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Effects of chilling tolerance induced by spermidine pretreatment on antioxidative activity, endogenous hormones and ultrastructure of *indica-japonica* hybrid rice seedlings

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Abstract

Spermidine (Spd) is known to be involved in the regulation of plant responses to chilling stress and counteract the adverse effect of stress conditions. Antioxidant activities, endogenous hormones and ultrastructure change under chilling stress were investigated in *indica-japonica* hybrid rice seedlings. 12-d-old seedlings were subjected to exogenous Spd (1 mmol L⁻¹) and then a chilling stress (6°C, 4 d) was induced, followed by a subsequent recovery (25°C, 4 d). Results showed that malondialdehyde (MDA) and proline content were enhanced significantly, whereas shoot fresh and dry weights decreased during chilling stress and after recovery; chlorophyll content of chilling-stressed seedlings increased slightly but declined after recovery; additionally, total soluble sugar, sucrose, fructose and starch contents increased significantly during chilling stress, and only soluble sugar and fructose contents were observed in increase after recovery; chilling stress-induced increases in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities, but declined after recovery, and the level of ascorbate peroxidase was lower during chilling stress and after recovery; however, endogenous indole-3-acetic acid (IAA), zeatin riboside (ZR), gibberellic acid (GA₃), and abscisic acid (ABA) levels were induced decreased compared with Spd pretreatment. The microscopic analysis revealed that chilling stress-induced destruction of the chloroplast envelope during chilling stress and increased the number of plastoglobuli along with aberrations in thylakoid membranes after recovery. In contrast, exogenous Spd protected rice seedlings from chilling-induced injuries in terms of lower malondialdehyde, proline and carbohydrates accumulation coupled with increased endogenous hormones metabolism. After recovery, Spd pretreatment chilling-exposed seedlings showed higher activities of antioxidant enzymes and normal physiological function of chloroplasts. These results suggest that Spd could promote effectively chilling tolerance which might be largely attributable to the integrity of cell structure and normal metabolism of endogenous hormones in *indica-japonica* hybrid rice seedlings.

Keywords: polyamines, chilling stress, antioxidative activity, endogenous hormones, ultrastructure, *indica-japonica* hybrid rice (*Oryza sativa* L.)

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1. Introduction

Low temperature is a limiting environmental factor on plants that can cause significant growth and yield reductions in many agronomic crops, including rice (Boyer 1982). Gen-



erally, the mean lethal temperature for rice is $\sim 4.7^{\circ}\text{C}$, and when the ambient temperature goes below $5\text{--}10^{\circ}\text{C}$, the plant seedlings' growth and development is impeded (Sanchez et al. 2014). During chilling stress, cellular membranes can be attacked by free radicals, resulting in membrane lipid peroxidation and accumulating malondialdehyde (MDA) and proline (Dai et al. 2012). The generation of reactive oxygen species (ROS) has been associated with oxidases in plasma membrane and the electron transport of chloroplast (Laloi et al. 2004) and will affect membranes of cell ultrastructure (Taylor and Craig 1971). Plants exhibit several antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), which have been reported to be correlated with increased stress tolerance, engaged in protection of the cellular membranes and alleviation of ROS effects (Xu et al. 2011; Mostofa et al. 2014). Jouve et al. (2004) reported that carbohydrates are a major category of compatible solutes that include hexoses (mostly fructose and glucose), disaccharides (sucrose), sugar alcohols, all of which accumulate during stress. In previous studies, carbohydrates, such as sugars (glucose, fructose, sucrose and fructans) and starch, were reported to accumulate in plants under salt stress (Parida et al. 2002), drought stress (Sheoran and Saini 1996) and chilling stress (Morsy et al. 2007), yet data on polyamines application is limited under chilling stress of rice (Morsy et al. 2007). Chilling condition causes rapid changes in membrane structure of plant leaves (Xu et al. 2008), resulting from closure of stomata induced by osmotic stress. Moreover, endogenous hormones, such as cytokinins (ZR), abscisic acid (ABA), gibberellin (GA_3), and indole-3-acetic acid (IAA), regulate the growth and development of plant leaves by affecting cell division, elongation and differentiation within apical meristems (Luomala et al. 2005), are vitally sensitive to ambient environment although they are much little concentration *in vivo* of plant. When plants were encountered to chilling stress, it could adjust the changes of ambient environment in the regulation of the content of endogenous hormones (Wang et al. 2006a).

Polyamines (PAs), including putrescine, spermidine (Spd) and spermine, are small aliphatic, low molecular weight polycations nitrogenous compounds that are ubiquitous in prokaryotic and eukaryotic organisms and participate in the regulation of physiological and developmental processes. Pretreatment with PAs significantly enhanced the levels of antioxidant capacity and alleviated osmotic damage caused by membrane lipid peroxidation (Mostofa et al. 2014). Meanwhile, PAs mediated in ABA inducing in stomata closure by protecting cell structure to avoid swollen chloroplast (Konstantinos et al. 2010). It has been demonstrated that exogenously applied PAs can rapidly enter the intact

chloroplast (He et al. 2002) and play a role in protecting the photosynthetic apparatus from adverse effects of environmental stresses. There is a close relationship between PA and hormones in the regulation of plant growth (Liu et al. 2005). The function of PAs and hormones (CTK) was superposition, because of chloroplast being rich in PAs, but when it was lacked, it might affect the chloroplast structure and function (Shu et al. 2012). In recent studies, exogenous Spd applications successfully alleviated various abiotic stresses, and protected cell structure such as salinity (He et al. 2008; Shu et al. 2012), drought (Kubis 2008), heat (Mostofa et al. 2014), and chilling (Yamamoto et al. 2012).

Plants are subject to changes in temperature, both during changes in season and more rapidly within individual days. *indica-japonica* hybrid rice had been widely applied in the field practice owing to the powerful heterosis and super-high yield potential, but it is severely affected by aberrant changes in temperature (Li et al. 1997). This fluctuation in temperature might affect the physiology of rice plants and can cause severe damage, especially during the seedling stage. Mostofa et al. (2014) reported that foliar application of Spd enhanced heat tolerance during heat stress and after recovery of rice. However, the effect of Spd application on rice seedlings during chilling stress and after recovery is unclear. Moreover, exogenous Spd applications were seldom tested as a way to alleviate chilling stress, particularly in oxidative damage and carbohydrate accumulation, endogenous hormones and ultrastructure of chloroplasts in *indica-japonica* hybrid rice (Yamamoto et al. 2012). We hypothesize that low temperature may cause carbohydrate, endogenous hormones and ultrastructure changes in leaves of rice and higher activities of antioxidants with Spd application may alleviate those changes and protect cell membranes from lipid peroxidation. Therefore, in this study, we examined the effect of chilling stress with or without application of exogenous Spd on the carbohydrate content, endogenous hormones, ultrastructure of chloroplasts, and the activity of the antioxidant system in rice plants. The results will be helpful to understanding the roles of Spd applications on enhancing chilling tolerance in *indica-japonica* hybrid rice.

2. Results

2.1. Plant growth

In the present study, the plant height, shoot fresh weight (FW), shoot dry weight (DW), and relative water content decreased significantly during chilling stress and after recovery in the non-Spd-pretreated chilling-stressed seedlings (6°C) as compared with the control, while the Spd pretreatment seedlings showed significantly lower reductions in these

indices during chilling stress and they did not increase during the recovery period (Table 1). In the control (Spd pretreatment non-chilling stressed) seedlings, there was a slight increase in the above indices except plant height. Similarly, the relative growth rate (RGR) decreased significantly in the chilling stress after recovery. However, the Spd pretreatment significantly increased the RGR after recovery. The application of 1 mmol L⁻¹ exogenous Spd greatly alleviated the chilling-mediated growth reduction in the rice seedlings.

2.2. Chl content

The chilling stress-induced seedlings showed significant increases in the total Chl content and Chl *b* during chilling stress, while Spd pretreatment seedlings showed significant reductions (Table 2), and the trends in CK and CK+Spd were similar to that. In contrast, the Chl *a* content and Chl *a*/Chl *b* value decreased in chilling stress-induced seedlings during chilling stress and increased to high levels in the Spd pretreatment chilling-stressed seedlings. There was a similar trend among all the treatments after recovery and chilling stress, but no difference was found in Chl.

