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The protective effect of cold acclimation on the low temperature stress of the lotus (*Nelumbo nucifera*)

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Abstract: This study compared the protective effect of cold acclimation on the cold tolerance in the lotus (*Nelumbo nucifera*). The cold acclimation increased the sprouting rate and leaf expansion rate of the lotus by about 36% at 0 °C, and the cold acclimation could enhance the levels of the stress related osmolytes including higher proline, soluble protein, and soluble sugar contents. The electrolyte leakage and lipid peroxidation level of the control samples increased significantly, but these indices did not change significantly in the cold acclimation group during low temperature stress. Furthermore, the cold acclimated rhizomes had higher antioxidant enzyme activities and a more stable ROS homeostasis response to the low temperature stress. Some stress-related genes were significantly up-regulated after the cold acclimation, especially the antioxidase related genes (*CAT1, GPX, APX* and *MSD*) were up-regulated nearly five times higher than that of the control group at the 0 °C condition. Additionally, the *ICE1-CBF-COR* pathway was involved in the lotus cold acclimation process. These results suggested that cold acclimation can obviously improve the stress tolerance of the lotus by the stable ROS homeostasis, enhance the antioxidant enzyme activity, regulate the stress-related gene expression and alleviate the stress damage.

Keywords: cold tolerance; reactive oxygen species; antioxidant system

Low temperatures are a serious abiotic stress that can severely impair plant growth and agricultural production (Gao et al. 2009), but plants can enhance their ability to resist cold stress by cold acclimation (Theocharis et al. 2012). Plants have evolved their physiological and molecular responses to cold tolerance. Reactive oxygen species (ROS) have been reported to be involved in the processes leading to plant acclimation to various stresses. Plants alter their defence system components, such as compatible osmolytes and antioxidant enzymes, to alleviate stress damage (Suzuki et al. 2011). Some cold-sensitive plants show metabolic disorders and structural injury under cold stress (Atici, Nalbantoglu 2003). However, others are well adapted to low temperatures through cold acclimation (Theocharis et al. 2012). Some physiological changes during cold acclimation are important for increasing their cold tolerance ability (Hsieh et al. 2004).

The lotus is an aquatic plant. It is considered an important economic species with excellent ornamental, edible and medicinal value (Sinha et al. 2000).

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The lotus is sensitive to low temperatures as growth ceases below 15 °C (Wang, Zhang 2005). Recently, some studies have shown that low temperatures during the growing season can trigger the activation of the lotus' physiological defences. However, the defence mechanisms of the lotus responding to low temperatures is not clear.

MATERIAL AND METHODS

Plant materials and treatments. The rhizomes of *N. nucifera* 'Huohua' were potted in plastic pots (26 cm in diameter) on February 15, 2017, and cultivated at 28 °C/25 °C (day/night) as the control group. Another pre-treated group was placed in a growth chamber (18 °C/15 °C, day/night) and treated for six days for cold acclimation. For the cold treatment, all the rhizomes were transferred to a growth chamber and the temperature was decreased from 10 °C to 0 °C, reducing it by 5 °C each time, at 2-day intervals, and then recovered at 28 °C/25 °C (day/night) for six days. The rhizome samples were collected after the cold acclimation, low temperatures (10 °C, 5 °C and 0 °C) and recovery stage for the electrolyte leakage (EL), malondialdehyde (MDA) and H_2O_2 detention. Some samples were frozen in liquid nitrogen immediately and maintained at –80 °C until the subsequent assays. Another sample was directly held at 28 °C/25 °C (day/night) to calculate the sprouting rate and the leaf expansion rate. All the experiments were performed with three replicates.

Determination of the sprouting rate and leaf expansion rate. Sprouting is defined as when the bud growth is more than 1 cm. Leaf expansion is defined as when the leaf is fully developed. One hundred bud samples were examined per treatment.

Determination of the proline, soluble protein, and soluble sugar. The proline content was determined as described by Yang et al. (2019), the soluble protein and soluble sugar contents were determined as described by Li (2000).

Determination of the EL, MDA, H_2O_2 and antioxidant enzyme activity. Assay kits (Nanjing Jiancheng Bioengineering Institute, China) were used for the detection of the MDA, EL and H_2O_2 levels, and the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were determined according to the Yang et al. (2019) method.

Quantitative gene expression analysis. The quantitative gene expression analysis used the TB Green™ Premix Ex Taq™ II kit (TaKaRa, Japan) according to the Zhang et al. (2013) method. *Actin* was selected as the housekeeping gene. The relative quantitative expression of each gene was calculated via the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in Table S1 in electronic supplementary material (see the electronic version).

