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EFFECTS OF EXOGENOUS ABSCISIC ACID ON EXPRESSION OF COLD-REGULATED GENES IN WINTER WHEAT UNDER LOW TEMPERATURE STRESS

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Abstract

This study aimed to explore the effects of exogenous abscisic acid (ABA) on expression of cold-regulated genes in winter wheats under low-temperature (LT). Vars. Dongnongdongmai 1 (DNDM1) and Jimai22 (JM22) were treated with ABA at 4°C for 0, 0.5, 2, 8, 24, 48, 72, 96, 120, 144 and 168 h, and at 4, 0, -10 and -25°C. RNA was extracted by Trizol and the expression levels of genes were assessed by Quantitative real-time PCR. Moreover, the levels of ABA, MDA, SOD, soluble glucose and proline were also measured at each time points. After treating with ABA, no significant changed trends were identified in the gene expression levels at 4°C in both DNDM1 and JM22. However, significant increased variation trends were observed in the expression of *MYB80*, *Wrabi5*, and *Wrab17* after treating with ABA at 4, 0 and -10°C in DNDM. The expression of *CBF1VD-22D* was also upregulated after treating with ABA before 0°C in DNDM1. But no significant changed trends were identified in the genes expression in JM22 both in the control and ABA treated groups at 4, 0 and -10°C. *Wcs120* were significant increased at 4, 0 and -10°C, but no significant difference was identified between the control and ABA treated groups. SOD and soluble glucose were obviously accumulated after treating with ABA outdoor in DNDM1. ABA could promote the adaptation of DNDM under LT condition via increasing the expression of *MYB80*, *Wrabi5*, and *Wrab17* and accumulations of SOD and soluble glucose.

Key words: Abscisic acid; Low temperature stress; Cold-regulated genes; Winter wheats.

Introduction

Low temperature (LT) is an important natural stress that can affect the quality and production of crops (Riaz-ud-Din *et al.*, 2010; Gupta and Deswal, 2012). Previous study has documented that LT can increase the permeability and decrease the integrity of the cell membrane, which can disrupt the normal metabolism of crop and result in reduction of output (Campos *et al.*, 2003). Moreover, crops also can develop a variety of mechanisms to response LT challenges, such as alterations in accumulation of sugar and osmolytes, and lipid composition (Catalá *et al.*, 2014). Affecting transcriptional factors in regulating the expression of downstream effectors is the most direct way for crops to cope with cold-stress (Korn *et al.*, 2008). However, the variations of genes related to the ability of surviving under extremely LT are still not well elucidated (Chen *et al.*, 2014). Therefore, it is very essential to investigate the changes of survival related genes under LT.

Abscisic acid (ABA) is a common phytohormone which can regulate the expression levels of several genes in response to abiotic stresses, such as cold, drought, and salinity (Sui *et al.*, 2012). ABA participates in several aspects of plant growth, including seed germination, embryo maturation, photosynthesis, and adaptation to cultural condition changes (Chinnusamy *et al.*, 2006; Hartung, 2010; Hu *et al.*, 2010; Min & Gui-Qing, 2014). According to previous literatures, cold stress response pathways can be divided two aspects: ABA-denpendent and ABA-independent pathways under LT (Agarwal & Jha, 2010; Hu *et al.*, 2010). In the ABA-dependent pathway, ABA can activate the ABA-responsive element binding factor (ABF) to regulate the expression of downstream *Cor/Lea* genes (Talanova *et al.*, 2011). While in the ABA-independent pathway, another transcriptional factor family C-repeat binding factor (CBF) was triggered by LT to regulate the expression of *COR* gene to improve the tolerance of plants (Yamaguchi-Shinozaki and Shinozaki, 2005). Thus, both pathways are essential for plants responding to LT.

According to above evidences, two breeds of winter wheats: Dongnongdongmai (DNDM) 1 and Jimai (JM) 22 were selected to investigate the expression changes of LT responses genes in ABA-dependent and independent pathways. Based on these investigation, we hope to provide some new insights in understanding the LT response.

Materials and Methods

Materials: DNDM1 and JM22 wheat breeds were utilized in the current study. For DNDM1, it is the only wheat planted with more than 85% winter survival rate in Heilongjiang (45°7'N, 126°6'E), China. JM22 is another wheat breed planted in northern China. Although it has been planted over a wide area of northern China, it is still not able to survive at cold temperature of Heilongjiang (lowest is -30°C).

Planting and grouping: Research on DNDM1 and JM22 were carried out at outdoor in glasshouse. For the glasshouse, wheat seedlings were planted with natural black soil in a 9×9 cm^2 plastic pots in temperature controlled room for 30 d. The conditions of the controlled room were set as follows: (1) temperature was 25/20°C for day/night; (2) the photoperiod was 14/10h and the light intensity was 500

$\mu molm^{-2}s^{-1}$; and (3) the relative air humidity was 70%. Meanwhile, the following chemicals were added by ratios to the soil as fertilizers for plants: KH_2PO_4 1 g kg^{-1}soil and $(NH_4)_2SO_4$ 1g kg^{-1} soil and KCl 1 g kg^{-1} soil. Then, the plants were irrigated daily with distilled water. When seedlings grown to three leaf stage, the plants were divided into two groups: the control and ABA treated groups with three replicates for each. Plants in the control group were treated with a spray of distilled water, and in the ABA treated group were treated with a spray of 1×10^{-5}mol L^{-1} ABA. After 24 h of treatment, the temperature was lowered to 4/2°C (day/night) for cold-stress. Then the leaf samples were collected at 0, 0.5, 2, 8, 24, 48, 72, 96, 120, 144, and 168 h after cold stress conducted. Finally, leaf samples were frozen in liquid nitrogen and then store at -80°C for the further analysis.

