



Seed Priming with Spermidine and Trehalose Enhances Chilling Tolerance of Rice via Different Mechanisms

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Abstract

Chilling stress is an important limiting factor for rice growth and development. To examine the effect of spermidine (Spd) and trehalose (TH) priming in response to chilling stress of rice seed, we investigated the effects of seed priming with Spd, TH, and a mixture of Spd + TH on response and resilience of rice plants to chilling stress. Priming with Spd, TH and Spd + TH resulted in better seed vigor and seedling growth than hydro-priming and non-priming under low temperature (15 °C). In addition, Spd-, TH-, and Spd + TH-priming notably decreased malondialdehyde content but increased the proline and soluble sugar contents and antioxidant enzymes activities. Interestingly, spermine content and the expression of *spermine-synthase* (*SPMS*) gene were improved by Spd-priming. However, lower spermine content and *SPMS* expression was observed in the TH-primed plants. Moreover, expressions of *trehalose-6-phosphate-phosphatase* (*TPP*) genes was upregulated by TH-priming but downregulated by Spd-priming. It could be concluded from our results that (1) both Spd- and TH-priming could increase the contents of proline and soluble sugar, which were involved in osmotic adjustment, and antioxidant enzymes activities; (2) Spd-priming promoted the accumulation of endogenous spermine by upregulating *SPMS1* and *SPMS2*, but inhibiting the expressions of *TPP1* and *TPP2*; and (3) TH-priming increased the expressions of *TPP1* and *TPP2* but downregulated the expressions of *SPMS1* and *SPMS2*. These findings suggested that seed priming with Spd and TH might enhance the chilling tolerance of rice seedling via different mechanisms.

Keywords Seed priming · Chilling stress · Spermidine · Trehalose

Introduction

Chilling is one of the major environmental stresses restricting plant growth and development. Rice yield reduction caused by chilling injury is a common problem worldwide. Rice varieties distributed in different regions are often subjected to varying degrees of low temperature hazards during the growing period (Cruz et al. 2013). The previous study showed that low temperature stress always delayed seed germination and caused poor growth of rice seedlings (Saddam et al. 2016). Therefore, it was necessary to develop novel methods to enhance the chilling tolerance of rice seed.

Polyamines (PAs), a class of physiologically active substances, widely exist in biological cells. Studies have shown

that low temperature stress easily induced the accumulation of endogenous PAs in many plants, such as mustard (Mo and Pua 2010), watermelon (Akiyama et al. 2004), rice (Nayyar et al. 2005), wheat, barley and oats (Gondor et al. 2016). It suggested that PAs might involve in the cold tolerance of plants. PAs in higher plants mainly include putrescine (Put), spermidine (Spd) and spermine (Spm). Different kind of PAs often had different responses to chilling injury across plant species. Kushad and Yelenosky (1987) found that the resistance to low temperature stress of three citrus varieties was related to the Spd content. The exogenous application of Spd and Spm in water nutrient solution could effectively alleviate the damage of chickpea seedlings under low temperature, and the Spd had better effect (Nayyar and Chander 2004). Besides, Spd priming showed a more significant effect than Put and Spm on chilling tolerance of *Lolium perenne* seed (Wang et al. 2012a, b). The embryos of two maize inbred lines, Huang and Mo 17, were less susceptible to cold injury by Spd priming than Spm priming (Zheng et al. 2008). As above, in terms of improving plant cold tolerance, Spd might

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have more significant effect among three PAs to some extent. Zeng et al. (2016) found that Spd priming could promote effectively chilling tolerance by maintaining the integrity of cell structure and normal metabolism of endogenous hormones in indica-japonica hybrid rice seedlings. However, it was still not clear about the mechanism of Spd involving in the improvement of chilling tolerance of hybrid rice seed.

Trehalose (TH) is a non-reducing sugar composed of two glucose molecules (Wingler 2002). It was reported to act as an osmotic regulator in many plants under various stresses, such as chilling and osmotic stress (Luo et al. 2008; Elbein et al. 2003; Jang et al. 2003). Williams et al. (2015) reported that TH was widely detected in low temperature tolerant crops, suggesting TH involving in the establishment of cold tolerance in plants. However, the effect of TH priming on the cold resistance of rice seed was still unclear.

In our previous study, exogenous Spd increased the expression level of trehalose synthesis-related genes under high-temperature stress, indicating that there was a relationship between Spd and TH on stress resistance of rice seed. However, few reports focused on the combined effect of Spd and TH on rice chilling-resistance, and the mechanism involved in their combination effect remained completely unclear. In this experiment, we found that both rice seed vigor and seedling quality enhanced under chilling stress after Spd or TH single-priming. Therefore, antioxidant enzymes activities, soluble sugar content, polyamine contents, the corresponding genes expressions of Spd or TH biosynthesis-related genes were determined after priming with Spd, TH, and Spd + TH combination to acquire better understandings on the potential interaction of Spd + TH priming in rice seed chilling resistance.

Materials and Methods

Seed Priming

Spermidine (Spd) and trehalose (TH) solutions of different concentrations (0, 0.5, 1, and 2 mM) were prepared. Rice seeds were disinfected with 2% NaClO for 15 min, then rinsed with distilled water and soaked in the Spd and TH solutions for 24 h in the dark (25 °C). After soaking, the seeds were rinsed with distilled water and the seeds were dried off to their initial moisture.