Statistical analysis. Results were analysed by an analysis of variance (ANOVA) using the SPSS 17 software (SPSS Inc., Chicago, USA). Duncan's new multiple range test was applied for the separation of the means to determine the significant differences.

RESULTS

Effect of cold acclimation on the sprouting rate and leaf expansion rate. The low temperature environment inhibited the lotus sprouting rate and leaf expansion rate, while the cold acclimation pre-treatment can obviously improve this inhibitory effect (Table 1). In the control group, the sprouting rate of the lotus rhizomes was 83.67%, 70.67% and 57.33% under the 10 °C, 5 °C and 0 °C growth conditions, respectively; after the cold acclimation treatment, the sprouting rate rose to 96.67%, 86.0% and 78.33%, respectively. The leaf expansion rate of the cold acclimation group increased by 24% (10 °C), 19.1% (5 °C) and 36.63% (0 °C) compared to the control group. This result indicated that the cold acclimation can effectively improve the cold tolerance of the lotus.

Effect of cold acclimation on the compatible osmolyte changes. The cold acclimation pre-treatment led to a significant increase in the proline content, with an approximately 2.2-fold higher proline level in the pre-treated group than that in the con-

Table 1. The sprouting rate and the leaf expansion rate of the pre-treated and control groups

Rate	10 °C	5 °C	0 °C
Sprouting rate			
Pre-treated	96.67 ± 2.89^{a}	86.00 ± 5.57^{a}	78.33 ± 3.05^{a}
Control	83.67 ± 5.51^{a}	70.67 ± 6.02^{bc}	57.33 ± 7.23^{b}
Leaf expansion rate			
Pre-treated	91.33 ± 3.51^{a}	83.00 ± 4.58^{ab}	76.00 ± 4.00^{a}
Control	73.67 ± 7.57^{b}	69.67 ± 6.11^{c}	55.33 ± 4.51^{b}

$^{a–c}$Different letters indicate the significant differences between the different samples in the same temperature condition

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trol rhizomes (Figure 1A). Furthermore, the proline content in the pre-treated rhizome was significantly higher than that in the control group during the subsequent low temperature treatment. Under cold stress at 10 °C, the highest proline levels were observed in both the pre-treated and control rhizomes, and a significant decrease occurred thereafter. After six days of recovery, the proline content returned to a lower level similar to that before applying the cold stress.

The cold acclimation pre-treatment led to a significant accumulation of the soluble protein content prior to the recovery (Figure 1B). There was a significant difference in the soluble protein content between the cold acclimation and control groups, the soluble protein first increased and then decreased in the cold acclimation pre-treated rhizomes, while a significant decrease occurred in the control groups during the continuous low temperature treatments (Figure 1B). Under cold stress at 10 °C, the highest soluble protein content was observed in the pre-treated rhizomes.

The cold acclimation also caused a highly significant increase in the soluble sugar content after the low temperature treatments (Figure 1C). The soluble sugar was significantly higher in the pre-treated rhizomes than that in the control groups when exposed to cold stress, and there was no significant difference between the two groups after the six days recovery. In both groups, the soluble sugar content initially decreased at 10 °C and sharply increased at 5 °C and 0 °C until the end of the cold stress, and the highest soluble sugar content level was observed after exposure to 0 °C (Figure 1C).

Effect of cold acclimation on the membrane lipid peroxidation. The cold acclimation significantly reduced the membrane lipid peroxidation of the lotus rhizomes under the low temperature