For outdoor trial, the seeds were sown in an experiment farmland with 5 m rows and 0.6 m spacing at September 8, 2015 (average temperature was 18°C) in Qiqihar University, Qiqihar, Heilongjiang. The pH for the planted soil was 6.9, and the 21 $g\cdot m^{-2}$ of pure nitrogen and 10 $g\cdot m^{-2}$ of P_2O_5 were used as fertilizers. When seedlings grew to three leaf stage (September 29, 2015), the plants were divided into two groups: the control and ABA treated groups with three replicates for each. Plants in the control group were treated with a spray of distilled water, and in the ABA treated group were treated with a spray of 1×10^{-5} mol L^{-1} ABA. Leaf samples were harvested at 4°C, 0°C, -10°C and -25°C, respectively. After collection, the leaf samples were snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

Quantitative real-time PCR (RT-qPCR): Total RNA of wheat leaf samples were extracted by Trizol Reagent (Invitrogen, Carlsbad, CA) fully following the manufacturer's instruction. A PrimeScript RT Kit (Takara Biotechnology, Otsu, Japan) was utilized to synthesize cDNA template with 0.2 μg total RNA. Then amplication were conducuted as follow system: 12.5 μl of 2×SYBR Premix Ex Taq II (Takara), 0.8 μl, 10 μM of forward and reverse primers, respectively, 0.5μl of Cdna. Then the transcript levels of interest genes were quantified by real-time PCR using an Agilent Technologies Mx3000P Real-Time PCR System (Genetimes Technology, Inc. China). *TaRPII36* was used as internal reference and the relative expression of genes were computed by the 2(-ΔΔCt) method. Premiers of interested genes were designed using primer 5 according to the wheat gene sequences from GenBank.

Assessment of ABA in leaf samples: With liquid-nitrogen, the leaf sample was ground to powder and 1.0 g was taken out for the ABA assessment. Then, 2 ml of extraction buffer was added and the sample was transferred to 10 ml tubes. Meanwhile, 2 ml of extraction buffer was utilized to wash mortar and to add the remains to 10 ml of tube. Subsequently, the tissue homogenate was blending and standing at 4°C for 4 h. After this procedure, the sample was centrifuged at 3500 rpm for 8 min. The supernatant was collected and the sediment was extracted by 1 ml of extraction buffer once again. Then, the supernatant of these two extractions was mixed and volume was recorded. The supernatant was extracted by C-18 solid phase extraction. After the column balanced by 80% of methanol, sample was loaded into column and the first sample was collected. The column was eluted by the cycle of 100% methanol, 100% diethyl ether, and 100% methanol. Then, the elutropic sample was transferred to 5 ml of centrifuged tube and methanol was removed by nitrogen and sample was sizing to 1 ml by dilution buffer.

The maximum concentration of ABA was 500 ng/ml and eight concentration degrees of ABA were diluted by 2 fold in sequence (including 0 ng/ml). Then, samples were loaded at the 96-well plate with two duplications and 50μl for each. Antibodies were diluted by dilution buffer and added to 96-well plate with 50 μl for each. Then, the plate was placed into wet box for reaction at 37°C for 30 min. After reaction, plate was washed with buffer for 4 times. Then, 100 μl of diluted secondary (1 μl : 1 ml) was added and reaction was performed in a wet box at 37°C for 30 min. Same as above, the plate was washed and 100 μl of substrate (10 mg o-phenylenediamine, 10 ml substrate buffer and 4 μl 30% H_2O_2) was added to each for developing. Reaction was terminated by 50 μl of 2 mol/l sulfuric acid and optical density (OD) at 490 nm was assessed using an enzyme-linked immuno-spectrophotometer. According to the standard samples, the regression equation was obtained as:

$$y = -1.077x + 1.297,\ R^2 = 0.98,$$

$$\text{Logit (B/B0)} = \text{In}\frac{B/B_0}{1-B/B_0} = \text{In}\frac{B}{B_0-B}$$

x represents the logarithm of concentration; y represents the logit value of OD; B_0 represents the OD value of 0; B represents the OD values of other concentration. Then, the content of ABA (ng/g·fw) was calculated by the formula below:

$$\text{Content (ng/g)} = \frac{N\times V_2\times V_3\times B}{V_1\times W}$$

V_1 represents the volume of supernatant after drying by liquid nitrogen; V_2 represents the total volumes of supernatant after sample extracted; V_3 represents the sizing volume of sample by dilution buffer after nitrogen; W represents the fresh weight of leaf; N represents the concentration of ABA; B represents the diluted fold of sample.

Evaluation of MDA in leaf sample: About 0.5 g of leaf powder was taken out for Malondiadehyde (MDA) assessment. First, 2 ml of 10% trichloroacetic acid (TCA) was added to grind leaf to homogenate. Subsequently, 8 ml of 10% TCA was utilized to grind further, and then the homogenate was centrifuged 4000g at 4°C for 10 min. Supernatant was collected, and 2 ml of it was mixed with 2 ml of 0.6% thibabituric acid (TBA in 10% TCA) to react in boiled water for 15 min. After reaction, the mixture was rapid cooling and centrifuged at 2000 g for 10 min. Then, the OD values at 532 nm and 450 nm were recorded.