Seed Germination

All primed rice seeds were subjected to a 14-day germination experiment. The primed seeds were incubated in growth chambers with a photosynthetic photon flux density of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a photoperiod of 12 h light: 12 h dark (Ji et al. 2010). The chilling temperature was set

to 15 °C. The treatments in this study were, respectively, referred to as CKn (untreated dry seeds incubated at 25 °C), CKc (untreated dry seeds incubated 15 °C), H₂O (distilled water-primed seeds incubated at 15 °C), Spd_{0.5} (0.5 mM Spd-primed seeds incubated at 15 °C), Spd₁ (1 mM Spd-primed seeds incubated at 15 °C), Spd₂ (2 mM Spd-primed seeds incubated at 15 °C), TH_{0.5} (0.5 mM TH-primed seeds incubated at 15 °C), TH₁ (1 mM TH-primed seeds incubated at 15 °C), TH₂ (2 mM TH-primed seeds incubated at 15 °C), Spd_{0.5} + TH_{0.5} (0.5 mM Spd + TH-primed seeds incubated at 15 °C), Spd₁ + TH₁ (1 mM Spd + TH-primed seeds incubated at 15 °C), Spd₂ + TH₂ (2 mM Spd + TH-primed seeds incubated at 15 °C).

The number of germinated seed (the seed whose radicle visibly protruded through the seed coat and reached to the half-length of the diameter of the whole seed) was counted every day. The germination energy (GE) and germination percentage (GP) were calculated at the 5th and 14th days, respectively. After 14 days of germination, root length (RL) and shoot height (SH) were measured, and the seedling dry weight (SDW) was determined after drying at 80 °C for 48 h. Each treatment has three replications. All the measurements were based on ten randomly selected normal rice seedlings for each replication. We also calculated the Germination Index (GI) and Vigor Index (VI) following the method described by Hu et al. (2016).

Measurements of Physiological Parameters

0.3 g of fresh rice seeds was taken, and added 3 ml of phosphoric acid buffer with pH 7.8, grinded in ice bath until homogenized, and transferred to 5 ml centrifugal tube. Then, the mixture was centrifuged at 4 °C at $10,000 \times r \text{ min}^{-1}$ for 20 min, the supernatant was the crude enzyme extract for the determination of enzyme activity.

Malondialdehyde (MDA) concentration was determined using the thiobarbital (TBA) reaction, as described by Jing et al. (2016) with some modifications. 1.5 ml of the crude enzyme solution was mixed with 2.5 ml of TBA-TCA solution, boiled in water for 15 min, rapidly cooled to room temperature, centrifuged at $1800 \times r \text{ min}^{-1}$ for 10 min, and the supernatant was taken. The absorbance at 532 nm (OD_{532}) and 600 nm (OD_{600}) was measured for the mixtures, and the content of MDA was calculated. For each sample, the measurements were carried out in triplicate.

The peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and peroxidase (APX) activities were performed according to the method described by Zhu et al. (2016) with some modifications. For POD, the assay mixture consisted of 1.35 ml of 25 mM phosphate buffer (pH 7.0), 100 μl of 1.5% guaiacol, 100 μl of 100 mM H₂O₂, and 100 μl of enzyme extract. The increase in 470-nm absorbance was measured spectrophotometrically at 470 nm for 2 min. The

enzymatic activity was calculated as nmol of guaiacol oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$ (fresh weight) and was expressed as $\text{nmol g}^{-1} \text{FW min}^{-1}$. For each sample, the measurements were carried out in triplicate.

For SOD, the reaction mixture was composed of 50 mM phosphate buffer (pH 7.8), 1.22 mM riboflavin, 13 mM methionine, 56 μM NBT, 78.2 μM EDTA, and 100 μl enzyme extract. Distilled H_2O was added into the mixture to bring the final volume to 3 ml. A complete reaction mixture without enzyme extract was used as a control. The reaction was initiated by placing the tubes with the mixture under 4000 Lux for 15 min. The reaction was measured spectrophotometrically at 560 nm for inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). In this assay, 1 unit was defined as the amount of SOD required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as SOD g^{-1} protein.

For CAT, the enzyme assay contained 1.4 ml of phosphate buffer (25 mM pH 7.0 + 2 mM EDTA), 200 μl of 300 mM H_2O_2 , and 100 μl enzyme extract in a total volume of 1.7 ml. The CAT activity was estimated by monitoring the 240-nm absorbance of the mixture for 2 min and was expressed as $\text{nmol g}^{-1} \text{FW min}^{-1}$.

For APX, the enzyme assay contained 1.4 ml of phosphate buffer (25 mM pH 7.0 + 2 mM EDTA), 100 μl of 7.5 mM ascorbic acid, 100 μl of 300 mM H_2O_2 , and 100 μl enzyme extract in a total volume of 1.7 ml. The APX activity was estimated by monitoring the 290-nm absorbance of the mixture for 2 min and was expressed as $\text{nmol g}^{-1} \text{FW min}^{-1}$.

Determination of Proline Content

Proline content was determined using a method described by Bates et al. (1973). 100 ml of leaf samples were taken and ground in 2 ml of 3% sulfosalicylic acid. The homogenate was heated in a boiling water bath for 15 min and centrifuged at $12,000 \times g$ for 10 min. 200 μl of the supernatant was added into a mixture of 200 μl of tri-distilled water, 200 μl of glacial acetic acid, and 400 μl of acid ninhydrin. Then, the mixture was placed in the boiling water bath for 1 h. The absorbance at 520 nm was measured, and the proline content was expressed as $\text{nmol g}^{-1} \text{FW}$.

Determination of Soluble Sugar Content

The content of soluble sugar was determined by anthrone colorimetry (Oscar et al. 2010). Briefly, 0.1 g of leaves were taken. Distilled water was added into the leaf samples, and the mixtures were incubated in a water bath pot at 100 °C for 30 min. After that, the supernatant was separated, added with 5 ml distilled water and reheated for 30 min. Then, the supernatant was removed, the glass tube was cleaned, the solution was transferred to a 25-ml volumetric flask, then to

a 50-ml centrifuge tube, and centrifuged at $5000 \times g$ for 15 min. Afterwards, 0.5 ml of centrifuged solution was taken, diluted, and 0.5 ml solution was transferred to a 20-ml test tube. A mixture of 1.5 ml of distilled water, 0.5 ml of anthrone, and 5 ml of concentrated sulfuric acid was added to the solution. After that, we fully vibrated the tubes and immediately put the test tubes into boiling water bath. The test tubes were taken out from boiling water bath and allowed to cool naturally in room temperature with the temperature of distilled water as a reference. Finally, we determined the absorbance at 620 nm.