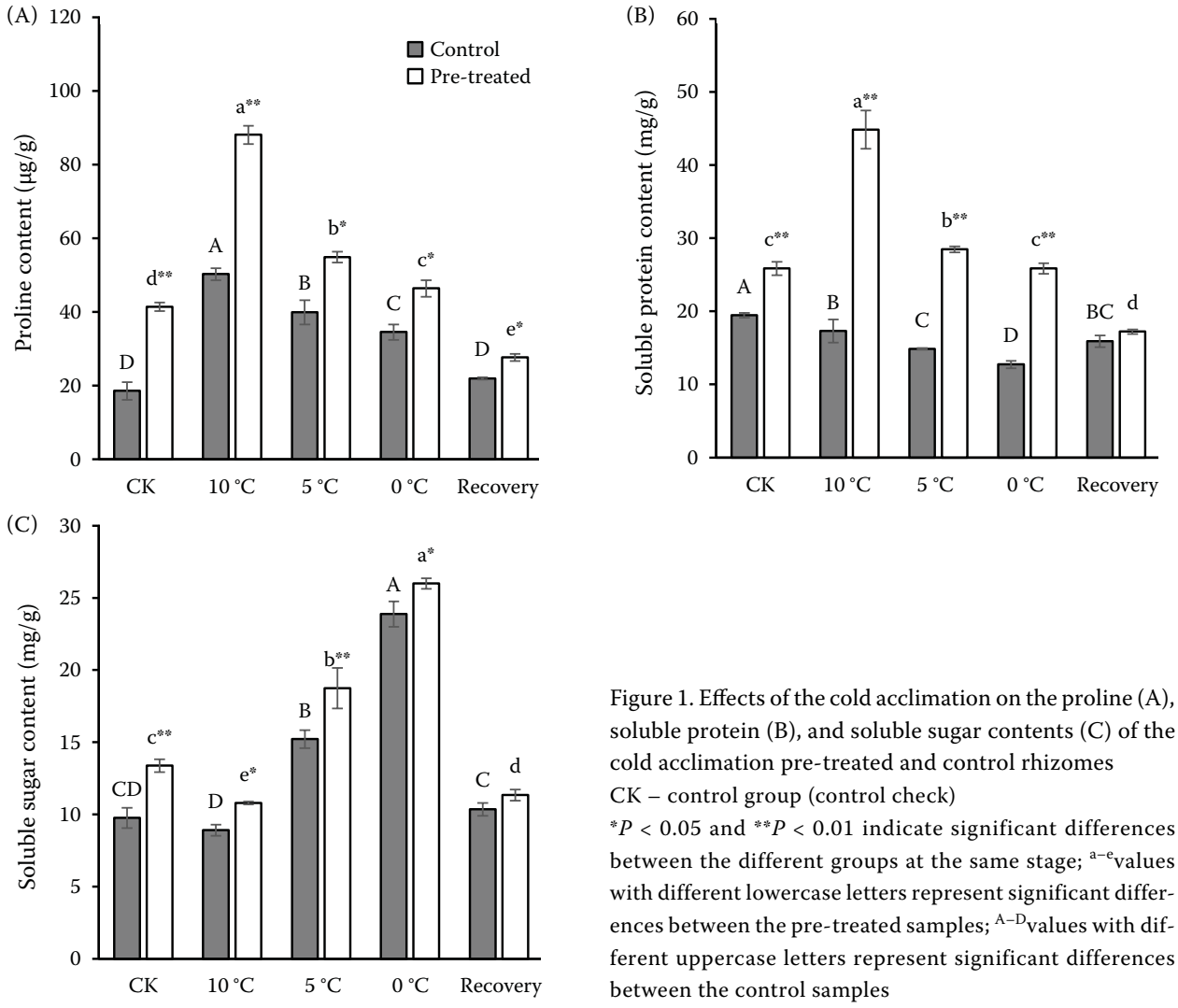

Figure 1. Effects of the cold acclimation on the proline (A), soluble protein (B), and soluble sugar contents (C) of the cold acclimation pre-treated and control rhizomes

CK – control group (control check)

$^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the different groups at the same stage; $^{a-e}$values with different lowercase letters represent significant differences between the pre-treated samples; $^{A-D}$values with different uppercase letters represent significant differences between the control samples

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stress. The cold acclimation led to a significant increase both in the MDA content and the EL, which was approximately 1.2-fold higher in the pre-treated rhizomes than in the control ones (Figures 2A and 2B). Furthermore, the MDA contents and the EL had no significant differences in the pre-treated rhizomes during the continuous low temperature treatments; however, they were significantly increased in the control group (Figures 2A and 2B). Moreover, the H_2O_2 levels in the two groups showed a similar trend to that of the MDA and EL. The H_2O_2 level of all the samples continued to increase as the treatment temperature decreased, and the H_2O_2 of the control samples was significantly higher than that of the pre-treated rhizomes during the continuous low temperature and recovery treatments. The highest level of the H_2O_2 content was observed at the 0 °C treated stage in both groups (Figure 2C).

Effect of cold acclimation on the antioxidant enzyme activity. The cold acclimation pre-treatment resulted in higher antioxidant enzyme activity levels (Figure 3), The pre-treated rhizomes exhibited an increase in the SOD, POD and CAT activities by 10.6, 38.8, and 48.1% over the control rhizomes, respectively. The changes in the SOD and POD activities showed a similar pattern (Figures 3A and 3B), the minimum and maximum appeared at 5 °C and 0 °C, respectively. As the temperature decreased, higher SOD and POD activity levels were observed in the pre-treated rhizomes. No significant difference was observed between the pre-treated and control rhizomes after the six days recovery. The trend for the CAT activity was the opposite of that for the SOD and POD activities in the pre-treated rhizomes, and the change pattern was similar in both the pre-treated and control rhizomes. A maximum was observed at the 5 °C treated stage (Figure 3C).