Measurement for SOD: About 0.5 g of leaf powder was taken out for SOD assessment. The leaf powder was further grinded and mixed with 5 ml of pre-cooled extraction buffer (50 mM pH 7.8 phosphate buffer contained with 1% polyvinylpyrrolidone). The homogenate was filtrated with four layers of gauze and centrifuged at 10500 rpm for 20 min for three times. Then, 4 ml supernatant was mixed 3 ml reaction mixture (14.5 mM methonine, 3 μM EDTA, 2.25 mM NBT) and 1.6 ml extraction buffer away from light. Then, the mixture was exposed to light with 4000 × for 10 min. After this procedure, mixture was taken out away from light and colorimetric value was assessed. The activity of SOD was computed using followed forluma:

$$\text{SOD activity (U/mg)} = \frac{(A_{CK} - A_E) \times V}{0.5 \times A_{CK} \times W \times V_1}$$

ACK represents the OD value of control; AE represents the OD value of the sample; V represents the total volume of the sample; Vt represents the volume of the sample using measurement; W represents the fresh weight of the sample.

Measurement for proline: Before sample measurement, the standard curve of proline solution was produced. First, total 25mg proline was dissolved in water and sized to 250 ml to obtain a 100 μg/ml proline solution. Then, six concentrations of proline: 1, 2, 3, 4, 5 and 6 μg/ml were diluted by water to 50 ml for each. Standard solutions of proline were mixed with 2ml of glacial acetic acid and 2ml of acidic-ninhydrin and heated in boiled water for 30 min. After cooling, 4ml of methylbenzene was added and shaked for 30s. After standing for layering, the upper proline-methylbenzene solution was transferred to cuvette with injector, and OD value of solution was measured at 520 nm with methylbenzene as control. Finally, the regression equation of OD value (Y) and concentration of proline (X) was obtained.

About 0.5 g of leaf powder was taken out for proline assessment. Sample was mixed with 5 ml of 3% sulfosalicyclic acid and placed in boiled water for 10 min with shaking. After this, sample was cooled and infiltrated to a new tube and filtrate was the extraction of proline. Then, 2 ml of proline extraction was mixed with 2 ml glacial acetic acid and 2 ml of acidic-ninhydrin in a new tube with a stopper and boiled in water for 30 min to obtain a red mixture. After cooling, 4 ml of methylbenzene was added and blended. After standing for layering, the upper solution was removed to 10 ml tube with a injector and centrifuged at 3000 rpm for 5 min. Following this, the upper proline-methylbenzene solution was sucked with injector to cuvette and OD value was measured at 520 nm. Methylbenzene was set as control. The content of proline in 2 ml sample solution was computed according to standard proline solution curve. The percentage of proline in the sample was calculated via the follow formula: proline percentage (μg/g) = [X × 5/2]/ weight (g). X represents the content of proline in 2 ml solution.

Measurement for soluble glucose: First, the glucose standard curve was produced. According to specification, a series of glucose concentration was prepared in 7 tubes with stoppers. Then, 4 ml of anthranone was added in each tube and heated with boiled water at the same time for 10 min. After this procedure, the tubes were quick – cooled by ice to room temperature. The first tube was set as control and the OD values of different samples were assessed at 625 nm. Then, the standard curve of glucose was produced with OD in Y and glucose (μg) in X.

Subsequently, soluble glucose of leaf and subterraneous stem was assessed. Sample was cut into pieces less than 2 mm and dried in a drying oven at 105°C. Then, 0.3 g of the dried sample was collected and mixed with 5 ml distilled water in tube. Tube was bathing in water at 80°C for 30 min and infiltrated. The sediment was re-extracted using distilled water at 80°C for 20 min and infiltrated to obtain a new solution. Then, the infiltrated solution for the first and second times were mixed and sized to 25 ml by distilled water. Before measurement, the stoste was diluted. Then, 1 ml of diluted extracted solution was mixed with 4 ml of anthranone in tube, and distilled water was used as control. Then, similar to the production of glucose standard curve, the soluble sugar in the tissue sample was detected. Finally, the average OD value at 625 nm was utilized for further calculation. The formula is presented as follows:

$$\text{Soluble sugar (mg/g)} = \frac{A \times V \times n}{1000 \times v \times W}$$

A represents the sugar content computed by standard curve (μg); V represents the volume of extracted solution (ml); v represents the volume utilized for measurement (ml); n represents the diluted fold; W represents the weight of sample (g).

Statistical analysis: In the current study, DPS7.05 statistical analysis software (http://ishare.iask.sina.com.cn/f/7771768.html) was used to perform statistical analysis. Mean ± standard deviation was utilized to present effect size and comparison between groups was assessed using Duncan's test. $p \leq 0.05$ was set as the threshold for the significant difference.