Measurement of Endogenous Polyamine Content

PAs extraction from seedlings was analyzed by HPLC according to the testing method published by Huang et al. (2017). 2 g of fresh leaves from each treatment were homogenized with 2 ml of 5% (w/v) cold perchloric acid. The mixtures were kept in ice bath for 1 h and then centrifuged at $10,000 \times g$ for 30 min at 4 °C. The supernatant was transferred and stored at -80 °C for PAs quantification. 5 ml of the supernatant was taken and mixed with 1 ml of 2 mM NaOH solution and 10 μl of benzoyl chloride. The mixtures were incubated separately for 20 min at 37 °C. 2 ml of saturated NaCl solution and 2 ml of diethyl ether were added into the mixture, which was then centrifuged at $1500 \times g$ for 15 min at 4 °C. From the centrifuged mixture, 1 ml of diethyl ether phase was extracted, dried by nitrogen and redissolved in 100 μl of methanol for the following test. The extracts were filtered through a 0.22 μm membrane filter and then eluted through a 6.0 mm \times 150 mm, 5 mm particle size reverse-phase (C18) column (Shim-Pack CLC-ODS) at room temperature. PAs peaks were detected by an SPD-20A (Shimadzu) absorbance detector at 254 nm. The mobile phases consisted of water and methanol (35/65, v/v) at a flow rate of 1.0 ml min^{-1} . The polyamine standards (Sigma) of Put, Spd, and Spm were used for the generation of standard curves. For each sample, the measurements were carried out in triplicate.

Real-time Quantitative Polymerase Chain Reaction (PCR)

Total RNA of each sample was extracted and 500 ng of RNA were reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit (Vazyme, China). Real-time PCR reaction was carried out with CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Primer sets were designed with the Primer5 software and the rice actin gene was used as an internal reference gene (Table 1). The 20 μl reaction system contained 1 μl of diluted cDNA, 0.6 μl of reverse and forward primers, 7.8 μl of ddH_2O , and 10 μl of the AceQ qPCR SYBR

Table 1 Primer sequences used in RT-PCR

Genes	Primer sequence
<i>ACTIN</i>	L:agtgtctggattgaggat R:tcttgcttagcattcttg
<i>ADC1</i>	L:ctatggcggaggtagaccag R:ctcgcgaactccacaat
<i>ADC2</i>	L:acaactaccacatgaacctctec R:ggtcggcttctcgttcag
<i>ODC</i>	L:tcgtgttcgacgacatgg R:cagtggtgatctttatcgtga
<i>SAMDC</i>	L:gtctttgctgacctgatgg R:cgtgcaagatccagaacagag
<i>SPDS</i>	L:ggtgtttcagtcctccacgta R:tcctctcagtgacctgaatc
<i>SPMS1</i>	L:atgctttctatctgctgaaaca R:aacgcttgctcaggcataat
<i>SPMS2</i>	L:atgctttctatctgctgaaaca R:aacgcttgctcaggcataat
<i>TPP1</i>	L:ggctcgtaaacgaagtgtctg R:cggacgaacctctaaacca
<i>TPP2</i>	L:agtggagatctcgtgataagggt R:gcccatgatattccattcc

Green Master Mix (Vazyme, Nanjing, China). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ analytical method. All the reactions were carried out in 3 repeats and 3 independent biological replicates. The data were expressed as mean \pm SD (standard deviation) after normalization.

Table 2 Effect of spermidine (Spd) and trehalose (TH) priming on seed germination under chilling stress

Treatment	GE/ %	GP/ %	GI	VI
CKn	91.11 \pm 2.94ab	91.11 \pm 2.94ab	13.44 \pm 0.242a	1.13 \pm 0.020a
CKc	48.89 \pm 1.11d	58.89 \pm 1.11d	4.71 \pm 0.082d	0.19 \pm 0.003f
H ₂ O	75.56 \pm 2.94 cd	75.56 \pm 2.94c	10.56 \pm 0.338c	0.58 \pm 0.010e
Spd _{0.5}	88.89 \pm 2.94ab	88.89 \pm 2.94ab	12.44 \pm 0.309b	0.90 \pm 0.040d
Spd ₁	93.33 \pm 3.85a	93.33 \pm 3.85a	13.61 \pm 0.309a	0.96 \pm 0.013cd
Spd ₂	88.89 \pm 1.11ab	88.89 \pm 1.11ab	13.33 \pm 0.167ab	0.99 \pm 0.027c
TH _{0.5}	85.56 \pm 2.94b	85.56 \pm 2.94b	12.83 \pm 0.441ab	0.88 \pm 0.049d
TH ₁	93.33 \pm 1.25ab	93.33 \pm 0.21ab	13.72 \pm 0.278a	1.09 \pm 0.027ab
TH ₂	90.00 \pm 3.33ab	90.21 \pm 3.33ab	13.33 \pm 0.441ab	1.02 \pm 0.040bc
Spd _{0.5} + TH _{0.5}	87.33 \pm 1.51a	90.11 \pm 1.51a	13.65 \pm 0.334a	0.79 \pm 0.012d
Spd ₁ + TH ₁	92.11 \pm 1.11ab	92.33 \pm 1.12ab	13.66 \pm 0.278a	1.05 \pm 0.037ab
Spd ₂ + TH ₂	93.33 \pm 1.93a	93.33 \pm 1.93a	13.72 \pm 0.294a	0.89 \pm 0.028d