Correlation analysis. MDA had a significant positive correlation to the H_2O_2 content in the control and pre-treated rhizomes (Tables 2 and 3). In the

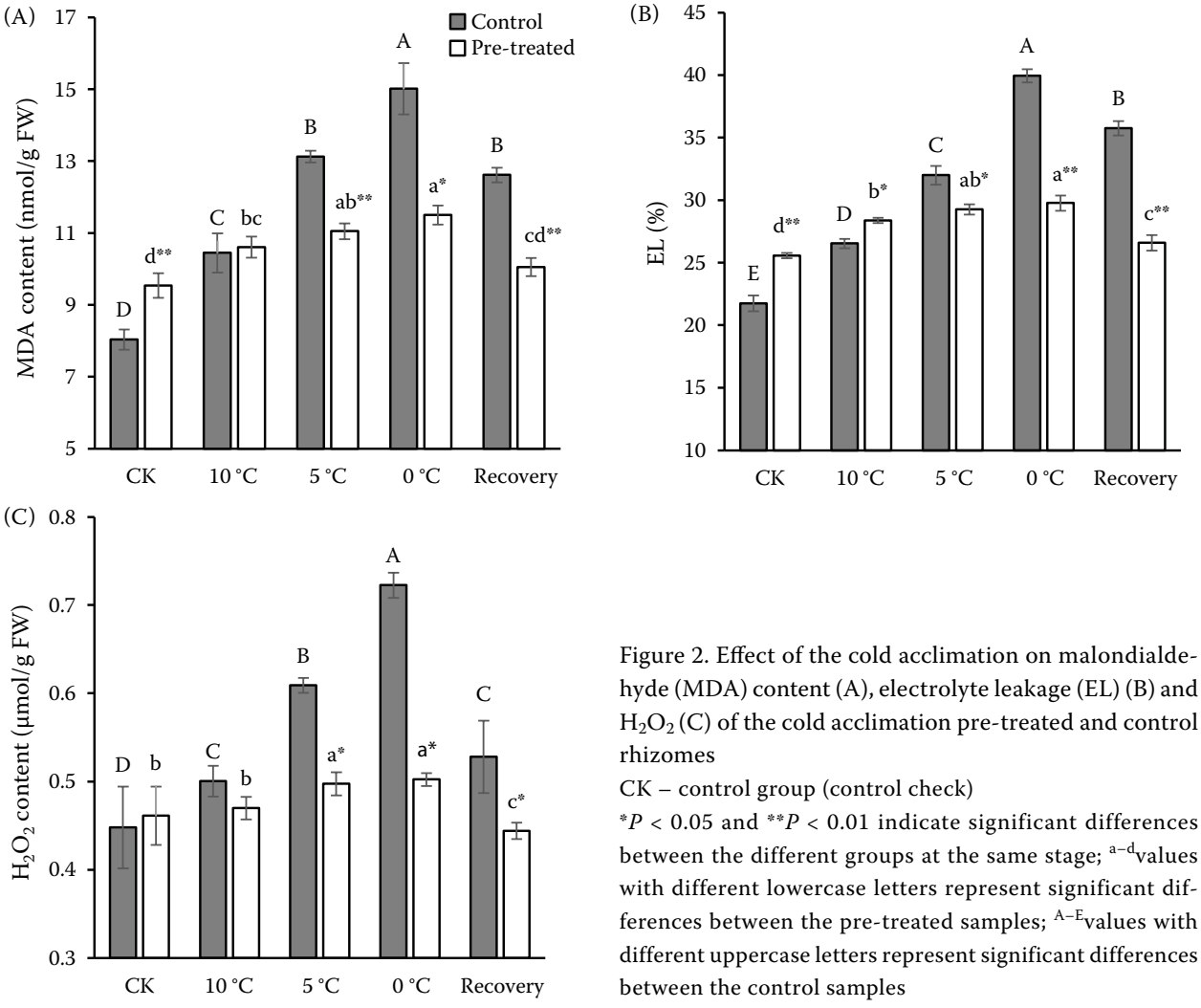

Figure 2. Effect of the cold acclimation on malondialdehyde (MDA) content (A), electrolyte leakage (EL) (B) and H_2O_2 (C) of the cold acclimation pre-treated and control rhizomes

CK – control group (control check)

$^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the different groups at the same stage; $^{a–d}$values with different lowercase letters represent significant differences between the pre-treated samples; $^{A–E}$values with different uppercase letters represent significant differences between the control samples

