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Effects of molybdenum on expression of cold-responsive genes in abscisic acid (ABA)-dependent and ABA-independent pathways in winter wheat under low-temperature stress

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• *Background and Aims* Molybdenum (Mo) is an essential trace element for higher plants. It has been shown that application of Mo enhances the cold resistance of winter wheat. In order to improve our understanding of the molecular mechanisms of cold resistance arising from application of Mo in winter wheat, investigations were made regarding the transcription of cold-responsive (COR) genes in abscisic acid (ABA)-dependent and ABA-independent pathways in winter wheat regulated by Mo application under low-temperature stress.

• *Methods* Two cultivars of winter wheat (*Triticum aestivum*), Mo-efficient cultivar '97003' and Mo-inefficient cultivar '97014', were grown in control (−Mo) and Mo fertilizer (+Mo) treatments for 40 d at 15/12 °C (day/night), and the temperature was then reduced to 5/2 °C (day/night) to create low-temperature stress. Aldehyde oxidase (AO) activities, ABA contents, the transcripts of basic leucine zipper (bZIP)-type transcription factor (TF) genes, ABA-dependent COR genes, *CBF/DREB* transcription factor genes and ABA-independent COR genes were investigated at 0, 3, 6 and 48 h post cold stress.

• *Key Results* Mo application significantly increased AO activity, ABA levels, and expression of bZIP-type TF genes (*Wlip19* and *Wabi5*) and ABA-dependent COR genes (*Wrab15*, *Wrab17*, *Wrab18* and *Wrab19*). Mo application increased expression levels of *CBF/DREB* transcription factor genes (*TaCBF* and *Wcbf2-1*) and ABA-independent COR genes (*Wcs120*, *Wcs19*, *Wcor14* and *Wcor15*) after 3 and 6 h exposure to low temperature.

• *Conclusions* Mo might regulate the expression of ABA-dependent COR genes through the pathway: Mo → AO → ABA → bZIP → ABA-dependent COR genes in winter wheat. The response of the ABA-dependent pathway to Mo was prior to that of the ABA-independent pathway. Similarities and differences between the Mo-efficient and Mo-inefficient wheat cultivars in response to Mo under cold stress are discussed.

Key words: Molybdenum, cold resistance, cold responsive gene, low-temperature stress, ABA-dependent pathway, ABA-independent pathway, aldehyde oxidase.

INTRODUCTION

Molybdenum (Mo) is an essential element for higher plants and plays a vital role in many physiological and biochemical processes. More than 40 Mo-enzymes catalysing diverse redox reactions have been found in all organisms. However, only four of these enzymes have been found in plants (Schwarz and Mendel, 2006), namely nitrate reductase (NR), aldehyde oxidase (AO), xanthine dehydrogenase (XDH) and sulfite oxidase (SO; a list of abbreviations is given in Table 1). These Mo-enzymes participate in diverse metabolic processes, such as nitrate assimilation, phytohormone synthesis, purine catabolism and sulfite detoxification in plants (Mendel and Hansch, 2002). Among them, AO has been shown to catalyse the final steps in the conversion of indole-3-acetaldehyde to indole-3-abscisic acid (IAA), and the oxidation of abscisic aldehyde to abscisic acid (ABA; Kaiser *et al.*, 2005). Mutations in either the AO apoprotein or enzymes involved in Mo-cofactor (Moco) biosynthesis and Moco activation (sulfuration) disrupt ABA synthesis (Sagi *et al.*, 2002; Schwarz, 2005). A low ABA level results in a wilty appearance on plants as a result of excessive transpiration, loss of stomatal control, altered seed dormancy and impaired defence responses to environmental stress (Mendel and Hansch, 2002; Kaiser *et al.*, 2005).