2.3. Carbohydrate and starch concentrations

As shown in Fig. 1, the soluble sugar, sucrose, fructose, and starch contents in leaves of rice seedlings increased significantly during chilling stress as compared with the control. They are ~86% (Fig. 1-A), 42% (Fig. 1-B), 272% (Fig. 1-C), and 16% (Fig. 1-D) higher, respectively, than the control, but the significantly increasing trend during chilling recovery was only found in total soluble sugar and fructose, and no significant difference was found in sucrose and starch. The Spd pretreatment significantly decreased the total soluble sugar, sucrose and fructose contents during chilling stress and sustained significant increase except sucrose after recovery. There was little significant difference in starch, although it was similar to that during chilling stress and after recovery.

2.4. MDA and proline content

Under the non-Spd-pretreated chilling-stress, the MDA level increased significantly during chilling stress and after recovery in the leaves of rice seedlings (Fig. 2-A), while

Table 1 Effect of exogenous spermidine (Spd) on chilling-induced changes in the growth parameters of rice seedlings

Treatments ¹⁾	Plant height (cm)	Shoot fresh weight (FW, mg seedling ⁻¹)	Shoot dry weight (DW, mg seedling ⁻¹)	Relative water content (%)	RGR ²⁾ (mg g ⁻¹ d ⁻¹)
4 d L					
CK	21.73±2.48 a	151.22±14.24 a	22.26±2.09 a	85.28±0.19 a	
CK+Spd	21.09±1.57 a	162.22±3.15 a	23.12±3.16 a	85.77±1.71 a	
T	17.35±0.57 c	94.78±6.83 c	18.03±1.70 b	80.97±1.21 b	
T+Spd	19.32±0.35 b	123.67±12.55 b	21.31±1.98 ab	82.76±0.16 b	
4 d R					
CK	27.29±0.46 a	186.00±6.93 ab	26.19±1.32 a	85.92±0.21 a	44.95±0.32 b
CK+Spd	26.97±0.27 a	201.67±16.37 a	27.78±2.13 a	86.22±0.16 a	52.35±9.04 a
T	22.48±0.74 b	116.67±4.98 c	20.76±1.36 b	82.22±0.68 c	30.85±6.54 c
T+Spd	22.26±0.97 b	164.78±21.89 b	25.92±3.68 a	84.29±0.22 b	41.67±6.25 b

¹⁾ 4 d L, low temperature treatment for four consecutive days; 4 d R, normal temperature recovery for four consecutive days. CK, control plants under 25°C; CK+Spd, plants under 25°C with Spd foliar spraying; T, plants under 6°C; T+Spd, plants under 6°C with Spd foliar spraying. The same as below.

²⁾ RGR, relative growth rate.

Data are represented as means±SD from three separate experiments as 30 replicates. Different letters indicate significant differences among various treatments in the same period by Duncan's multiple range test at *P*<0.05. The same as below.

Table 2 Effect of exogenous Spd on chilling-induced changes in the chlorophyll (Chl) content in rice seedlings

Treatments	Total Chl (mg g ⁻¹ FW)	Chl <i>a</i> (mg g ⁻¹ FW)	Chl <i>b</i> (mg g ⁻¹ FW)	Chl <i>a</i> /Chl <i>b</i>
4 d L				
CK	3.35±0.04 bc	1.43±0.02 ab	1.92±0.05 bc	0.75±0.03 b
CK+Spd	3.50±0.09 ab	1.38±0.03 bc	2.13±0.12 ab	0.65±0.05 bc
T	3.72±0.15 a	1.31±0.04 c	2.41±0.19 a	0.55±0.06 c
T+Spd	3.16±0.13 c	1.49±0.04 a	1.67±0.17 c	0.90±0.11 a
4 d R				
CK	3.11±0.27 a	1.53 ±0.06 a	1.59 ±0.33 a	1.00 ±0.27 a
CK+Spd	3.14±0.11 a	1.52±0.03 a	1.61 ±0.14 a	0.95 ±0.10 a
T	2.88±0.14 a	1.59±0.01 a	1.29 ±0.14 a	1.24 ±0.15 a
T+Spd	2.99±0.05 a	1.56±0.01 a	1.42 ±0.06 a	1.10 ±0.05 a

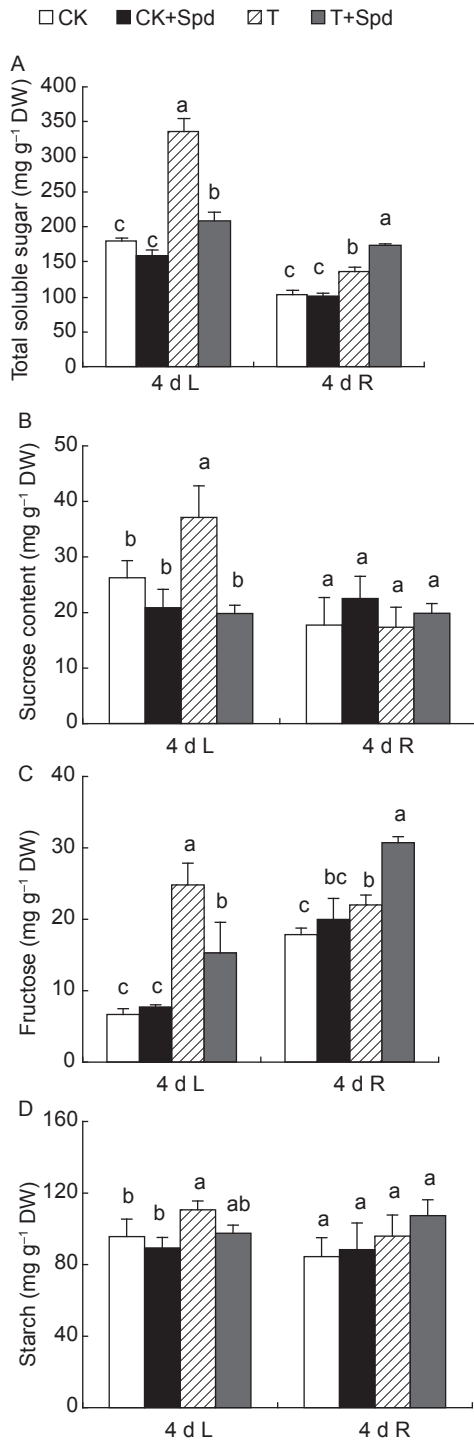


Fig. 1 Effects of exogenous spermidine (Spd) on chilling-induced changes in soluble sugar (A), sucrose (B), fructose (C), and starch (D) in the leaves of rice seedlings. Vertical bars represent SD of the mean ($n=3$). Different letters indicate significant differences among various treatments in the same period at $P<0.05$, according to Duncan's multiple range test. 4 d L, low temperature treatment for four consecutive days; 4 d R, normal temperature recovery for four consecutive days. CK, control plants under 25°C; CK+Spd, plants under 25°C with Spd foliar spraying; T, plants under 6°C; T+Spd, plants under 6°C with Spd foliar spraying. The same as below.

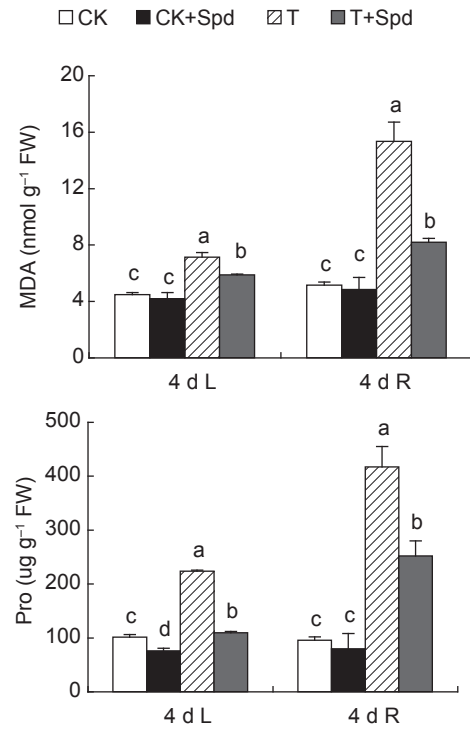


Fig. 2 Effects of exogenous Spd on chilling-induced changes in malondialdehyde (MDA) (A) and proline (Pro) (B) contents in the leaves of rice seedlings.

the MDA level decreased significantly in the Spd pretreatment seedlings during chilling stress and did not increase after the recovery period compared with chilling-stressed seedlings. The MDA level of chilling-stressed seedlings increased significantly during chilling stress and after recovery as compared with the control. Similar to MDA, the proline level increased significantly during chilling stress and remained at this high level after recovery (Fig. 2-B). The exogenous Spd application decreased significantly the proline level during chilling stress and stayed at a similar level after recovery. Spd application also decreased significantly the proline level under the control seedlings, and maintained the trend after recovery but no significant difference.