Results

Effects of exogenous ABA in ABA-dependent pathway: To further investigate the effect of ABA, the expression levels of several ABA-dependent genes were measured. Following with ABA treated time, no significant variations were identified in the expression levels of *CBF1VD-D22* in both DNDM1 and JM22 at 4°C (Fig. 1A). However, with the downregulated gene expression in the control group, the expression of *CBF1VD-D22* was remarkably decreased after ABA treatment and presented an increased trend after treating with ABA at 0 and -10°C in DNDM1 (Fig. 1B). But the expression of *CBF1VD-D22* in JM22 was significantly increased after treating with ABA since 0°C. Similarly, the expression levels of *MYB80* did not show significant

correlations with the increased ABA treating time in both DNDM1 and JM22 at 4°C (Fig. 1C). However, with the decreased ABA treated temperature, the expression levels of *MYB80* were significantly increased at 4, 0, and -10°C in both DNDM1 and JM22 (Fig. 1D). Following the increased ABA treated time at 4°C, the expression levels of *Wabi5* were markedly increased at 0.5 and 2 h, but decreased since 8 h in DNDM1 (Fig. 1E). However, the expression levels of *Wabi5* in JM22 did not perform significant correlation with the increased ABA treating time at 4°C. Following the decline of ABA treated temperature, the expression levels of *Wrabi5* were significantly increased at 4, 0 and -10°C in DNDM1 and JM22 , except at 0°C in JM22 (Fig. 1F). Same with the expression of *Wabi5*, the expression levels of *Wab17* were also significantly increased at 0.5 and 2h, but obviously decreased at 8 and 24 h after treating with ABA at 4°C in DNDM1 (Fig. 1G). Meanwhile, significant upregulations of *Wrab17* transcripts were detected at 0, 0.5 and 2 h, but downregulated at 8 and 24 h in JM22 after treating with ABA at 4°C. Moreover, the expression levels of *Wrab17* were also obviously increased after treating with ABA at 4, 0 and -25°C in DNDM1, but no significant variation trend was found in JM22 after treating with ABA at 4, 0, -10 and -25°C (Fig. 1H).

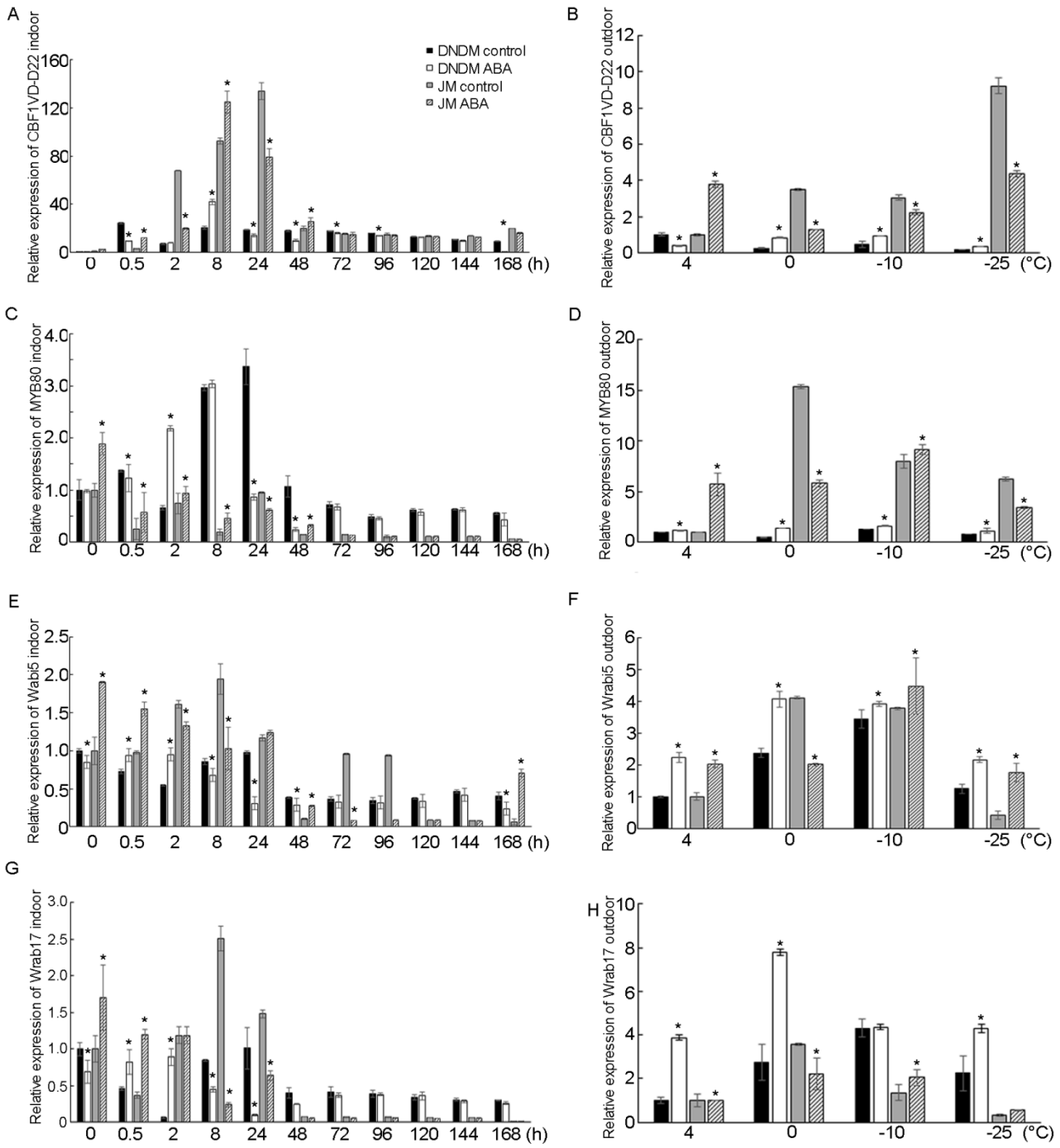

Fig. 1. The gene expression levels involved in ABA-dependent pathway determined by quantitative real time PCR in DNDM1 and JM22. ABA, abscisic acid; DNDM1, dongnongdongmai 1; JM22, Jimai 22.

