.72- and 5.64-fold, respectively; and *OsRAB16* increased by 12.47- and 12.29-fold, respectively (Fig. 6). These findings indicate that these drought response genes increased the drought tolerance observed in the *OsTPP3*-overexpressing lines.

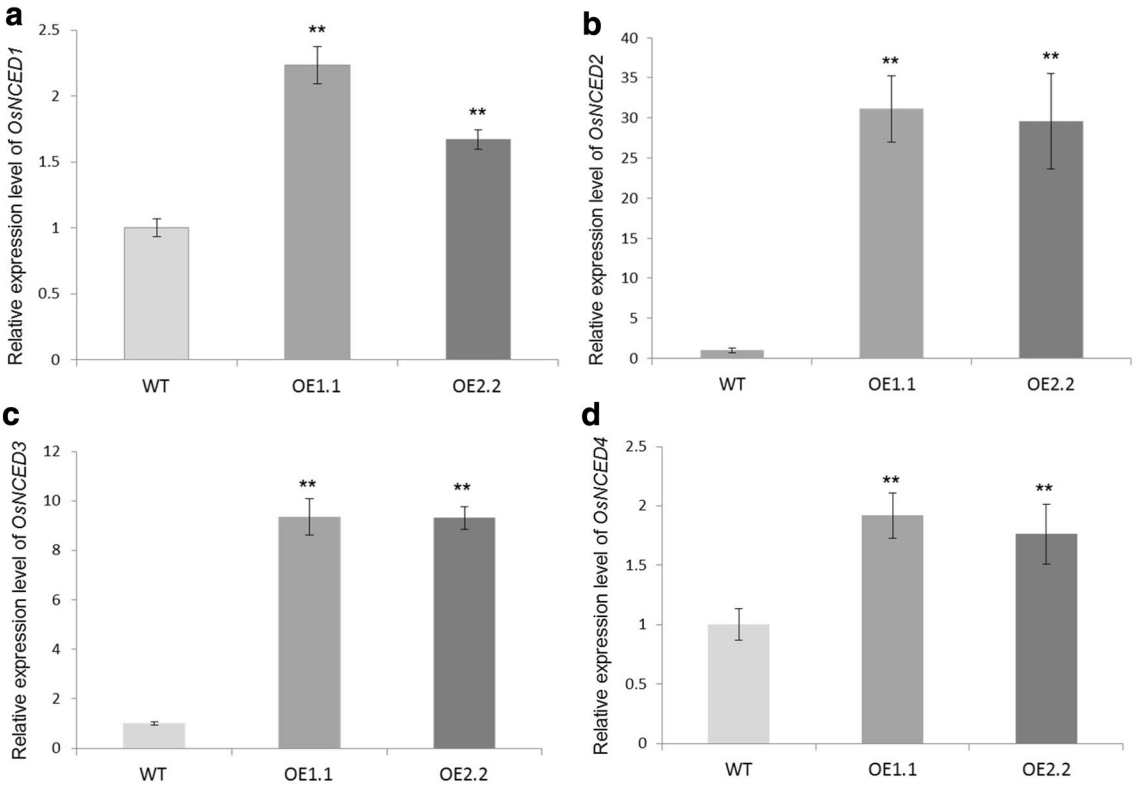

Fig. 5 Expression of *OsNCED1*, *OsNCED2*, *OsNCED3*, and *OsNCED4* genes. WT, ZH11; OE1.1 and OE2.2, *OsTPP3* overexpression lines. Two-week-old seedlings were used. RT-qPCR data were normalized to the rice *UBI* gene and are shown relative to WT. Error bars represent the mean ± SD of three biological replicates

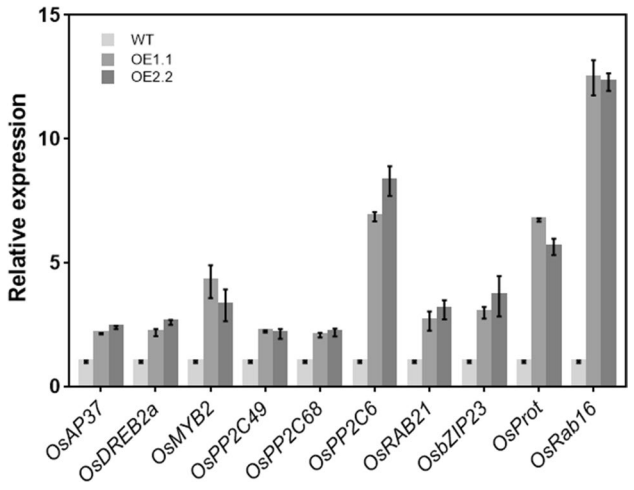

Fig. 6 Expression of abiotic stress-related genes. WT, ZH11; OE1.1 and OE2.2, *OsTPP3* overexpression lines. Two-week-old seedlings were used. RT-qPCR data were normalized to the rice *UBI* gene and are shown relative to WT. Error bars represent the mean ± SD of three biological replicates