Wheat has been regarded as being insensitive crop to Mo deficiency, and thus few studies were reported until 1954 (Mulder, 1954). It was reported that approximately 446 million hectares of arable land was Mo-deficient in China. Mo deficiency in soil is becoming a limiting factor for crop production in many provinces of China, such as Shanxi, Shandong, Jiangxi, Jiangsu and Sichuan (Hu *et al.*, 2002). Wang *et al.* (1990) found that Mo deficiency was a major factor causing leaf-yellowing and tiller death on wheat in winter, and low-temperature stress accelerated the development of Mo-deficiency symptoms. Further studies showed that application of Mo improved the cold-resistance of winter wheat (Wang *et al.*, 1995). Vankova-Radeva *et al.* (1997) and Li *et al.* (2001) also found that Mo application resulted in the increase of frost tolerance of winter wheat grown in acidic soil. Further research showed that Mo application affected the lipid composition of winter wheat leaves (Yaneva *et al.*, 1995) and increased the activities of Mo-containing enzymes (Yaneva *et al.*, 1996; Vunkova-Radeva *et al.*, 2003) and antioxidative enzymes (Sun *et al.*, 2006*c*). Moreover, nitrogen-containing

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TABLE 1. *List of main abbreviations used in the text*

COR	Cold-responsive or cold-regulated
NR	Nitrate reductase
AO	Aldehyde oxidase
XDH	Xanthine dehydrogenase
SO	Sulfite oxidase
TF	Transcription factor
CBF	C-repeat binding factor
bZIP	Basic leucine zipper

compounds (Hu *et al.*, 2002), chlorophyll biosynthesis and photosynthetic characteristics (Yu *et al.*, 2006; Sun *et al.*, 2006*a*) were also found to be closely related to Mo application in winter wheat under low-temperature stress. These studies mainly focused on the physiological basis of cold resistance enhanced by Mo application in wheat, but little is known about its molecular mechanism.

Low temperature is one of the most common adverse environmental factors affecting the growth of winter wheat in central China. Various mechanisms have been suggested to account for chilling injury or freezing tolerance in plants (Lee *et al.*, 1999). In the past decade, many reports have investigated the molecular mechanisms of low-temperature signal transduction and cold acclimation. A number of cold-responsive (COR) genes have been identified and characterized from both dicotyledonous and monocotyledonous plants (Sharma *et al.*, 2005). Genetic analysis indicated that COR gene expression is mediated by both ABA-dependent and ABA-independent pathways (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Sharma *et al.*, 2005). In the ABA-dependent pathway, endogenous ABA might activate basic leucine zipper (bZIP) transcription factors, and then regulate ABA-dependent COR genes through ABA-responsive elements (ABREs; Uno *et al.*, 2000; Xiong *et al.*, 2002), whereas in the ABA-independent pathway, low temperature triggers the expression of the CBF (C-repeat binding factor) family of transcription factors (TFs), which in turn activate downstream COR genes that confer or enhance freezing tolerance in plants (Thomashow, 1999). The ABA-dependent COR genes in wheat include *Wrab15* (Kobayashi *et al.*, 2004), *Wrab17* (Tsuda *et al.*, 2000; Kobayashi *et al.*, 2008*c*), *Wrab18* (Kobayashi *et al.*, 2004) and *Wrab19* (Tsuda *et al.*, 2000; Egawa *et al.*, 2006), whereas the ABA-independent COR genes include *Wcs19* (Fowler *et al.*, 2001), *Wcor14* (Tsvetanov *et al.*, 2000) and *Wcor15* (Takumi *et al.*, 2003).

Molybdenum regulates ABA biosynthesis via AO, and the phytohormone ABA is involved in mediating expression of COR genes. In order to improve our understanding of the molecular mechanisms of cold resistance enhanced by Mo application in winter wheat, the present study focuses on the effects of Mo application on COR gene transcription in winter wheat, and, in particular, compares the differential responses of ABA-dependent and ABA-independent pathways in COR gene expression to Mo under low-temperature stress.