Effects of exogenous ABA in ABA-independent pathway: After treating with ABA, the expression levels of *Wcor15*, *Wcs120* and *COR39* in DNDM1 and JM22 were examined. The expression levels of *Wcor15* were remarkably increased within 8 h at 4°C in both DNDM1 and JM22 in the control groups, but expression levels of *Wcor15* did not present significant variation trends in both DNDM1 and JM22 at 4°C in the ABA treated groups (Fig. 2A). Meanwhile, expression levels of *Wcor15* did not show significant variations after treating with ABA at 4, 0, -10 and -25°C (Fig. 2B). Although the expression level of *Wcs120* was significantly increased in DNDM1 after treating with ABA within 8 h, the expression level was significantly decreased since 24 h after treating with ABA at 4°C (Fig. 2C). Meanwhile, the expression levels were also increased treated with ABA at 4°C and 0°C (Fig. 2D). Unlike with DNDM1, no obvious variation trends were identified in JM22 after treating with ABA both indoor and outdoor. The expression levels of *COR39* were significantly changed after treating with ABA at 4°C within 8h, but no significant trends were identified in the variations of *COR39* in both DNDM1 and JM22 with the ABA at 4°C ranged from 0 to 168 h (Fig. 2E). Meanwhile, no obvious changed trends were also found in the expression of *COR39* after treating with ABA at decreased temperature in both DNDM1 and JM22 outdoor (Fig. 2F). Besides, the expression of *TaCBFII-5.2* was also determined. With the increase of ABA treated time at 4°C, no significant variation trends were identified in the expression levels of *CBFII-5.2* in both DNDM1 and JM22 (Fig. 2G). Meanwhile, with the decrease of ABA treated temperature, no significant variation trends were observed in the expression levels of *CBFII-5.2* in both DNDM1 and JM22 (Fig. 2H).