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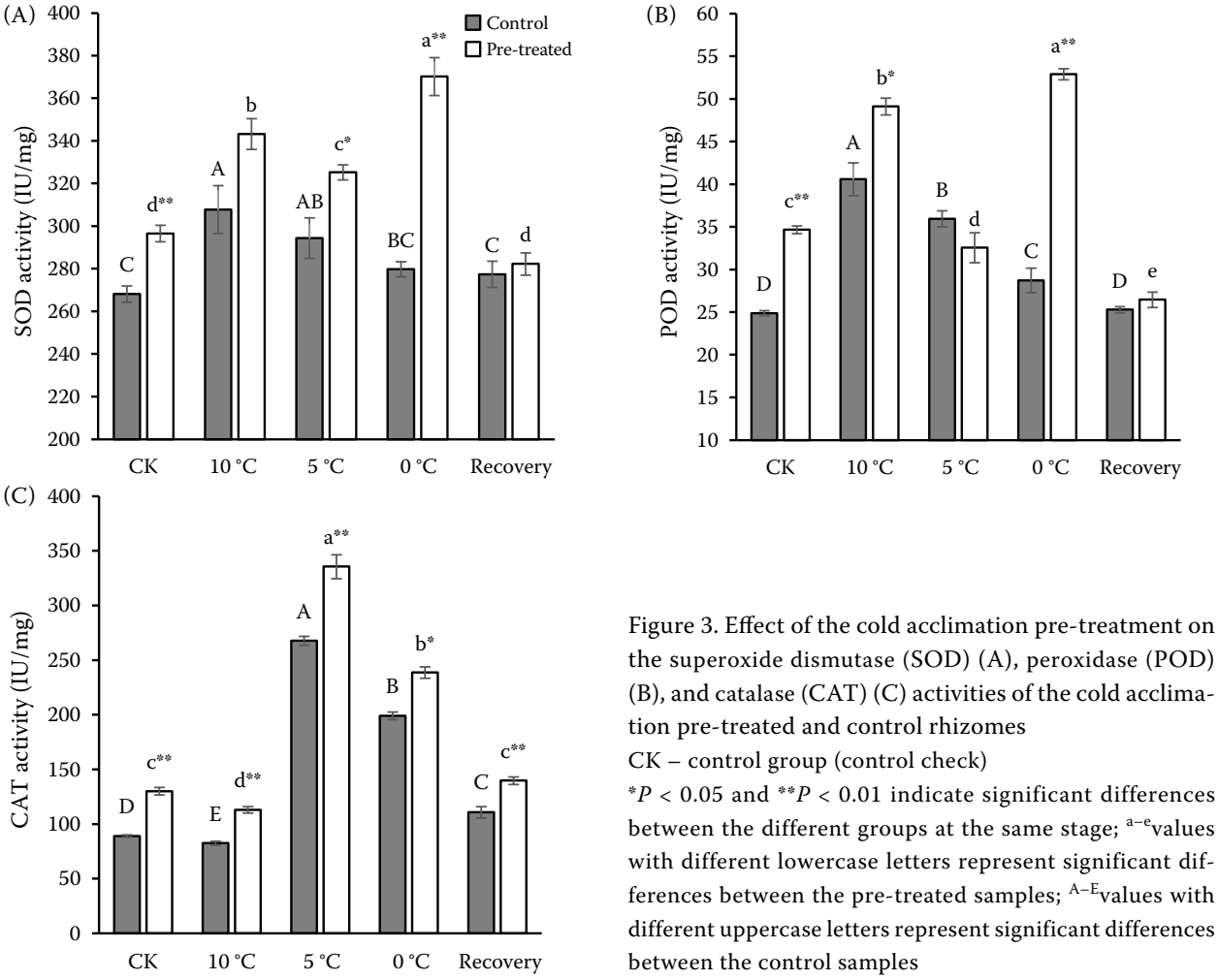

Figure 3. Effect of the cold acclimation pre-treatment on the superoxide dismutase (SOD) (A), peroxidase (POD) (B), and catalase (CAT) (C) activities of the cold acclimation pre-treated and control rhizomes

CK – control group (control check)

$^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the different groups at the same stage; $^{a–e}$values with different lowercase letters represent significant differences between the pre-treated samples; $^{A–E}$values with different uppercase letters represent significant differences between the control samples

control rhizomes, the MDA content had a significant positive correlation to the CAT activity and the sugar content. Furthermore, the CAT activity and the sugar content also had a significant positive correlation. It was concluded that the lotus rhizomes resist chilling injury through the accumulation of osmolytes and CAT antioxidant enzymes. In the pre-treated rhizomes, MDA had positive correla-