Discussion

Since the discovery of trehalose, its function as a sugar signaling molecule has been extensively investigated (Ramon and Rolland 2007; Smeekens et al. 2010). Trehalose can increase the tolerance of plants to drought, salt, low temperature, and other abiotic stress conditions. For instance, *OsTPP1* overexpression improves resistance to different stresses (Ge et al. 2008) and increases trehalose levels to improve the resistance of transgenic rice to chilling (Zhang et al. 2017). In another example, *OsTPP7* overexpression increases germination of seeds under submergence and anaerobic conditions (Kretzschmar et al. 2015). In this study, no significant phenotypic changes were observed in the *OsTPP3*-overexpressing rice variety ZH11. Drought simulated by PEG treatment showed that overexpressing plants were more tolerant than WT, which is consistent with previously reported functions of *TPP* family genes in rice (Ge et al. 2008; Kretzschmar et al. 2015; Shima et al. 2007; Zhang et al. 2017). Researchers have conducted functional studies on *TPP* and *TPS* gene

families in rice, wheat, and maize that indicate that these genes are intimately associated with growth and development under stress conditions (*OsTPS1*; Li et al. 2011a; Nuccio et al. 2015; Xie et al. 2015).

ABA is a central regulator of abiotic stress responses in plants. Here, we show that expression levels of genes known to play a role in ABA biosynthesis (*OsNCED1*, *OsNCED2*, *OsNCED3*, and *OsNCED4*) are significantly higher in plants overexpressing *OsTPP3* than in WT. Among these genes, expression levels of *OsNCED2* and *OsNCED3* were much higher than the WT plants, which may increase ABA contents and lead to an increase in drought tolerance compared to WT plants. In addition, the expression levels of various genes involved in drought responses were significantly higher in *OsTPP3*-overexpressing plants, particularly *OsPP2C6* in the ABA signal transduction pathway (Han et al. 2017), the ABA marker gene *OsRAB16* (Hong et al. 2009), and *OsProt*, which is responsible for proline transport (Igarashi et al. 2000). These findings suggest that enhanced drought resistance in *OsTPP3*-overexpressing plants may be due to an increase in ABA content and transport, and may be associated with higher levels of proline and other antioxidant substances.

In previous reports, the expression level of *OsTPP1* and *OsTPP2* were induced by exogenous ABA. Both of them confer stress tolerance in rice (Ge et al. 2008; Shima et al. 2007). Sweet potato *IbMIPS1* (Zhai et al. 2016), cotton *GhTPS11* (Wang et al. 2016), were induced too under stress treatment. *Oropetium thomaeum* under exogenous ABA stress application can increase trehalose accumulation (Zhang et al. 2018b). ABA biosynthesis and signaling genes were generally up-regulated and trehalose synthesis-related genes were up-regulated in tea plant (*Camellia sinensis*) (Liu et al. 2016), in switchgrass (*Panicum virgatum* L.) (Zhang et al. 2018a), and in *IbMIPS1* overexpression of sweet potato (Zhai et al. 2016). These results suggest ABA and trehalose have some connection when plants encounter stress. One trehalose gene, *OsTRE1*, its overexpression of transgenic plants showed remarkable increases in trehalase activity, while had no morphological alterations or growth defects, except enhanced salt tolerance (Islam et al. 2019). It is similar with the results in our study. Arabidopsis *TPS1* gene product plays an essential role in regulating the growth of vegetative, as well as embryogenic tissue in a mechanism involving ABA and metalose metabolism (Avonce et al. 2004; Gómez et al. 2010). *OsTPS8* may regulate suberin deposition and trehalose in rice through ABA signaling to confer salinity tolerance (Vishal et al. 2019). All these studies suggest that trehalose and ABA have intimate connection in stress tolerance and growth development. These data will serve as a valuable resource for stress tolerance breading through genetic engineering.

TPP and *TPS* gene families have important functions in plants (Ramon and Rolland 2007), and research on these genes may reveal possible downstream applications to improve abiotic stress tolerance in plants. When rice *TREHALOSE-6-PHOSPHATE PHOSPHATASE1* (*OsTPP1*) was overexpressed in developing maize ears using a floral promoter, kernel set and harvest index increased (Nuccio et al. 2015). Field data from several sites over multiple seasons showed that the activity of *OsTPP1* improved yields from 9 to 49% under non-drought or mild drought conditions and from 31 to 123% under more severe drought conditions, relative to non-transgenic controls (Nuccio et al. 2015). In another example, transgenic rice expressing a fusion of *TPS* and *TPP* from *E. coli* that produces a bifunctional enzyme showed an increase in trehalose levels in leaves and seeds, which resulted in improved tolerance to drought, salt, and cold (Jang et al. 2003). These examples suggest that trehalose protects against abiotic stress and increases yield in extreme environmental conditions. In this study, the phenotype of *OsTPP3*-overexpressing plants appeared wild type under normal conditions but showed improved tolerance under drought conditions. This finding underscores the importance of further studies of *OsTPP3* and the *TPP* and *TPS* gene families to develop molecular tools that might benefit future agricultural applications.

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