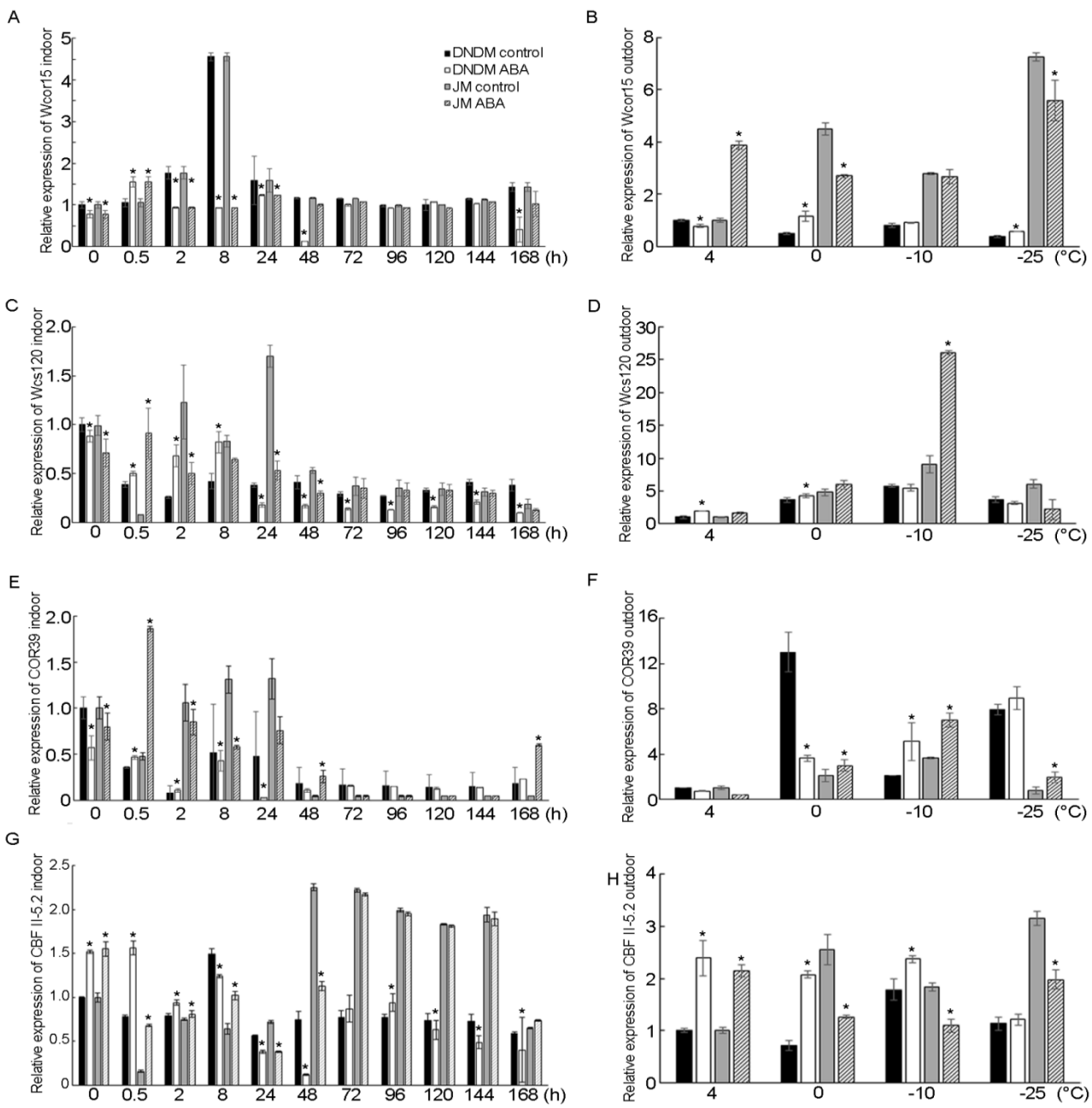

Fig. 2. The gene expression levels involved in ABA-independent pathway determined by quantitative real time PCR. ABA, abscisic acid; DNDM1, dongnongdongmai 1; JM22, Jimai 22.

Alterations of ABA, MDA, SOD and proline after treated with exogenous ABA: The levels of ABA in DNDM1 and JM22 were also investigated. With the extended ABA treatment time at 4°C, the levels of ABA were significantly increased compared with the control groups since 24 h in DNDM1 and since 2 h in JM22 (Fig. 3A). Meanwhile, after treating with ABA, the ABA levels were also significant upregulated at 0 and -10°C in both DNDM1 and JM22 (Fig. 3B). However, no significant differences were identified in the levels of MDA in both DNDM1 and JM22 at 4°C after treating with ABA (Fig. 3C). Simultaneously, no obvious differences were detected in the levels of MDA after treating with ABA at 4, 0, -10 and -25°C in both DNDM1 and JM22, except at -25°C in DNDM (Fig. 3D). SODs were also measured in both DNDM1 and JM22 during the treated condition. Same as the changes of MDA, there were also no remarkable differences identified in the levels of SOD in ABA treated groups compared with the control group in both DNDM1 and JM22 at 4°C (Fig. 3E). However, levels of SOD were increased significantly after treating with ABA at 4, 0, -10 and -25°C in DNDM1 (Fig. 3F).

Changes in metabolism of DNDM1 and JM22 were also estimated after treating with ABA. The levels of soluble glucose were significantly increased since 72 h after treating with ABA at 4°C compared to the control groups in DNDM1, but no significant difference was identified in JM22 (Fig. 4A). Meanwhile, soluble glucose levels were obviously increased at -10 and -25°C in the ABA treated group as compared with the control group in DNDM1, but no significant differences were identified in JM22 (Fig. 4B). After treating with ABA, the levels of proline in DNDM were significantly upregulated as compared with the control groups since 72 h at 4°C, and proline in JM22 were also obviously increased as compared with the control groups since 24 h at 4°C (Fig. 4C). Moreover, improvement of proline level was also identified at -10 and -25°C after treated with ABA in both DNDM1 and JM22 (Fig. 4D).