Table 2. The correlation of the physiological indices of the control rhizomes under cold stress

Indices	Proline	Soluble protein	Soluble sugar	MDA	H_2O_2	EL	SOD	POD	CAT
Proline	1.00	–0.28	0.11	0.26	0.26	0.07	0.83**	0.95**	0.24
Soluble protein	–	1.00	0.83**	–0.95**	–0.87**	–0.89**	–0.17	–0.11	–0.69**
Soluble sugar	–	–	1.00	0.78**	0.93**	0.74**	–0.12	–0.11	0.68**
MDA	–	–	–	1.00	0.88*	0.95**	0.14	0.07	0.69*
H_2O_2	–	–	–	–	1.00	0.84**	0.05	–0.05	0.73**
EL	–	–	–	–	–	1.00	–0.04	–0.14	0.53*
SOD	–	–	–	–	–	–	1.00	0.91**	0.12
POD	–	–	–	–	–	–	–	1.00	0.18
CAT	–	–	–	–	–	–	–	–	1.00

CAT – catalase; EL – electrolyte leakage; MDA – malondialdehyde; POD – peroxidase; SOD – superoxide dismutase

$^{*}P < 0.05$; $^{**}P < 0.01$

All the data were correlation coefficients

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Table 3. The correlation of the physiological indices of the pre-treated rhizomes under cold stress

Indices	Proline	Soluble protein	Soluble sugar	MDA	H_2O_2	EL	SOD	POD	CAT
Proline	1.00	0.98**	–0.17	0.28	0.23	0.41	0.53*	0.60*	–0.09
Soluble protein	–	1.00	–0.20	0.21	0.16	0.32	0.50	0.61*	–0.17
Soluble sugar	–	–	1.00	0.73**	0.71**	0.68**	0.69**	0.47	0.69**
MDA	–	–	–	1.00	0.64**	0.89**	0.81**	0.58*	0.64**
H_2O_2	–	–	–	–	1.00	0.73**	0.69**	0.48	0.66**
EL	–	–	–	–	–	1.00	0.82**	0.59*	0.67**
SOD	–	–	–	–	–	–	1.00	0.90**	0.35
POD	–	–	–	–	–	–	–	1.00	–0.04
CAT	–	–	–	–	–	–	–	–	1.00

CAT – catalase; EL – electrolyte leakage; MDA – malondialdehyde; POD – peroxidase; SOD – superoxide dismutase
*$P < 0.05$; **$P < 0.01$
All the data were correlation coefficients

tions to the sugar content, and the MDA also had significant positive correlations to the SOD, POD and CAT activities. The data suggested that the cold acclimation may enhance the antioxidant enzyme activity and help the lotus to resist chilling injuries.

Identification of differentially expressed genes by qRT-PCR analysis. *CAT, GPX* and *MSD* exhibited the same expression mode, which were significantly up-regulated after the cold acclimation, and continuously up-regulated in the control group and down-regulated in the cold acclimation group during the initial cooling stage (10 °C to 5 °C); however, the expression of these genes in the cold acclimation group was maximised at the 0 °C stage (Figure 4). Furthermore, the expression of *CAT1*, *GPX*, *MSD*, *CSD*, *CBF2*, *CRP2* were significantly up-regulated after the cold acclimation treatment, which indicated that the cold acclimation may activate these stress-related genes expression. In the control group, most of the gene expression trends were initially present at lower levels, then obviously up-regulated at the 10 °C and 5 °C conditions, and rapidly down-regulated at 0 °C. However, these genes showed relative higher levels after the cold acclimation treatment, then down-regulated at 10 °C and 5 °C, and the expression level peaked at 0 °C (Figure 4).

DISCUSSION

Membranes are the primary sites of cold-induced injury. The process of cold acclimation promotes membrane stabilisation and prevents damage that would lead to cell death (Matteucci et al. 2011). ROS-induced membrane lipid peroxidation reflects the stress-induced damage at the cellular level (Jain et al. 2001), and the EL and MDA indicate the extent of the stress-induced damage in plants (Tang et al. 2014). In this study, cold acclimation obviously improved the inhibition of the sprouting rate and leaf expansion rate, and also alleviated the membrane damage of the lotus during the subsequent cold stress. This result was consistent with the findings of Garbero et al. (2011).