CKn: normal temperature control; CKc: low temperature control; H₂O: water priming; Spd_{0.5}: 0.5 mM Spd priming; Spd₁: 1 mM Spd priming; Spd₂: 2 mM Spd priming; TH_{0.5}: 0.5 mM TH priming; TH₁: 1 mM TH priming; TH₂: 2 mM TH priming; Spd_{0.5} + TH_{0.5}: 0.5 mM Spd + TH priming; Spd₁ + TH₁: 1 mM Spd + TH priming; Spd₂ + TH₂: 2 mM Spd + TH priming. The same as follows. The data were presented as the mean \pm standard deviation values (SD) and different letters indicated significant differences between treatments ($\alpha=0.05$, LSD)

GE germination energy, GP germination percentage, GI germination index, VI vigor index

Statistical Analysis

We used analysis of variance (ANOVA) to analyze the data in the Statistical Analysis System (SAS) followed by calculation of the least significant difference (LSD, $\alpha=0.05$). Percentage data were arc-sin-transformed prior to analysis.

Results

Seed Priming Enhanced Seed Vigor and Seedling Growth Under Chilling Stress

The seed germination was inhibited under low temperature, but improved by priming treatments (Table 2). Under the chilling stress, GP, GE, GI, and VI were significantly improved after all priming treatments (Table 2). Spd- and TH-priming had better effects than water priming. Specifically, compared with water-primed seeds, Spd₁-priming improved the GI and VI by 28.88% and 65.52%, respectively; and TH₁-priming improved the GI and VI by 29.92% and 87.93%, respectively. Spd₁ and TH₁-primed seeds recorded the highest GP and GE under chilling conditions compared with the other two concentrations (Table 2). Seeds primed with Spd₁ + TH₁ showed no significant differences in GP, GE, and GI compared with Spd_{0.5} + TH_{0.5} and Spd₂ + TH₂. However, VI was significantly higher in Spd₁ + TH₁-primed seeds compared with the seeds primed with other two combined concentrations.

Similar changes were found in seedling quality (Table 3). Compared with water-primed seeds, priming

Table 3 Effect of Spd and TH priming on root growth under chilling stress

Treatment	RL/cm	SH/cm	FL/cm	SDW/g
CKn	8.22 ± 0.294a	5.9 ± 0.127bc	14.11 ± 0.288a	0.08 ± 0.003a
CKc	2.30 ± 0.289g	1.93 ± 0.203e	4.23 ± 0.484g	0.04 ± 0.001g
H ₂ O	3.51 ± 0.368f	3.87 ± 0.053d	7.38 ± 0.415f	0.05 ± 0.001f
Spd _{0.5}	4.72 ± 0.044def	6.42 ± 0.110a	11.15 ± 0.071cde	0.07 ± 0.002cd
Spd ₁	5.59 ± 0.126bc	6.22 ± 0.24ab	11.81 ± 0.366bc	0.07 ± 0.001d
Spd ₂	4.25 ± 0.123e	6.13 ± 0.012ab	10.38 ± 0.127e	0.07 ± 0.001bcd
TH _{0.5}	5.25 ± 0.064cd	6.07 ± 0.092ab	11.33 ± 0.03cd	0.07 ± 0.002d
TH ₁	5.83 ± 0.170b	6.3 ± 0.108a	12.13 ± 0.115b	0.08 ± 0.001b
TH ₂	5.42 ± 0.044bc	5.56 ± 0.078c	10.98 ± 0.102def	0.08 ± 0.001bc
Spd _{0.5} + TH _{0.5}	5.41 ± 0.011bc	5.32 ± 0.139c	10.95 ± 0.149cde	0.06 ± 0.002e
Spd ₁ + TH ₁	5.83 ± 0.170b	6.2 ± 0.125a	12.01 ± 0.125b	0.08 ± 0.001b
Spd ₂ + TH ₂	5.51 ± 0.021bc	5.54 ± 0.139c	11.05 ± 0.149cde	0.06 ± 0.003e

The data were presented as the mean ± standard deviation values (SD) and different letters indicated significant differences between treatments ($\alpha=0.05$, LSD)

RL root length, SH shoot height, FL full length, SDW seedling dry weight

with Spd₁ improved the full length (FL) and SDW by 60.03% and 40%, respectively; and priming with TH₁ improved the FL and SDW by 64.36% and 60.00%, respectively. The results also showed that priming with Spd₁ and TH₁ produced the highest seedling quality under normal and chilling conditions compared with the other two concentrations (Table 3). Priming with Spd₁ + TH₁ had no significant effects on root length, but the treatment significantly increased the SH, FL, and SDW compared with the other two concentrations.

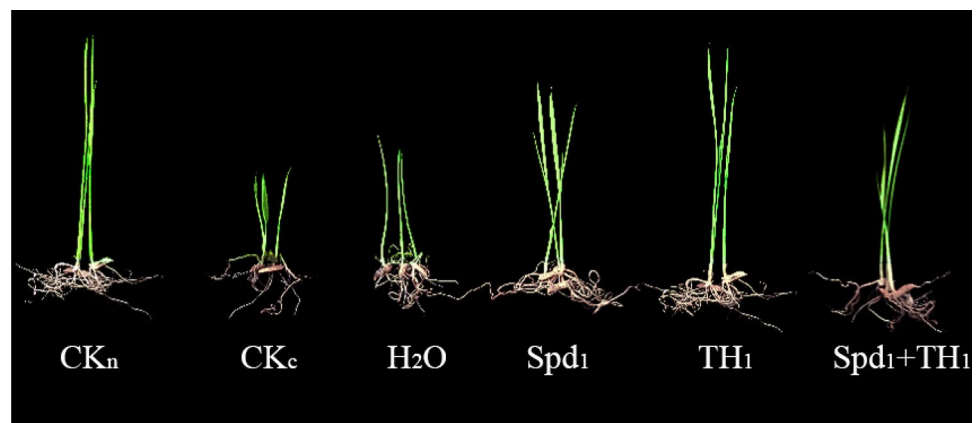
According to the effects of priming treatments on seed vigor and seedling growth (Fig. 1), unprimed seeds under normal temperature (CKn), unprimed seeds under low temperature (CKc), water-, Spd₁-, TH₁-, and Spd₁ + TH₁-primed seeds were chosen for the following experiments.