2.5. Antioxidant enzyme activities

The treatments showed significant effects on the four antioxidant enzymes (Fig. 3). After exposure to chilling stress for 4 d, SOD activity increased significantly; however, after recovery significantly decreasing differences were found (Fig. 3-A). Spd pretreatment seedlings had significantly increased SOD activities during chilling stress and after recovery. Chilling stress created different effects on CAT activity. CAT activity during chilling stress was significantly

higher than the control and it also increased after recovery (Fig. 3-B). In contrast, CAT activity increased significantly in the Spd pretreatment seedlings irrespective of chilling stress or not. A slight increase was found in the POD activity of chilling-stressed seedlings during chilling stress (Fig. 3-C), and after recovery POD activity was significantly lower than the control plants, but POD activity increased significantly under the Spd pretreatment. APX activity showed a significant decrease during chilling stress and maintained a significant decreasing trend after recovery compared with the control (Fig. 3-D). However, the exogenous Spd application enhanced the APX activity during chilling stress and it did not decrease after recovery compared with taht in the chilling-stressed seedlings, but a significant decline was observed in contrast to the control.

2.6. Endogenous hormones

Endogenous hormone contents of seedlings were apparently affected by the treatments (Fig. 4). ABA content, exposure to chilling stress, increased significantly during chilling stress and after recovery compared with control (Fig. 4-A), moreover, ABA content was high significantly in Spd pretreatment. Spd-treatment enhanced significantly ($P < 0.05$) IAA content during chilling stress, but it did not decrease during recovery (Fig. 4-B). Similarly, cytokinin content (ZR) was decreased significantly by chilling stress treatment during chilling stress and after recovery compared with control (Fig. 4-C), whereas the exogenous Spd application enhanced the ZR content during chilling stress and was still maintained the higher level after recovery. In contrast, GA_3 content reduced significantly during chilling stress (Fig. 4-D) while it remained the same level after recovery. However, Spd pretreatment significantly increased the content of GA_3 during chilling stress and it did increase during recovery.

2.7. Changes in cell ultrastructure

Under control condition, the chloroplasts showed thylakoids with grana during chilling stress (Fig. 5) and after recovery (Fig. 6). The ultrastructure of the chloroplasts seemed to show no difference between the control plants and plants treated with Spd. However, low temperature caused remarkable structural changes during chilling stress and after recovery. Specifically, some chloroplasts were swollen, and internal lamellae of the stromal thylakoids were damaged to induce partly granal thylakoid layers to loosen during chilling stress. Furthermore, the chloroplast membranes were damaged and became indistinct, but Spd pretreatment enhanced recovery of the chloroplast shape in that the stromal lamellae seemed to form formally the grana ultrastructure and the chloroplasts were observed higher distinct membranes.

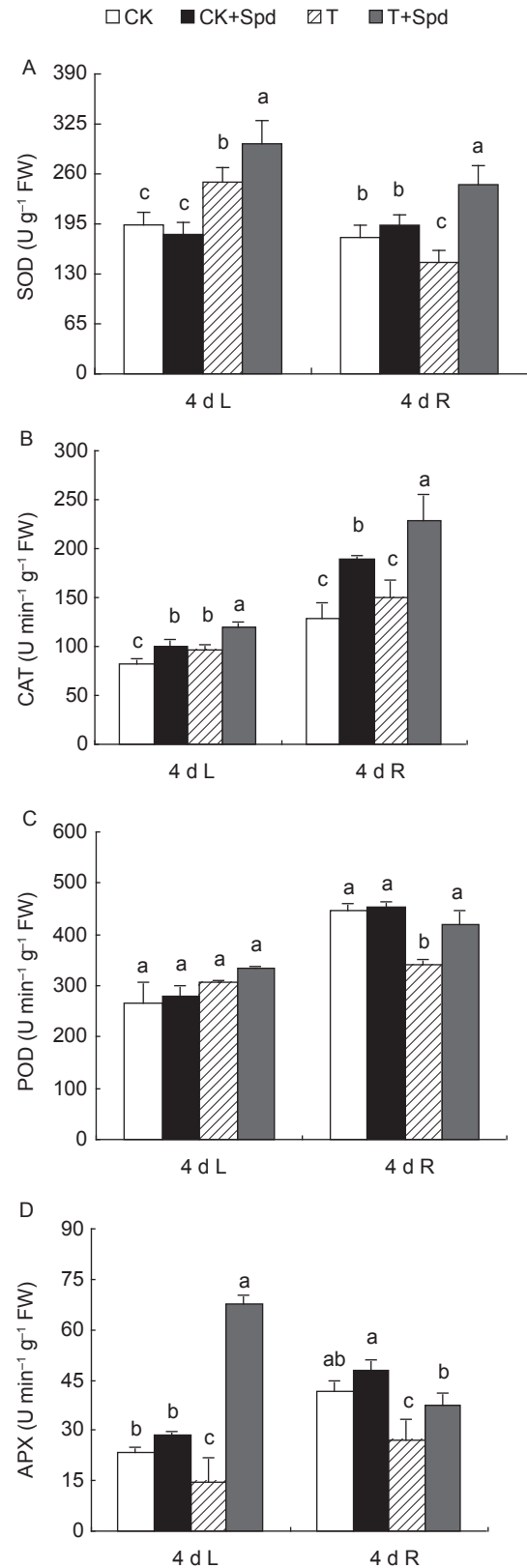


Fig. 3 Effect of exogenous Spd on chilling-induced changes in superoxide dismutase (SOD) (A), catalase (CAT) (B), peroxidase (POD) (C), and ascorbate peroxidase (APX) (D) activities in the leaves of rice seedlings.

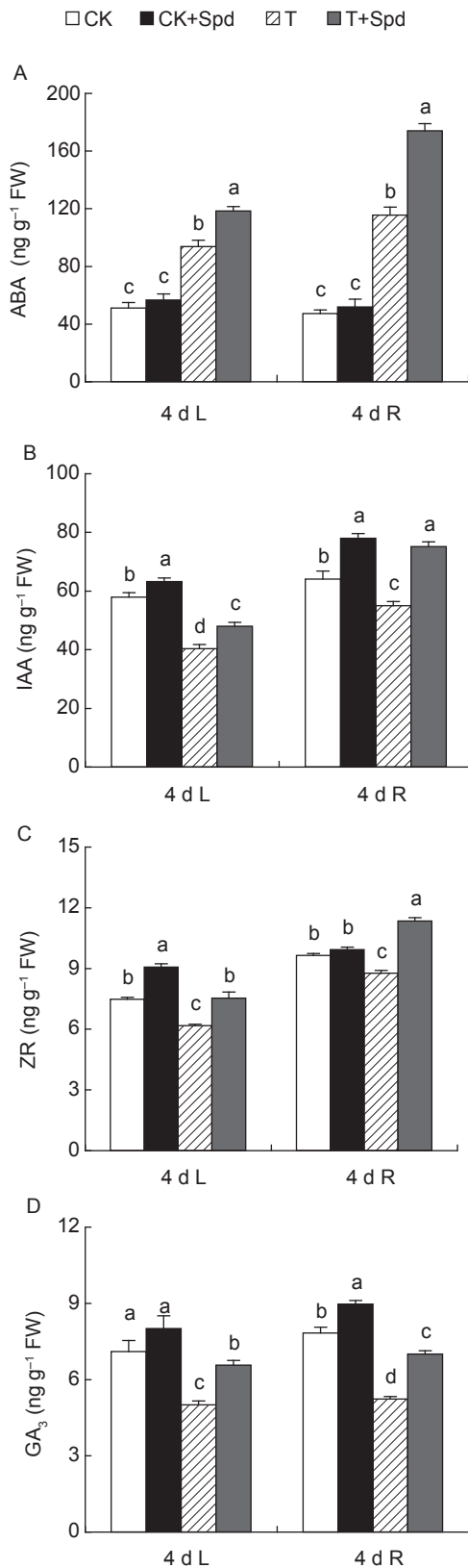


Fig. 4 Effect of exogenous Spd on chilling-induced changes in abscisic acid (ABA) (A), indole-3-acetic acid (IAA) (B), cytokinins (ZR) (C), and gibberellin (GA₃) (D) contents in the leaves of rice seedlings.