Cold acclimation involves a number of physiological changes including the increased generation of osmolytes, modifications to the composition of the membrane lipids, and the antioxidant enzyme activity (Walker et al. 2010). Proline plays multiple roles in plant stress tolerance by mediating the osmotic adjustment, stabilising membranes, inducing the osmotic stress-related genes, and scavenging the ROS (Khedr et al. 2003; Kaur et al. 2011). Similarly, soluble proteins are especially associated with cold hardiness (Guy 1990; Couée et al. 2006). Soluble sugars are typical compatible osmolytes that protect plant cell membranes during cold-induced dehydration, preventing protein aggregation and degeneration during freezing and thawing (Ruelland et al. 2009). In this study, the cold acclimation increased the proline, soluble protein and soluble sugar contents by about 2.2, 1.3 and 1.4-fold, which suggested that the cold acclimation triggered the accumulation of the compatible osmolytes enhancing the osmotic adjustment capacity of the lotus as a defence strategy. Furthermore, the proline and soluble protein had similar content pattern changes, and the proline and soluble sugar contents showed an opposite trend

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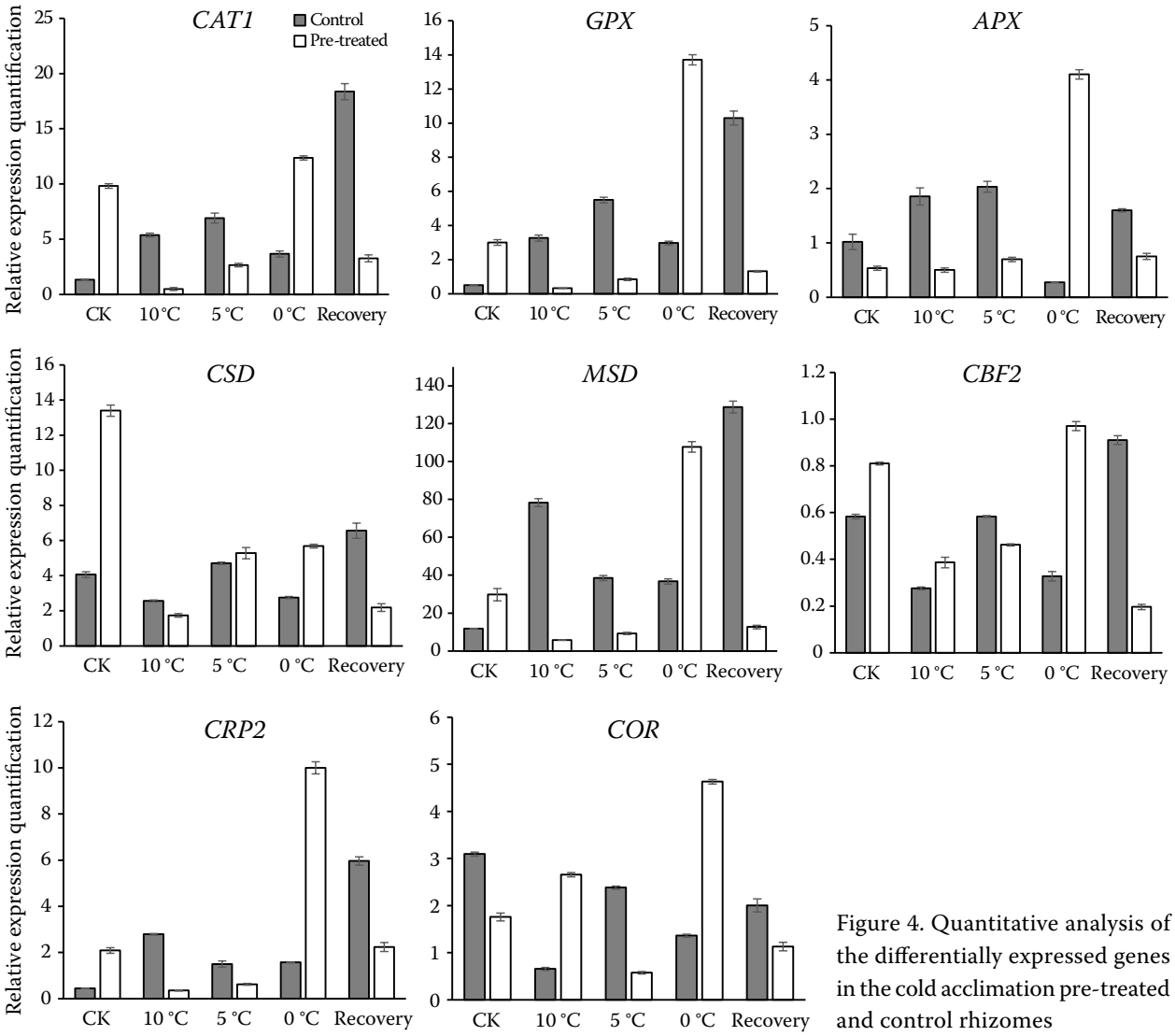

Figure 4. Quantitative analysis of the differentially expressed genes in the cold acclimation pre-treated and control rhizomes

during the low temperature treatments; the proline significantly increased at the beginning (10 °C and 5 °C) of the low temperature treatment, and the highest soluble sugar level appeared at later stages (0 °C), which suggested that these osmotic compounds synergistically interact to improve the lotus tolerance under low temperature stress.