Seed Priming Decreased MDA Content and Increased Proline and Soluble Sugar Contents Under Chilling Stress

Compared with the CKc seeds, water priming significantly decreased MDA content by 8.33%. Furthermore, Spd₁-, TH₁-, and Spd₁ + TH₁-priming decreased MDA content by 40.63%, 44.80%, and 55.99%, respectively (Fig. 2a).

The proline and soluble sugar contents after CKc treatment increased significantly compared with those in CKn seeds (Fig. 2b, c). Water priming enhanced the accumulation of proline and soluble sugar. Compared with the CKc treatment, Spd₁-, TH₁-, and Spd₁ + TH₁-priming increased the proline content by 27.22%, 13.99%, and 29.01%, respectively and increased the soluble sugar content by 34.44%, 73.69%, and 51.98%, respectively.

Fig. 1 Effect of seed priming with Spd₁, TH₁ and Spd₁ + TH₁ on rice seedling growth under chilling stress (15 °C). CKn: normal temperature control (25 °C); CKc: low temperature control (15 °C); H₂O: water priming + low temperature; Spd₁: 1 mM Spd priming + low temperature; TH₁: 1 mM TH priming + low temperature; Spd₁ + TH₁: 1 mM Spd + 1 mM TH priming + low temperature



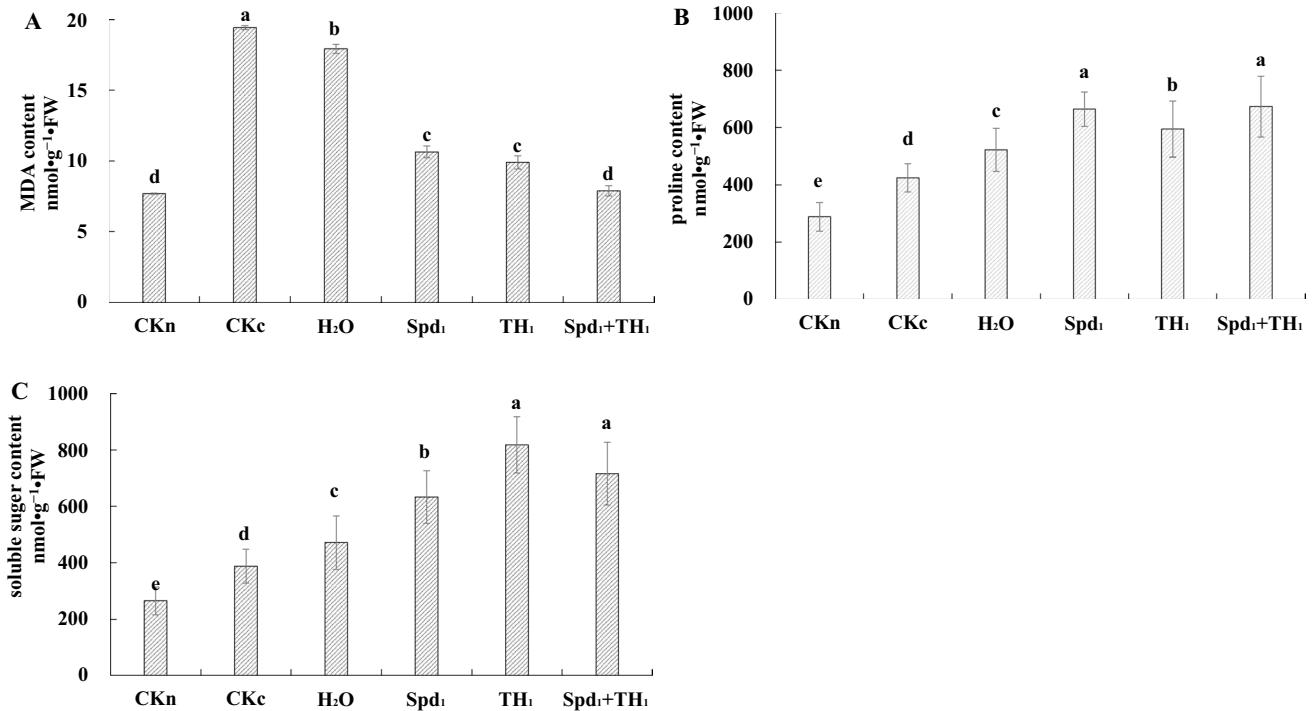


Fig. 2 Effects of Spd₁, TH₁ and Spd₁ + TH₁ on MDA (a), proline (b) and soluble sugar (c) content in rice seedlings under chilling stress. The bars mean standard deviation and different letters indicate signifi-

cant differences between treatments ($\alpha=0.05$, LSD). The treatment methods were the same as those in the Fig. 1 notes

Seed Priming Stimulated Antioxidant Enzymes Activities in Response to Chilling Stress

The priming treatments elevated the activities of antioxidant enzymes, including CAT, POD, and APX (Fig. 3). Compared with water priming, Spd₁-, TH₁-, and Spd₁ + TH₁-priming significantly increased the CAT activity under chilling stress by 50.53%, 30.42%, and 27.33%, respectively. Furthermore, Spd₁-, TH₁-, and Spd₁ + TH₁-priming significantly improved the POD activity irrespective of chilling stress by 62.21%, 67.89%, and 40.33%, respectively, compared with the water priming. Similarly, APX activities in Spd₁-, TH₁-, and Spd₁ + TH₁-primed seeds were significantly improved under chilling stress by 6.44%, 83.44%, and 78.86%, respectively, compared with that of water-primed seeds. In contrast, SOD decreased significantly after Spd₁-, TH₁-, and Spd₁ + TH₁-priming treatments under chilling stress compared with water priming (Fig. 3).