Moreover, many ribosomal particles were released after recovery under chilling stress (Figs. 5 and 6)

2.8. Phenotypic performance

Generally, phenotypic performance was affected, along with the morphological characteristics, when the plant seedlings were subjected to chilling stress (Fig. 7). Phenotypical observations showed that the Spd pretreatment chilling-stressed seedlings had a better visual performance of leaves (less dehydration and frizzle) compared with the seedlings subjected to the chilling stress without Spd pretreatment (Fig. 7-A and B). After recovery a significant decline, including wilting, yellowing and marginal burning in the leaf apex, was observed (Fig. 7-C and D).

3. Discussion

Chilling stress leads to adverse effects on crop growth and development, resulting in the reduction of grain yield (Yamamoto *et al.* 2012). Growth and morphological analyses are widely used tools for characterizing plant growth. In this study, the results showed that chilling exposure significantly decreased the plant height, aboveground fresh and dry biomass accumulation and relative water content during chilling stress and after recovery, resulting in a lower RGR of the rice seedlings after recovery (Table 1). However, the exogenous 1 mmol L⁻¹ Spd foliar spray pretreatment on rice leaves significantly alleviated the growth inhibition caused by chilling stress and increased the aboveground biomass accumulation. Our findings is corroborated those of Tian *et al.* (2012) who stated that foliar spray with Spd effectively enhanced the tolerance of the plants to high-temperature stress. Higher plant heights and greater plant biomasses are beneficial for maintaining the advantages of plant growth and physiological activity. This might be because of the involvement of Spd in the regulation of plant growth and development under chilling stress (Yamamoto *et al.* 2012).

Chloroplasts are the major source of reactive oxygen species (ROS) under both stressed and non-stressed conditions (Nakano and Asada 1981). A low temperature treatment decreases the chlorophyll content by inhibiting protochlorophyllide oxidoreductase and Chl synthesis (Liu *et al.* 2012). However, in our study, chilling stress slightly increased the total Chl content by significantly increasing Chl *b* during chilling stress as compared with the control, while in Spd pretreatment seedlings the total Chl content declined significantly (Table 2) which is not consistent with the findings of Mostofa *et al.* (2014) under the heat stress. This could be possible ascribed that the chilling-stressed rice seedlings may be excessively dehydrated, resulting to a lower level of relative water content, and then leading to a higher

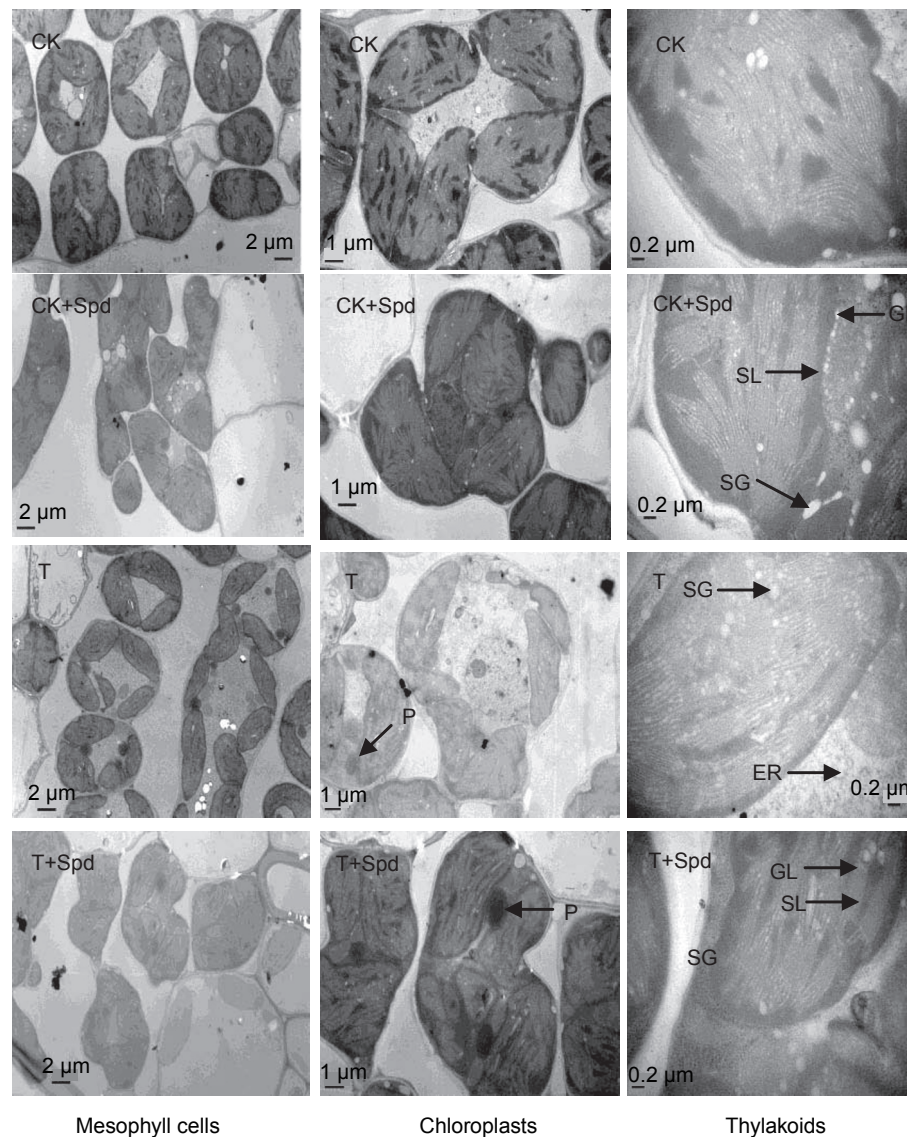


Fig. 5 Changes in ultrastructure of mesophyll cells, chloroplasts and thylakoids of rice seedlings during chilling stress. The second fully expanded leaves, numbered basipetally, were sampled for ultramicroscopic observation during chilling stress and after recovery, respectively. SL, stroma lamella; GL, grana lamellae; SG, starch grain; P, plastoglobulus; ER, endoplasmic reticulum. The same as below.

green status than the seedlings under a non-chilling stress treatment (Fig. 7-A and B). Therefore, the performance of withered but maintaining green status could be appeared in the chilling-stressed rice seedlings. After recovery, the chilling-stressed seedlings' performance showed irreversible trends suggesting that the photosynthetic apparatus were irreparably injury and unable to synthesize Chl (Fig. 7-C and D) and the Chl content decreased compared with that of the Spd chilling-stressed treatment.

Soluble carbohydrates, as an osmosis substance, are important factors related to chilling adaptation in plants, and it was recently proposed that sugars can act as real ROS scavengers in plants, especially when it was presented at

higher concentrations (Keunen *et al.* 2013). In our experiment, chilling stress significantly increased the total soluble sugar, sucrose, fructose, and starch in the chilling-stressed leaves of rice seedlings compared with the control, which indicated serious osmosis of membrane damage due to electrolyte leakage, and after chilling recovery this significant increase was maintained only in total soluble sugar and fructose. The most abundant soluble sugar that accumulates is usually sucrose during the exposure of plants to chilling temperature (Palonen *et al.* 2000), yet fructose may play important roles during chilling stress (Purvis and Grierson 1982). The highest increase was found in fructose, followed by total soluble sugar. The results were in agreement with