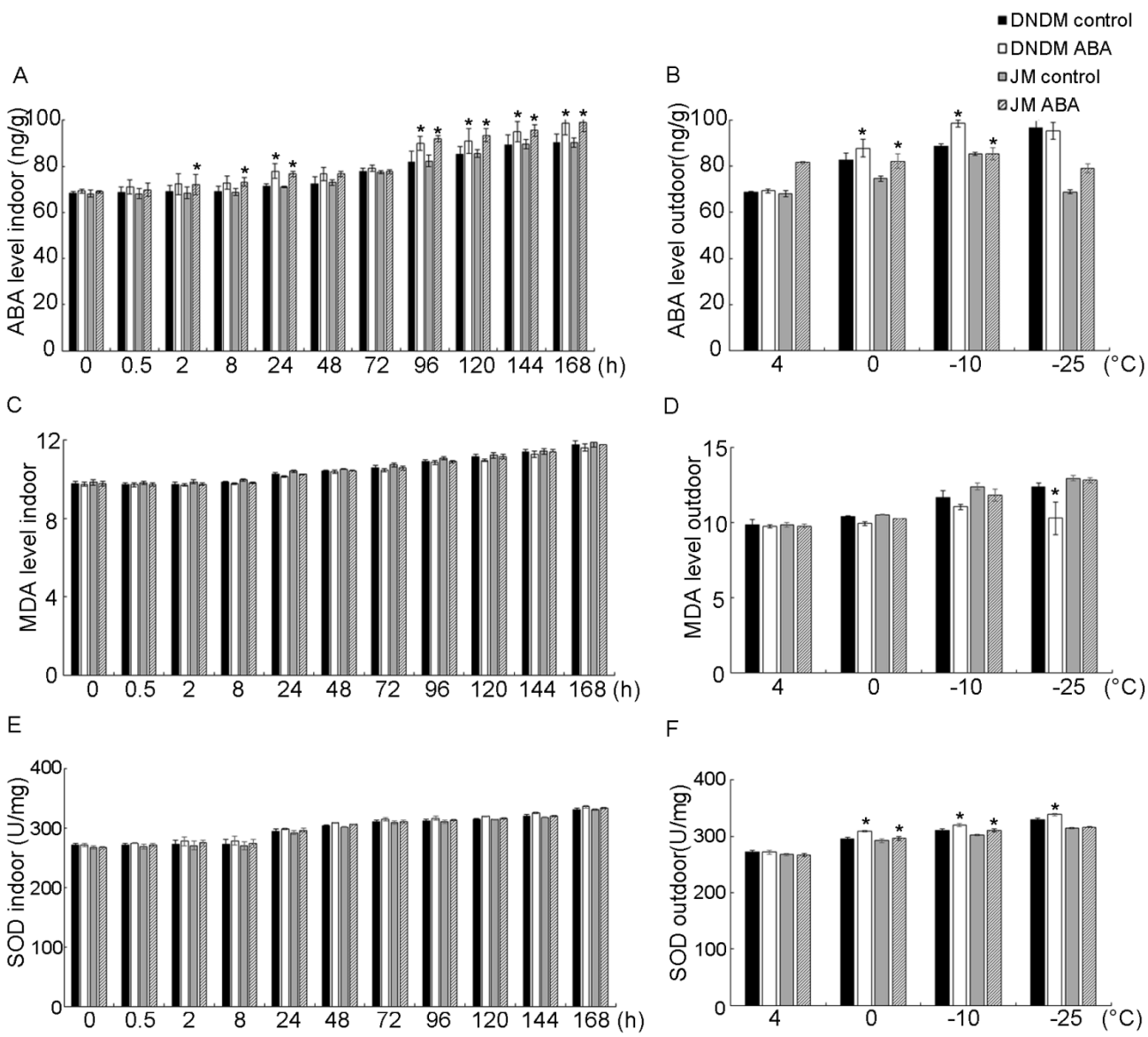

Fig. 3. Levels of ABA, MDA and SOD in DNDM1 and JM22. ABA, abscisic acid; DNDM1, dongnongdongmai 1; JM22, Jimai 22; MDA, Malondiadehyde.

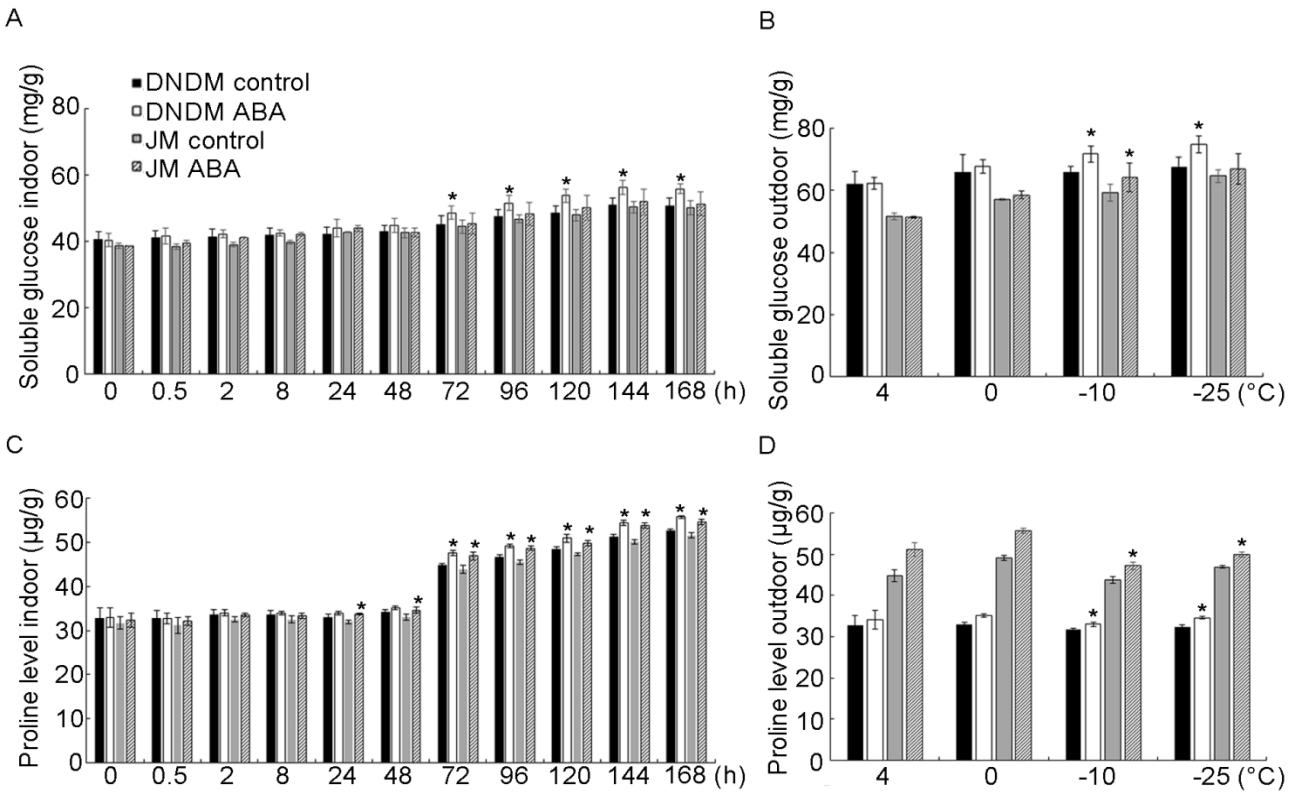

Fig. 4. Levels of soluble glucose and proline in DNDM1 and JM22. ABA, abscisic acid; DNDM1, dongnongdongmai 1; JM22, Jimai 22.