Seed Priming Regulated Polyamine Metabolism in Response to Chilling Stress

Put and Spd contents in the seedlings significantly increased under chilling stress compared with those in CKn seedlings (Fig. 4a, b). Spd₁- and Spd₁ + TH₁-priming significantly increased the content of Spm under chilling stress compared

with unprimed seeds. However, TH₁-priming showed no significant effects (Fig. 4c). Compared with CKc, priming with water, Spd₁, TH₁, and Spd₁ + TH₁ resulted in a significant decrease in the Put content under chilling stress, but there was no significant difference in Put content among seedlings underwent these treatments. The Spd content in seedlings primed with water is significantly lower than that of CKc seedlings, but is significantly higher than those in seedlings primed with Spd₁, TH₁, and Spd₁ + TH₁. Priming with TH and Spd + TH substantially decreased Spd content. However, Spm content increased substantially under chilling stress across the priming treatments. Particularly, after Spd treatment, the Spm content in seedlings reached the highest level. Interestingly, Spd + TH-priming increased the Spm content to a greater extent than TH-priming, but to a lesser extent than Spd-priming (Fig. 4).

The results showed that *ADC2*, *SAMDC*, *SPMS1*, and *SPMS2* were downregulated, while *SPDS* was upregulated under chilling stress compared with the normal condition (Fig. 4). However, compared with CKc seeds, Spd₁-primed seeds showed increased *SPMS1* and *SPMS2* expressions under chilling stress, while TH₁-priming resulted in decreased expressions of the genes (Fig. 4). Interestingly, Spd₁ + TH₁-priming generated a higher *SPMS2* expression than TH-priming, but a lower *SPMS2* expression than Spd-priming (Fig. 4).

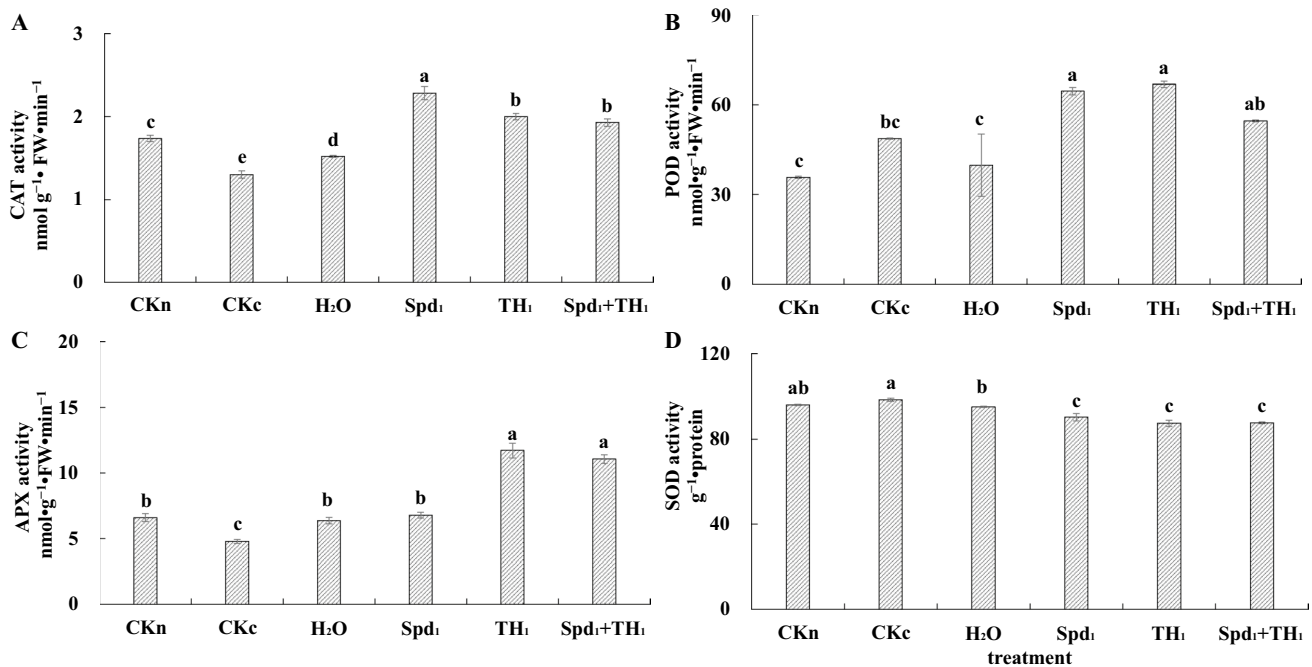


Fig. 3 Effects of seed priming with Spd₁, TH₁ and Spd₁+TH₁ on antioxidant enzyme activities of rice seedling under chilling stress. The bars mean standard deviation and different letters indicate significant differences between treatments ($\alpha=0.05$, LSD). The treatment methods were the same as those in Fig. 1 notes