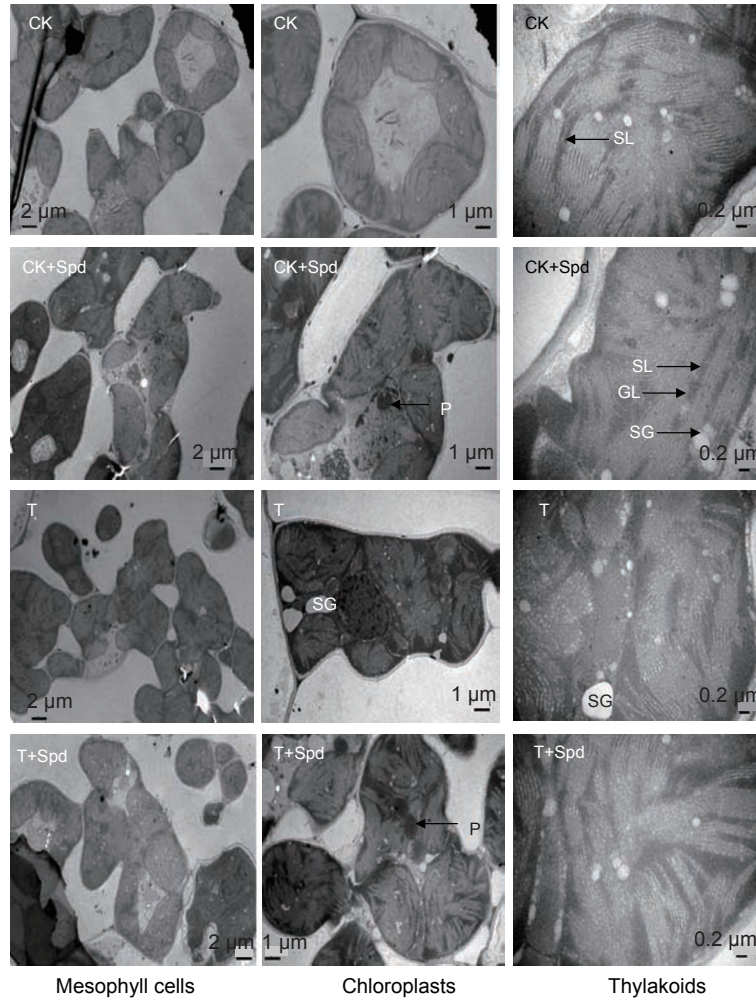


Fig. 6 Changes in ultrastructure of mesophyll cells, chloroplasts and thylakoids of rice seedlings after recovery.

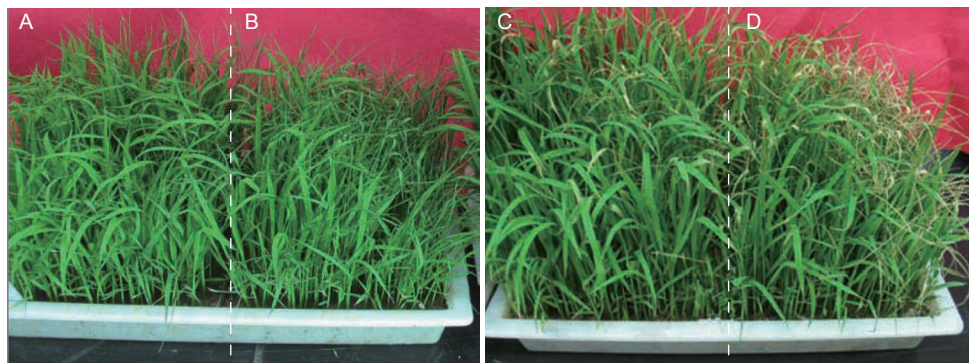


Fig. 7 Phenotypic appearance of chilling-stressed (B and D) and Spd pretreatment chilling-stressed seedlings (A and C). The photographs were taken 4 d after treatment (left) and 4 d after recovery (right) from chilling stress.

previous reports in cabbage (Sasaki *et al.* 2001), arabidopsis (Klotke *et al.* 2004) and olive (Gulen *et al.* 2009) under cold stress. The total soluble sugar, sucrose and fructose

decreased significantly in Spd pretreatment chilling-stressed seedlings during chilling stress, the reason might be that Spd application could alleviate osmosis of membrane

damage. However, increasing trends were observed after recovery supporting the well-established role of this sugar as an osmoprotectant that stabilizes cellular membranes and maintains turgor (Jouve *et al.* 2004), which indicated that Spd application alleviated the osmotic injury caused by chilling stress, and maintained a certain osmotic pressure to sustain normal physiology activity and metabolism. Certainly, the result of Spd application was also as that in the control with non stress.

MDA is the ultimate product of membrane peroxidation, and its content is related to the damage extent of ROS (Dai *et al.* 2012). Our results showed that induced chilling increases the MDA levels, which still elevated trend after recovery. This phenomenon showed high membrane damage in the chilling-stressed seedlings and therefore resulted in poor symptoms of stress, such as changes in growth parameters and Chl content (Tables 1 and 2). In contrast, Spd pretreatment chilling-exposed rice seedlings maintained a low level of MDA suggesting weak lipid peroxidation (Fig. 2-A), concurring with Mostofa *et al.* (2014). Similar to MDA, the proline content in rice leaves significantly increased due to the chilling stress, which might be an adaptive response to lose water through much serious lipid peroxidation, and the exogenous Spd reduced chilling-induced proline accumulation (Fig. 2-B). These results support the evidence that exogenous PAs applied decreased proline and MDA level (Xu *et al.* 2011; Mostofa *et al.* 2014) and it is consistent with the observation that membranes ultrastructure in leaves of chilling stress were seriously affected by low temperature.

Chilling stress could result in changes in the activities of antioxidant enzymes, which may affect the physiological traits of plants and adjust stress conditions by reducing damage caused by ROS (Qiu *et al.* 2005; Guo *et al.* 2006). In a previous study, Guo *et al.* (2006) reported that the tolerance to chilling in rice was associated with the enhanced capacity of the antioxidative system under chilling conditions. Our results showed that rice seedlings could reduce ROS by enhancing the activities of SOD, POD and CAT during chilling stress (Fig. 3-A-C), which was in agreement with the findings of Kumar *et al.* (2011) and Xu *et al.* (2011). However, the trend was inverted after recovery from chilling stress owing to high oxidative stress such as MDA production in rice seedlings. In contrast, Spd pretreatment seedlings sustained high SOD, CAT and POD activities even after recovery, which might indicate reduced levels of MDA and proline. The APX activity of rice seedlings was significantly decreased during chilling stress and after recovery; nevertheless, Spd pretreatment chilling-stressed plants maintained higher APX activities compared with those under just the chilling-stress treatment. These results were corroborated by the findings of Mostofa *et al.* (2014) who suggested that Spd pretreatment enhances heat tolerance

in rice seedlings by increasing the activities of antioxidant enzymes. In the same way, most of antioxidant enzymes were high in Spd pretreatment seedlings of the control. Therefore, Spd application maintained plant normal growth through improving cold tolerance in related to the higher antioxidant capacity accompanying with lower oxidative stress under chilling stress.

Plant hormones play an important role in regulating plant growth and development to response for ambient environment stress (Sevillano *et al.* 2009). An increase in ABA levels was reported to relate to increased chilling tolerance (Wang *et al.* 2008), which could correlate with inducing stomata closure, reducing the transpiration rate and maintaining cell water levels, and increasing the expression of cold-induced genes (Wang *et al.* 2008). The results showed that Spd enhanced significantly chilling tolerance by raising ABA content during chilling stress, and then did sharp decrease after recovery (Fig. 4-A) which agrees with the findings in wheat (Wang *et al.* 2009). In contrast, higher ABA content was sustained even after recovery in chilling-stressed seedlings indicating that the structure of leaf was seriously damage and the physiological activities were irreversible (Fig. 3). Another notable difference between chilling stress and control was in their endogenous IAA, ZR and GA₃ levels (Fig. 4-B-D). There decreased significantly the IAA, ZR and GA₃ levels during chilling stress and after recovery compared with the control which might contribute to higher oxidative stress (Fig. 2) and trigger osmotic matters accumulation (Fig. 1). Wang *et al.* (2006b) reported that increasing the IAA and ZR levels might be feasible for alleviating chilling in plum fruit. Similarly, increasing the IAA and ZR levels in Spd pretreatment during chilling stress and after recovery might be beneficial to increase seedlings chilling tolerance which sustained integrity structure of cell. Pusittigul *et al.* (2012) reported that a decrease in GA₃ concentration was associated with plant resistance to chilling stress. This suggests that increase chilling tolerance in rice may be related to decrease endogenous GA₃ levels during chilling stress and it did not increase after recovery (Fig. 4-D), but Spd pretreatment sustained higher GA₃ levels during chilling stress and even after recovery suggesting that the synthesis of GA₃ was affected slightly by chilling stress.