Discussion

Previous studies have reported that ABA plays an important role in the adaptation of the plant under LT stress via regulating gene expression (Knight & Knight, 2012; Theocharis *et al.*, 2012). In the current study, two wheat breeds DNDM1 and JM22 were examined, and several genes expression levels in both ABA-dependent and independent pathways and metabolism indices were determined. Based on these analyses, we hope to identify relationships between gene expression levels and temperature variations which may be helpful to improve cold adaptations of wheats.

Cold-regulated (*Cor*) gene is a series of cold-induced genes involved in the cold adaptation and can be regulated by many transcription factors, including C-repeated binding factors/dehydration responsive elements-binding proteins (CEF/DREB), myeloblastosis/ myelocytomatosis (MYB/MYC) and bZIP (basic leucine zipper) (Kume *et al.*, 2005; Agarwal & Jha, 2010; Hu *et al.*, 2010). *Wrab17* is one of ABA dependent cor genes. Sun *et al.*, have reported that the expression of *Wrab17* in winter wheat var. 97003 and 97014 was always upregulated under LT stress (Sun *et al.*, 2009). Talanova *et al.*, have demonstrated that expression of *Wrab17* presents an ABA-dependent manner in both winter and spring wheat (Talanova *et al.*, 2011). However, in this study, the expression of *Wrab17* was only significantly increased at 4 and 0°C after treating with ABA in DNDM1 as compared with the control groups. There were no significant correlations between ABA treated time and expression of *Wrab17* at 4°C in both DNDM1 and JM22. These evidences might indicate that the expression of *Wrab17* showed a significant correlation with the decreased ABA treatment temperature before 0°C, but not changed with the increased ABA treated time at 4°C in DNDM1.

CBF1VD-D22 is a newly identified ABA-dependent transcriptional factors and belongs to C-repeated binding factor (CBF) family (Badawi *et al.*, 2007). In this study, the expression of *CBF1VD-D22* was significantly increased in DNDM1 after treating with ABA at 4, 0 and -10°C. Meanwhile, the expression of *MYB80* and *Wabi5* were also significantly increased after treating with ABA at 4, 0 and -10°C. Talanova *et al.*, also had pointed out that the expression of *MYB80* and *Wabi5* were increased rapidly in the cold adaptation, and coincided with the cold hardiness (Talanova *et al.*, 2009). All of these evidences suggested that the expression of *CBF1VD-D22*, *MYB80* and *Wabi5* might be induced by cold stress to improve the expression of *cor* genes, such as *Wrab17* in DNDM1.

Wcs120 is an important ABA-independet cor gene investigated in this study. In the current study, *Wcs120* had a significant upregulations as compared with the control group and performed an obvious increased expression trends at 4, 0 and -10°C after treating with ABA in DNDM1 but not in JM22. Consistent with our identification, Kosova *et al.*, have demonstrated that Wcs120 protein is significantly accumulated in frost-tolerant wheat (Kosová *et al.*, 2012). It was also reported that *Wcs120* played an important role in the cold adaptation, and the expression level of *Wcs120* was markedly consistent with the decreased cultured temperature (Ganeshan *et al.*, 2008). All of these identifications indicated that *Wcs120* might played a crucial role in the cold adaptation in DNDM1. *CBF II-5.2*

is an important member of CBF in cold adaptation (Campoli *et al.*, 2009), but its biofunction is not well elucidated till now. In the current study, although the expression of *CBF II-5.2* were significantly increased in both the control group and ABA treated group at 4, 0 and -10°C and the expression in the ABA group was significantly higher than the control group, there were no significant increased trends in both the control and ABA treated groups. This might indicate that the *CBF II-5.2* performed an ABA-independent manner in the cold adaptation in DNDM1.

Alterations in metabolism are the important reflects of gene expression changes. Therefore, several metabolic indices were measured in this study. LT may result in an accumulation of ROS, thus, ROS scavenging enzyme SOD also plays a key role in the adaptation of plants (An *et al.*, 2012). After treating with the ABA, the SOD level in DNDM was significantly increased, and level in ABA treated group was markedly higher than in the control group. This might indicate that ABA treatment might promote the cold adaptation via promoting the removal of ROS. Previous study also demonstrated that accumulation of SOD promoted the survival of DNDM below 0°C (Liu *et al.*, 2013). Soluble glucose was also accumulated in DNDM after treating with ABA as compared with the control group. Glucose is not only the main energy source for wheat growth, but also is a source of acid synthesis (Yun *et al.*, 2012). With the accumulation in soluble glucose, wheat would obtain more adaptive and survival abilities under LT stress.

Conclusion

In conclusion, several *cor* genes and TF were measured in this study to investigate the ABA-dependent and independent pathway involved in cold adaptation. In the ABA-dependent pathway, *Wrab17*, *Wabi5*, *WYB80*, and *CBF1VD-D22* showed a significant upregulations after treating with ABA at 4, 0 and -10°C in DNDM1. Meanwhile, *Wcs120*, which was a *cor* gene involved in the ABA-independent pathway, also presented significant upregulation at 4, 0 and -10°C, but no obvious changed trends were obtained. Besides, the SOD and soluble glucose were also upregulated in DNDM after treated ABA at 4, 0 and -10°C in DNDM1. However, due to the large time lag outdoor, the variations of gene expression levels are not well documented, especially in the freezing temperature. Therefore, further investigations are required in the future to confirm our findings.

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