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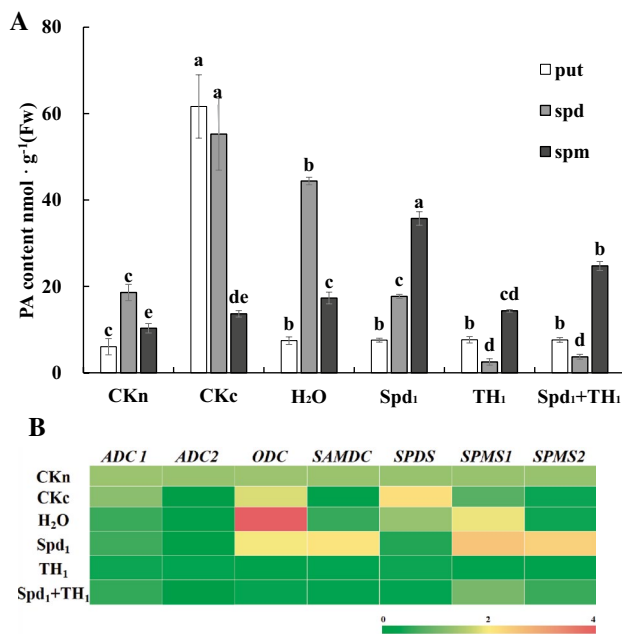


Fig. 4 Effects of priming with Spd₁, TH₁ and Spd₁+TH₁ on endogenous polyamines content (a) and polyamine synthesis-related genes (b) in rice seedlings under chilling stress. The bars mean standard deviation and different letters indicate significant differences between treatments ($\alpha=0.05$, LSD). The treatment methods were the same as those in the Fig. 1 notes

Seed Priming Regulated the Expression Levels of Trehalose-6-phosphate Phosphatase Genes in Response to Chilling Stress

OsTPP1 and *OsTPP2* were upregulated under chilling stress compared with the normal condition (Fig. 6). TH₁-priming significantly improved the *OsTPP1* and *OsTPP2* expressions under chilling stress compared with unprimed seeds. However, Spd₁-priming decreased the expressions of the genes (Fig. 6). Interestingly, the expression of *OsTPP2* in Spd₁+TH₁-primed seeds is higher than that of Spd₁-primed seeds, but lower than that of TH₁-primed seeds (Fig. 5).

Discussion

It is well known that rice originates from the tropics and subtropics and is extremely sensitive to chilling stress (Howarth and Ougham 2010). In this study, 15 °C was applied as chilling temperature according to the study of Ji et al. (2010), and results showed that seed vigor and seedling quality decreased substantially under chilling stress (Tables 2, 3). These results were consistent with Saddam et al. (2016), who found that rice seeds exposed to chilling stress (18 °C for 14 days) had considerably decreased germination speed, germination rate, and seedling growth. However, Spd-, TH-, and Spd+TH-priming alleviated the damage of chilling

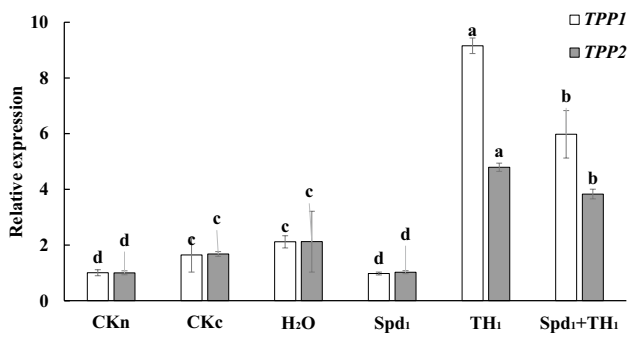


Fig. 5 Effects of priming with Spd₁, TH₁ and Spd₁+TH₁ on trehalose synthesis-related genes *OsTPP1* and *OsTPP2* in rice seedlings under low temperature stress. The expression level of different genes was measured by RT-qPCR analysis; the expression level of CKn was regarded as 1.0. The treatment methods were the same as those in the Fig. 1 notes

stress to rice seed vigor and seedling quality. Spd and TH treatments were effective under chilling stress, which was consistent with previous studies (Diao et al. 2015, 2016; Zhang et al. 2018; Kosar et al. 2018). In contrast, combined priming of Spd + TH did not further improve seed germination compared with separate Spd- and TH-priming.

Previous study indicated that there was a positive correlation between proline content and plant cold tolerance (Chen et al. 2010). Besides, the soluble sugar content of several plants, including cucumber (Wan et al. 2015) and Arabidopsis (Moustakas et al. 2011) increased under low temperature. In our study, Spd-priming significantly reduced MDA content but increased proline and soluble sugar contents in rice seedlings under chilling stress (Fig. 2b, c), which was in accord with the results of Diao et al. (2015)

and Nahar et al. (2015). The effects of TH-priming were similar to those of Spd-priming. Similar observations were reported by Xie et al. (2015), who found that exogenous TH helped to increase the contents of soluble protein and soluble sugar in wheat seedlings, improve the antioxidant capacity, reduce the degree of membrane lipid peroxidation injury, and therefore, enhanced the cold resistance in wheat. Moreover, the soluble sugar content in TH-primed seedlings was significantly higher than that in Spd-primed seedlings, but the effects of TH- and Spd-priming on proline content were just the opposite. It might be due to the fact that TH participates in sugar metabolism, while proline and Spd have the same synthetic precursor. The MDA content, as an index of oxidative damage degree, was consequently analyzed, and the results showed that priming treatments substantially down-regulated MDA accumulation during seed germination under chilling stress (Fig. 2a). The proline, soluble sugar, and MDA contents of Spd + TH-primed seedlings were just between those in seedlings primed with TH and Spd separately.