Under stress of low temperature, the membranes of individual stromal thylakoids are closed together, while the chloroplast as a whole begins to swell (Taylor and Craig 1971). Under chilling stress, the chloroplasts also swelled in leaves, and some thylakoids were swollen so that many vesicles were formed by lamella (Fig. 5). The same results have been observed in cucumber leaves under chilling stress (Xu *et al.* 2008). Compared with chilling stress treatment, chloroplasts of Spd pretreatment was not significantly during chilling stress (Fig. 5). This result suggests that Spd

might protect chloroplast membranes to avoid be damaged through the activated behavior of antioxidant system (Fig. 3) as observed by reduced the level of MDA in Spd pretreatment seedlings under chilling condition. However, few studies on structure of cell were reported after recovery from chilling stress. In this study, chloroplasts were isolated from the plasma membrane and the number of osmiophilic plastoglobuli was increased under chilling stress after recovery, which possibly due to breakdown of thylakoid membrane degradation. It has been indicated that the increased mass of osmiophilic plastoglobuli in thylakoid membranes is generally attributed to lipid peroxidation-mediated destruction of the membranes (Zhang *et al.* 2010). Changes in the lipid composition of chloroplasts are responsible for altered thylakoid membrane structures, such as disrupted chloroplast envelopes and swollen thylakoids (Yamane *et al.* 2003). Moreover, other organelles such as endoplasmic reticulum had ruptures under chilling stress, while that of Spd application kept the basic integrity. Chloroplast development derived from proplastid is mainly controlled by cytokinin (ZR) induced with Spd (Polanská *et al.* 2007), while SOD and APX are key enzymes for scavenging ROS of chloroplast, which could maintain redox equilibrium of chloroplast (Myouga *et al.* 2008). This might be responsible for higher endogenous hormones, higher SOD activity and APX and lower lipid peroxidation (Figs. 2–4).

4. Conclusion

Chilling-induced oxidative stress in rice seedlings was evident through higher levels of MDA and proline coupled with an increase on carbohydrate accumulation, Chl content, and lower plant height, FW, DW, and RGR. Chilling-exposed seedlings also displayed inhibition of the antioxidative system and poor induction of the endogenous hormones, particularly damage effect on structure of cell during chilling stress, but the appearance was observed after recovery of chilling stress owing to oxidative burden and inefficient non-enzymatic and enzymatic antioxidants as well as of endogenous hormones. However, exogenous Spd exhibited a beneficial role through sustained and simultaneous activation of the antioxidative systems and physiological function of chloroplasts and endogenous hormones metabolism which rendered rice plants more tolerant to chilling-induced oxidative and osmotic damage. The novel aspect of the present work is that exogenous Spd modulates the carbohydrate level which might be involved in the simultaneous induction of the antioxidant defense systems and endogenous hormones metabolism and thereby enhance chilling-stress tolerance in rice seedlings. However, these results came only from laboratory practices, and further studies should be needed

to clarify in the field conditions, to obtain sustainable crop production under extreme chilling conditions.

5. Materials and methods

5.1. Plant materials, growth conditions and treatments

indica-japonica hybrid rice (*Oryza sativa* L. cv. Yongyou 15, chill-sensitivity) seeds were surface sterilized with a H_2O_2 solution for 20 min, washed with distilled water and imbibed for 48 h. The seeds were sown on plastic nets floating on distilled water in plastic plates packed with towels and kept in the dark at $(32\pm 0.5)^\circ\text{C}$ for germination. After 48 h, uniformly germinated seeds were transferred to a seeding tray covered in a matrix containing roseite, charcoal, silver sand, controlled release fertilizer, microelements and other mixture materials. The seeding tray (length \times width \times height: 33.0 cm \times 21.5 cm \times 4.0 cm) was divided into two equal sections by a clapboard inserted in the matrix soil. The tray-sown seeds were placed in a greenhouse to grow.

Twelve days after sowing, when two fully expanded leaves and one tender leaf had emerged on the seedlings, one half of the seedlings in the plastic tray were subjected to a foliar spray application of Spd (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) every afternoon from 17.00 to 18.00 h for four continuous days. The result of preliminary experiments indicated that the effective concentration of Spd was 1.0 mmol L^{-1} to alleviate chilling injuries (date not shown). Tween-20 (0.5%, v/v; Haijiechem, Zibo, China) was used as a surfactant. The other half of the seedlings were subjected to a foliar spray application of distilled water as the control. Each half of the plastic tray contained ~ 300 – 500 rice seedlings, and 200 mL of 1 mmol L^{-1} Spd or distilled water was sprayed on each half seedlings. After 4 d of pretreatment, both Spd pretreatment and control seedlings were exposed to 6°C (day/night: $8^\circ\text{C}/4^\circ\text{C}$) for 4 d, followed by recovery at 25°C (day/night: $27^\circ\text{C}/23^\circ\text{C}$) for 4 d. The intensity of the chilling stress was determined on the basis of a preliminary experiment where 6°C caused visible injury. The seedlings were grown under an illumination incubator (photon density: $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$, relative humidity (RH): 70–80%). Control and Spd pretreatment non-stressed seedlings were kept at 25°C throughout the experimental period. The aboveground rice seedlings were harvested after 4 d of chilling stress and after 4 d of recovery to determine the weight, Chl, carbohydrate, and various biochemical parameters. This experiment included four treatments: control, control additive Spd (pretreated with Spd), 6°C (chilling stress), and 6°C additive Spd (Spd pretreatment followed by chilling stress). Each treatment was replicated three times

under the same experimental conditions.

5.2. Determination of plant biomass and chlorophyll (Chl) content

Thirty rice seedlings were harvested for each replicate. Plant height was measured by a ruler with an accuracy of 1 mm from stem-sheath to the apical point. Before determining the harvested aboveground fresh weight, the seedlings were washed using deionized water. The samples were weighed immediately and then oven-dried at 105°C for 15 min followed by 75°C for 72 h, until they reached a constant weight, and then they were weighed for dry shoot biomass. The dry plant tissues were ground into a powder for carbohydrate analysis. An additional ~1–2 g fresh leaf tissue of each sample was immersed in liquid nitrogen for 1 min and then stored at –80°C for the biochemical analysis. The relative water content was determined as the weight of fresh samples minus dried samples, divided by the samples' fresh weight. The relative growth rate (RGR) was measured according to the method of Hunt (2003). The total Chl content was measured according to the method of Arnon (1949). Fresh leaves (0.2 g) were sliced and added to 10 mL 95% ethanol in the dark at 25°C for 24 h. The supernatants were collected and the absorbance values were recorded at 665 and 649 nm.

5.3. Estimation of MDA and proline content

The MDA content was determined using the thiobarbituric acid reaction following the method of Heath and Packer (1968), it was calculated from the difference in absorbance values at 450, 532 and 600 nm using an extinction coefficient of 155 mmol L⁻¹ cm⁻¹. The proline content was determined according to the method of Bates *et al.* (1973), and it was calculated in absorbance values at 520 nm using the standard curve of L-proline.

5.4. Antioxidant enzyme extraction and enzymatic activity assays

Antioxidant enzyme activities were determined using ~0.3 g samples homogenized in 8 mL of extraction solution containing 50 mmol L⁻¹ Na-phosphate buffer (pH 7.8), 0.2 mmol L⁻¹ EDTA (ethylene diamine tetraacetic acid) and 1% insoluble polyvinyl pyrrolidone with a chilled mortar and pestle. The homogenate was centrifuged at 12 000×g for 20 min and the supernatant was collected to analyze enzyme activity and protein content. The entire extraction procedure was carried out at 4°C. All spectrophotometric analyses were conducted using a spectrophotometer (DU-800, Beckman

Coulter, Inc., Fullerton, USA). SOD activity was estimated by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) by 50% (Madhava *et al.* 2000). The photo-reduction of NBT was measured at 560 nm, and SOD activity was expressed as U g⁻¹ FW. CAT activity was measured according to the method of Cakmak and Marschner (1992) by determining the decrease in absorbance at 240 nm due to the decline of H₂O₂ extinction. The enzyme activity was calculated in terms of 1 mol of H₂O₂ g⁻¹ FW min⁻¹ at (25±2)°C. POD activity was determined with guaiacol by the method of Nickel and Cunningham (1969). The enzyme activity was calculated in terms of 1 mol of guaiacol oxidized g⁻¹ FW min⁻¹ at (25±2)°C. APX activity was measured according to Nakano and Asada (1981) by monitoring the decrease in ascorbate oxidation at 290 nm as ascorbic acid was oxidized.