H_2O_2 can act as a systemic signal in the plants' adaptation to abiotic stresses (Foyer, Noctor 2005). A certain amount of H_2O_2 may act as a signalling molecule under stress, which can induce the antioxidant system and alleviate the stress damage during cold stress. Numerous reports have suggested that antioxidants are associated with cold tolerance in various crops. The antioxidant system protects plants against chilling injuries through the scavenging of the ROS (Guo et al. 2006; Lu et al. 2013). SOD, POD and CAT are important antioxidant enzymes that eliminate superoxide anion free radicals. Superoxide radicals are catalysed by SOD to form H_2O_2, while H_2O_2 is broken down by POD and CAT in various cellular organs (Foyer, Noctor 2009). SOD forms the first line of defence against ROS. The initial higher levels of the SOD activity in the pre-treated rhizomes suggested that the cold acclimation triggered an antioxidant defence against the low temperatures to prevent any peroxidative damage. Previous studies showed that the POD and CAT activities increased to enhance the tolerance against chilling injuries by the effective detoxification of H_2O_2 in rice (Matsumura et al. 2002) and pumpkins (Sebnem et al. 2004). Many studies have reported a direct relationship between the increased antioxidant activity and stress tolerance (Hossain et al. 2009). In this study, the POD and CAT activities exhibited an obvious increase in the cold acclimation and chilling

https://doi.org/10.17221/62/2020-HORTSCI

stress stages (Figure 3), which indicated that POD and CAT play an important role in improving the lotus tolerance against cold stress. Additionally, the cold acclimation elevated *GPX, CAT1* and *MSD* expression levels, suggested these genes have a positive response in the cold acclimation induction and chilling-induced damage in the lotus.

Numerous transcription factors facilitate cold signalling. *CBF2* plays a central part in the tolerance to stress and activates the *COR* gene (Theocharis et al. 2012). The *COR* gene has been shown to be critical in plants for both chilling tolerance and cold acclimation (Xiong et al. 2002). Since the *ICE1-CBF-COR* is a well-known pathway involved in plant cold-tolerance (Miura et al. 2011), the *CBF2* gene is expected to facilitate the cold-tolerant pathway. A 4.7-fold increase found in the *CBF*2 gene of our gene expression analysis after the cold acclimation in the pre-treated rhizomes supports this opinion. The *CBF* gene was reported to be induced within a few hours of exposure to low temperatures (Xiao et al. 2008), and transcripts from the targeted *CBF/DRE*-regulated *COR* genes start to accumulate after the *CBF* expression (Mantyla et al. 1995). In the lotus, the *CBF* response to cold stress in the control sample is significantly slower than that in the cold acclimated group, and *COR* delayed response to the cold stress was also observed in the lotus. Thus, the *ICE1-CBF-COR* pathway is involved in the lotus cold acclimation process.

Among all the genes, most of them were up-regulated after the cold acclimation, especially at the 0 °C cold stress condition, which activated the antioxidant protection for the rhizomes from cold injury. However, these gene expression levels decreased at the 10 °C treatment stage, which indicated that some negative feedback regulation occurred in this process. Similar findings were reported by Yang et al. (2015). Additionally, only *APX*, *CRP2* had a significant positive correlation to both CAT and POD in the control rhizomes. However, *GPX*, *APX*, *MSD*, *CBF2*, *CRP2* had a significant positive correlation to both CAT and POD in the cold acclimated rhizomes, which indicated that the physiological and transcriptional changes caused by the cold acclimation resulted in the better cold tolerance of the lotus.

CONCLUSION

N. nucifera is sensitive to low temperatures, which often causes severe stress damage and affects the normal growth and development. An appropriate cold acclimation pre-treatment may improve the tolerance of the lotus to the chilling stress. The physiological parameters including the accumulation of the proline, soluble proteins, soluble sugars, activating the antioxidant enzymes, and inducing the stress-related gene expression play important roles in the tolerance acquisition of the lotus to chilling stresses. Cold acclimation is beneficial for the application of some tropical lotus species in cold regions.

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