To adapt various adverse conditions, the antioxidant defense system had been formed internally in the plants, which removed the excessive reactive oxygen species through enzymatic and non-enzymatic mechanisms. The enzymatic defense system includes SOD, CAT, POD, and APX. In our study, Spd-, TH-, and Spd + TH-priming significantly improved CAT, POD, and APX activities in rice seedlings under chilling stress, which was consistent with Li et al. (2017), who found an upregulation of antioxidant enzymes activities after priming treatment. In our study, the activities of SOD in Spd- and TH-primed seedlings were significantly decreased under chilling stress (Fig. 3). The difference in the response of SOD activity to Spd- and

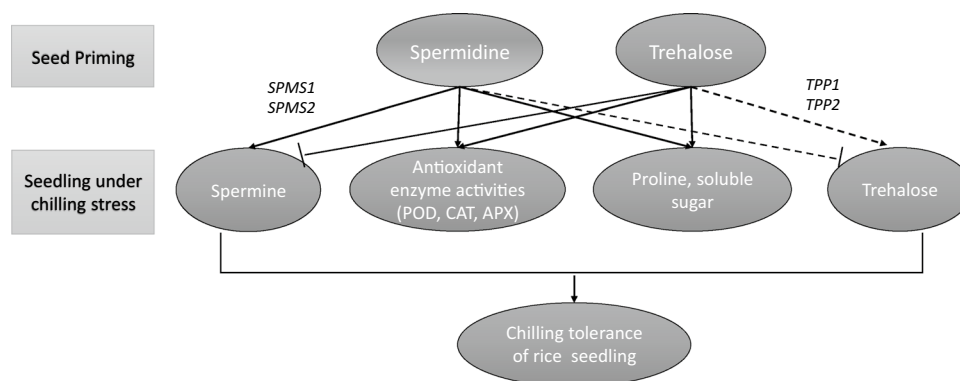


Fig. 6 Schematic presentation of main metabolic pathways regulated by spermidine and trehalose in rice seedling exposed to chilling stress. Both of spermidine and trehalose priming could increase osmotic adjustment substances (such as proline and soluble sugar) contents, antioxidant enzymes activities. The spermidine priming promoted the accumulation of endogenous spermine content by upregulating *SPMS1* and *SPMS2*, but inhibited the expression of

TPP1 and *TPP2*. On the contrary, trehalose priming increased the expression of *TPP1* and *TPP2* but downregulated *SPMS1* and *SPMS2* expression levels. Therefore, it was supposed that seed priming with spermidine and trehalose might enhance the chilling tolerance of rice seedling via different mechanisms. “→” indicated enhanced effect, “-” indicated inhibited effect, “- - -” indicated possible effect

TH-priming between different studies might be attributed to differences in plant individuals and chilling treatments. It was suggested that Spd and TH participate in the establishment of the plant's cold resistance through osmotic regulation and removal of reactive oxygen species.

Endogenous polyamine content was believed to be related to chilling tolerance in plants. In response to chilling stress, the contents of endogenous polyamines in different plants changed with similar trends, but the contents of Put, Spd, and Spm changed in different ways (Cuevas et al. 2008; Shen et al. 2000). After 24 h of low temperature treatment, the free Put content in *Arabidopsis* seedlings increased significantly, but there was no significant change as for Spd content (Cuevas et al. 2008). Shen et al. (2000) found that the Spd content in chilling-tolerant cucumbers increased significantly under low temperature, while that in chilling-sensitive ones did not change significantly; while the contents of Put and Spm in two types of varieties did not change significantly. In our study, Spm content significantly increased under chilling stress, especially after Spd-priming (Fig. 4). In addition, the polyamine synthesis-related genes include *arginine decarboxylase (ADC)*, *ornithine decarboxylase (ODC)*, *S-adenosylmethionine decarboxylase (SAMDC)*, *spermidine synthase (SPDS)*, and *spermine synthase (SPMS)*. The similar results also had been found by Imai et al. (2004) that low temperature significantly induced the expression of *SPMS1* in rice roots. Kasukabe et al. (2004, 2006) reported that upregulating *SPDS* gene of black-seeded pumpkin improved the cold tolerance of *Arabidopsis thaliana* and sweet potato. Under NaCl, drought and abscisic acid (ABA) treatments, the expression of *AtSPMS*, Put and Spm contents were all induced (Urano et al. 2003). Interestingly, *SPMS* expression of Spd + TH-primed seedlings in our study was higher than that of TH-primed ones, but lower than that of Spd-primed ones. It might be due to that TH inhibited the expression of polyamine biosynthesis genes, leading to decreased polyamine content.

The transcriptional levels of TH biosynthetic genes tend to increase under chilling stress. The expressions of several members of the *TPP* gene family were related to cold, salt, and ABA; and their expressions change in different organs and during different developmental stages (Elena et al. 2007). In rice plants, *OsTPP1* and *OsTPP2* all had TPP enzyme activity. In the roots and shoots of rice seedlings, *OsTPP1* and *OsTPP2* could be induced instantaneously by low temperature stress, drought, and exogenous ABA (Pramanik and Imai 2005). The expression of *OsTPP1* was upregulated rapidly by salt and ABA treatments, but was upregulated more slowly by cold stress (Ge et al. 2008), which is partially consistent with our findings (Fig. 5). Moreover, the *OsTPP2* expression induced by Spd + TH-priming is higher than that induced by Spd-priming but lower than that induced by TH-priming (Fig. 5). Based on

these results, it can be inferred that the Spd and TH may have antagonistic relationship and unable to enhance the cold tolerance of plants.

In this study, the Spd- and TH-priming significantly improved rice seed germination and seedling growth under chilling stress. The improved chilling tolerance was closely related with the increased contents of osmotic adjustment-related substances, the activities of antioxidant enzymes, PAs contents, and the expressions of PAs biosynthesis-related genes and trehalose-6-phosphate-phosphatase genes. The levels of endogenous Spm induced by Spd-priming and *TPP* genes induced by TH-priming might be the major factors responsible for chilling tolerance in rice (Fig. 6), which still needs further study.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest.

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