5.5. Estimation of carbohydrate and starch contents

Dried samples of ~50 mg were weighed into a 15-mL centrifuge tube; 10 mL of 80% ethanol (v/v) was added and gently shaken for 30 min. The homogenates were washed with ethanol, centrifuged at 4 000×g at 4°C for 10 min, and the supernatant was collected. This extraction was repeated three additional times to completely remove the residual glucose from the material. A total of 10 mg active carbon was added to the supernatant to absorb the Chl for 30 min at 80°C and diluted with water to 25 mL, filtering the supernatant. Then, the total soluble sugar, sucrose and fructose were determined for the filtered liquid, and the level of starch was determined in the residue. The carbohydrate content was expressed as mg g⁻¹ DW according to the method of Yoshida *et al.* (1976).

Total soluble sugar The reaction mixture of the total soluble sugar, consisting of 1 mL extraction liquid and 5 mL 0.2% anthranone sulfuric acid solution (g/v: 2/1 000, 72% sulfuric acid), was placed in a water bath for 15 min at 90°C. The reaction solution was taken out, rapidly cooled and developed for 10 min. The absorbance was recorded at 620 nm using a spectrophotometer (DU-800, Beckman Coulter, Inc., Fullerton, USA).

Sucrose determination The reaction mixture, consisting of 1 mL extraction liquid and 50 µL 12 N NaOH, was placed in a water bath for 5 min at 90°C, and then rapidly cooled. A total of 5 mL 0.2% anthranone sulfuric acid solution (g/v: 2/1 000, 72% sulfuric acid) was added for blending and the mixture was placed in a water bath for 10 min at 80°C. The reaction solution was developed for 10 min. The absorbance was recorded at 620 nm under the same conditions as above.

Fructose determination The reaction mixture, consisting of 1 mL extraction liquid and 5 mL 0.2% anthranone sulfuric

acid solution (g/v: 2/1 000, 72% sulfuric acid), was warmed at 40°C for 10 min, and then the absorbance was recorded at 620 nm under the same conditions as described above.

Starch determination The dry residue was left in the centrifuge tube in an oven at ~50–60°C for starch extraction. A total of 2 mL of distilled water was added to the centrifuge tube containing the dried residue and placed in a boiling water bath for 15 min and stirred occasionally. The tube was allowed to cool. Then, 2 mL 9.2 N HClO₄ was added, stirred constantly for 15 min, and then centrifuged 10 min before adding 2 mL of distilled water. The supernatant was collected and 2 mL 4.6 N HClO₄ was added to the residue. Then 5 mL of distilled water was added and the samples were centrifuged 10 min before adding 2 mL of distilled water. This was repeated twice and the supernatants were combined. The extracted starch was measured in the same manner as the soluble sugar.

5.6. Microscopic analysis

The ultrastructure of chloroplasts was analyzed using transmission electron microscope (Carl Zeiss, Göttingen, Germany). The second fully expanded leaves, numbered basipetally, were cut into pieces of approximately 1 mm². The cut leaves were then immersed in a solution containing 3% glutaraldehyde and 1% formaldehyde in a 0.1 mol L⁻¹ phosphate buffer (pH 7.4) for 2 h (primary fixation), then immersed in 2% osmic acid in the same buffer for 2 h (second fixation). After dehydration in acetone and embedding in Durcupan ACM (Fluka), the resulting leaves were cut to obtain ultrathin sections, stained with uranium acetate and lead citrate in series and examined using a HITACHI H-7650 transmission electron microscope (Carl Zeiss, Göttingen, Germany) at an accelerating voltage of 80 kV.

5.7. Hormone extraction and purification and quantification

Approximately 0.5 g FW of grain was sampled, and each endogenous hormone concentration was measured. The methods for extraction and purification of ZR, GA₃, IAA, and ABA were modified from those described by Yang *et al.* (2001). The samples were ground in a mortar (on ice) with 5 mL of 80% (v/v) methanol as the extraction medium, which contained 1 mmol L⁻¹ butylated hydroxytoluene (BHT) as an antioxidant. The methanol extracts were incubated at 4°C for 4 h and centrifuged at 10 000×g for 15 min at the same temperature. The supernatants were passed through Chromosep C₁₈ columns (C₁₈ Sep-Park Cartridge, Waters Corp., Millford, MA, USA). The columns were prewashed with 10 mL of 100% methanol and 5 mL of 80% methanol,

respectively. The hormone fractions were dried in a freeze dryer (Labconco, England), and dissolved in 1 mL phosphate buffer saline (PBS) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for the analysis by an enzyme-linked immunosorbent assay (ELISA).

The mouse monoclonal antigens and antibodies against ZR, GA₃, IAA, and ABA and the immunoglobulin G-horse-radish peroxidase (IgG-HRP) used in the ELISA were manufactured by the Phytohormones Research Institute, China Agricultural University. The quantification of ZR, GA₃, IAA, and ABA was performed by ELISA as previously described by Yang *et al.* (2001). The percentage recovery of IAA, ZR, ABA, and GA₃ in leaf was (90±3.7), (86±1.5), (98±5.2), and (85±4.1)%, respectively. The specificity of the monoclonal antibody and other possible nonspecific immunoreactive interference was checked previously and proved reliable (Yang *et al.* 2001).

5.8. Statistical analysis

The data were subjected to one-way analysis of variance and the mean differences were compared by Duncan's multiple range test using SAS ver. 9.1 (SAS Institute, Cary, NC, USA). Means were presented with standard deviations to indicate the variation of three replicates for each treatment. The differences between means were determined using *t*-tests (*P*<0.05).

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References

- Arnon D I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*, **24**, 1–15.
- Bates L S, Waldren R P, Teare I D. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil*, **39**, 205–207.
- Boyer J S. 1982. Plant productivity and environment. *Science*, **218**, 443–448.
- Cakmak I, Marschner H. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiology*, **98**, 1222–1227.

- Dai A H, Nie Y X, Yu B, Li Q, Lu L Y, Bai J G. 2012. Cinnamic acid pretreatment enhances heat tolerance of cucumber leaves through modulating antioxidant enzyme activity. *Environmental and Experimental Botany*, **79**, 1–10.
- Gulen H, Cansev A, Eris A. 2009. Cold hardiness of olive (*Olea europaea* L.) cultivars in cold-acclimated and non-acclimated stages: Seasonal alteration of soluble sugars and phospholipids. *Journal of Agriculture Science*, **147**, 459–467.
- Guo Z F, Ou W B, Lu S Y, Zhong Q. 2006. Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. *Plant Physiology and Biochemistry*, **44**, 828–836.
- Heath R L, Packer L. 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, **125**, 189–198.
- He L, Ban Y, Inoue H, Matsuda N, Liu J, Moriguchi T. 2008. Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple spermidine synthase in response to salinity and hyperosmosis. *Phytochemistry*, **69**, 2133–2141.
- He L X, Nada K, Kasukabe Y, Tachibana S. 2002. Enhanced susceptibility of photosynthesis to low temperature photoinhibition due to interruption of chill-induced increase of S-adenosylmethionine decarboxylase activity in leaves of spinach (*Spinacia Oleracea* L.). *Plant Cell Physiology*, **43**, 196–206.
- Hunt R. 2003. Growth analysis, individual plants. *Encyclopaedia of Applied Plant Sciences*, **2**, 579–588.
- Jouve L, Hoffmann L, Hausman J F. 2004. Polyamine, carbohydrate, and proline content changes during salt stress exposure of Aspen (*Populus tremula* L.): Involvement of oxidation and osmoregulation metabolism. *Plant Biology*, **6**, 74–80.
- Keunen E, Peshev D, Vangronsveld J, Van Den Ende W, Cuypers A. 2013. Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. *Plant, Cell & Environment*, **36**, 1242–1255.
- Klotke J, Kopka J, Gatzke N, Heyer A G. 2004. Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation — evidence for a role of raffinose in cold acclimation. *Plant, Cell & Environment*, **27**, 1395–1404.
- Konstantinos P A, Imene T, Panagiotis M N, Roubelakis-Angelakis K A. 2010. ABA-dependent amine oxidases-driven H₂O₂ affects stomata conductance. *Plant Signal Behavior*, **5**, 1153–1156.
- Kubis J. 2008. Exogenous spermidine differentially alters activities of some scavenging system enzymes, H₂O₂ and superoxide radical levels in water-stressed cucumber leaves. *Journal of Plant Physiology*, **165**, 397–406.
- Kumar S, Kaur R, Kaur N, Bhandhari K, Kaushal N, Gupta K, Bains T S, Nayyar H. 2011. Heat-stress induced inhibition in growth and chlorosis in mungbean (*Phaseolus aureus* Roxb.) is partly mitigated by ascorbic acid application and is related to reduction in oxidative stress. *Acta Physiology Plant*, **33**, 2091–2101.
- Laloi C, Apel K, Danon A. 2004. Reactive oxygen signaling: The latest news. *Current Opinon Plant Biology*, **7**, 323–328.
- Li H B, Wang J, Liu A M, Liu K D, Zhang Q F, Zou J S. 1997. Genetic basis of low-temperature-sensitive sterility in *indica-japonica* hybrids of rice as determined by RFLP analysis. *Theoretical and Applied Genetics*, **95**, 1092–1097.
- Liu J, Jiang M Y, Zhou Y F, Liu Y L. 2005. Production of polyamines is enhanced by endogenous abscisic acid in maize seedlings subjected to salt stress. *Journal of Integrative Plant Biology*, **47**, 1326–1334.
- Liu X G, Xu H, Zhang J Y, Liang G W, Liu Y T, Guo A G. 2012. Effect of low temperature on chlorophyll biosynthesis in albinism line of wheat (*Triticum aestivum*) FA85. *Physiologia Plantarum*, **145**, 384–394.
- Luomala E M, Laitinen K, Sutinen S, Kellomäki S, Vapaavuori E. 2005. Stomatal density, anatomy and nutrient concentrations of Scots pine needles are affected by elevated CO₂ and temperature. *Plant Cell and Environment*, **28**, 733–749.
- Madhava Rao K V, Sresty T V. 2000. Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. *Plant Science*, **157**, 113–128.
- Morsy M R, Jouve L, Hausman J F, Hoffmann L, Stewart J M. 2007. Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. *Journal of Plant Physiology*, **164**, 157–167.
- Mostofa M G, Yoshida N, Fujita M. 2014. Spermidine pretreatment enhances heat tolerance in rice seedlings through modulating antioxidative and glyoxalase systems. *Plant Growth Regulation*, **73**, 31–44.
- Myouga F, Hosoda C, Umezawa T, Iizumi H, Kuromori T, Motohashi R, Shono Y, Nagata N, Ikeuchi M, Shinozaki K. 2008. A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis*. *The Plant Cell*, **20**, 3148–3162.
- Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology*, **22**, 867–880.
- Nickel K S, Cunningham B A. 1969. Improved peroxidase assay method using leuco 2,3',6-trichloroindophenol and application to comparative measurements of peroxidatic catalysis. *Analytical Biochemistry*, **27**, 292–299.
- Palonen P, Buszard D, Donnelly D. 2000. Changes in carbohydrates and freezing tolerance during cold acclimation of red raspberry cultivars grown *in vitro* and *in vivo*. *Physiologia Plantarum*, **110**, 393–401.
- Parida A B, Das A, Das P. 2002. NaCl stress causes changes in photosynthetic pigments, proteins, and other metabolic components in the leaves of a true mangrove, *Bruguiera*

- parviflora*, in hydroponic cultures. *Journal of Plant Biology*, **45**, 28–36.
- Polanská L, Vicankova A, Novakova M, Malbeck J, Dobrev P I, Brzobohaty B, Vankova R, Machackova I. 2007. Altered cytokinin metabolism affects cytokinin, auxin, and abscisic acid contents in leaves and chloroplasts, and chloroplast ultrastructure in transgenic tobacco. *Journal of Experimental Botany*, **58**, 637–649.
- Purvis A C, Grierson W. 1982. Accumulation of reducing sugar and resistance of grapefruit peel to chilling injury as related to winter temperatures. *Journal of the American Society for Horticultural Science*, **107**, 139–142.
- Pusittigul I, Kondo S, Siriphanich J. 2012. Internal browning of pineapple (*Ananas comosus* L.) fruit and endogenous concentrations of abscisic acid and gibberellins during low temperature storage. *Scientia Horticulturae*, **146**, 45–51.
- Qiu J, Wang R M, Yan J Z, Hu J. 2005. Seed film coating with uniconazole improves rape seedling growth in relation to physiological changes under waterlogging stress. *Plant Growth Regulation*, **47**, 75–81.
- Sanchez B, Rasmussen A, Porter J R. 2014. Temperatures and the growth and development of maize and rice: A review. *Global Change Biology*, **20**, 408–417.
- Sasaki H, Ichimura K, Imada S, Yamaki S. 2001. Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings. *Journal of Plant Physiology*, **158**, 847–852.
- Sevillano L, Sanchez-Ballesta M T, Romojaro F, Flores F B. 2009. Physiological, hormonal and molecular mechanisms regulating chilling injury in horticultural species. Postharvest technologies applied to reduce its impact. *Journal of the Science of Food and Agriculture*, **89**, 555–573.
- Sheoran I S, Saini H S. 1996. Drought-induced male sterility in rice: Changes in carbohydrate levels and enzyme activities associated with the inhibition of starch accumulation in pollen. *Sex Plant Reproduction*, **9**, 161–169.
- Shu S, Guo S R, Sun J, Yuan L Y. 2012. Effects of salt stress on the structure and function of the photosynthetic apparatus in *Cucumis sativus* and its protection by exogenous putrescine. *Physiologia Plantarum*, **146**, 285–296.
- Taylor A O, Craig A S. 1971. Plants under climatic stress: II. Low temperature, high light effects on chloroplast ultrastructure. *Plant Physiology*, **47**, 719–725.
- Tian J, Wang L P, Yang Y J, Sun J, Guo S R. 2012. Exogenous spermidine alleviates the oxidative damage in cucumber seedlings subjected to high temperatures. *Journal of American Society Horticulture Science*, **137**, 11–19.
- Wang B G, Wang J H, Liang H, Yi J Y, Zhang J J, Lin L, Wu Y, Feng X Y, Cao J K, Jiang W B. 2008. Reduced chilling injury in mango fruit by 2,4-dichlorophenoxyacetic acid and the antioxidant response. *Postharvest Biology and Technology*, **48**, 172–181.
- Wang F, Cheng F M, Liu Y i, Zhong L J, Zhang G P. 2006a. Dynamic changes of plant hormones in developing grains at rice filling stage under different temperature. *Acta Agronomica Sinica*, **1**, 25–29. (in Chinese)
- Wang F, Tan X H, Gan L, Wu Y Y, Wang R Z, Li Q M. 2006b. Effects of cold shock treatments on endogenous phytohormones contents and proteins variations of navel orange fruits. *Journal of Food Science*, **1**, 71–74.
- Wang X, Yu J, Yang Y, Cang J, Li Z F. 2009. Changes of endogenous hormones of winter wheat varieties with different cold-resistances under low temperature. *Journal of Triticeae Crops*, **5**, 827–831. (in Chinese)
- Xu P L, Guo Y K, Bai J G. 2008. Effects of long-term chilling on ultrastructure and antioxidant activity in leaves of two cucumber cultivars under low light. *Physiologia Plantarum*, **132**, 467–478.
- Xu S C, Hu J, Li Y P, Ma W G, Zheng Y Y, Zhu S J. 2011. Chilling tolerance in *Nicotiana tabacum* induced by seed priming with putrescine. *Plant Growth Regulation*, **63**, 279–290.
- Yamamoto A, Shim IS, Fujihara S. 2012. Chilling-stress responses by rice seedlings grown with different ammonium concentrations and its relationship to leaf spermidine content. *Journal of Plant Biology*, **55**, 191–197.
- Yamane K, Kawasaki M, Taniguchi M, Miyake H. 2003. Differential effect of NaCl and polyethylene glycol on the ultrastructure of chloroplasts in rice seedlings. *Journal of Plant Physiology*, **160**, 573–575.
- Yang J C, Zhang J H, Wang Z Q, Zhu Q S, Wang W. 2001. Hormonal changes in the grains of rice subjected to water stress during grain filling. *Plant Physiology*, **127**, 315–323.
- Yoshida S, Forno D A, Cock J H, Gomez K A. 1976. Determination of sugar and starch in plant tissue. In: Yoshida S, ed., *Laboratory Manual for Physiological Studies of Rice*. International Rice Research Institute, Los Banos, Laguna, Philippines. pp. 46–49.
- Zhang M P, Zhang C J, Yu G H, Jiang Y Z, Strasser R J, Yuan Z Y, Yang X S, Chen G X. 2010. Changes in chloroplast ultrastructure, fatty acid components of thylakoid membrane and chlorophyll a fluorescence transient in flag leaves of a super-high-yield hybrid rice and its parents during the reproductive stage. *Journal of Plant Physiology*, **167**, 277–285.

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