MATERIALS AND METHODS

Plant preparation and sample collection

Two cultivars of winter wheat (*Triticum aestivum*), Mo-efficient cultivar '97003' and Mo-inefficient cultivar '97014', which differ in Mo uptake and distribution (Yu *et al.*, 2002), were grown in Mo-deficient yellow-brown soil in a controlled-climate growth chamber for 40 d at 15/12 °C (day/night) with a 14-h photoperiod at a light intensity of 400 μmol m^{-2} s^{-1} and 70 % air relative humidity. The properties of the soil were: Tamm reagents-extractable Mo 0·100 mg kg^{-1}, pH 5·02 (H_2O; water/soil = 1 : 1), organic matter 17·8 g kg^{-1}, alkaline hydrolysable nitrogen 80·5 mg kg^{-1}, Olsen-extractable phosphate 6·77 mg kg^{-1} and exchangeable potassium 121·3 mg kg^{-1}. Plants were irrigated daily with distilled water. The following chemicals were added to the soil as fertilizers: $(NH_4)_2SO_4$ 1 g kg^{-1} soil, KH_2PO_4 1 g kg^{-1} soil and KCl 1 g kg^{-1} soil; all chemicals were of analytical grade. The treatments were designed as control (−Mo, without addition of Mo) and Mo fertilization (+Mo, Mo 0·15 mg·kg^{-1} soil using molybdate [$(NH4)_6Mo_7O_{24}.4H_2O$]). The temperature of the growth chamber was reduced to 5/2 °C (day/night) for cold stress after 40 d growth from germination. The first fully expanded leaves from −Mo and +Mo treatments were collected after 0, 3, 6 and 48 h of cold stress, frozen in liquid nitrogen and then stored at −80 °C for further analysis. Four biological replicates were prepared for each treatment.

Tissue extraction and analysis of AO activity

AO activities were assayed according to Sagi *et al.* (1999) with some modification. Frozen leaf samples (1 g) were homogenized with ice-cold extraction medium containing 50 mM Tris-HCl (pH 6·8), 10 % (v/v) glycerin, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM flavin adenine dinucleotide and 3 % (w/v) polyvinylpolypyrrolidone (PVPP). The ratio of tissue to extraction buffer was 1:3 (w/v). The homogenized plant material was centrifuged at 27 000 *g*, 4 °C for 15 min. Ammonium sulfate was added to the supernatant to 60 % saturation. The resulting mixture was stirred for 30 min and then centrifuged at 15 000 *g* for 15 min. The precipitate was dissolved in a small volume of 50 mM K-phosphate buffer (pH 7·8) and desalted on Sephadex G-25 (Pharmacia) columns equilibrated with the same buffer. AO activity was assayed by monitoring the decrease of absorbance at 600 nm in a UV 2100 spectrophotometer (Shimadzu, Kyoto, Japan) using 2,6-dichloroindorphenol (DCIP) as an electron donor. The reaction mixture (3 mL) contained 100 μL of the enzyme extract, 200 mM Tris-HCl buffer (pH 7·4), 0·02 % DCIP, 1 mM phenazine methosulphate and 2 mM indole-3-aldehyde. AO activity was expressed as $nmol^{-1}$ DCIP mg^{-1} protein min^{-1}. Soluble proteins in the assays were measured (Bradford, 1976) using crystalline bovine serum albumin as a reference.

Total RNA extraction

Frozen leaves (200 mg) were ground in liquid nitrogen with a mortar and pestle. Total RNA was isolated from leaves by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Extracted total RNA was quantified with a UV 2100 spectrophotometer (Shimadzu). RNA quality was assessed by running 2 μg of the total RNA on a 1·2 % agarose gel. The total RNA was stored at −80 °C.

TABLE 2. *Sequences of primers used for real-time PCR amplification*

Gene	Primer type	Primer sequence 5′ to 3′
Wabi5	Forward	AACCAATGCCGTACTCGTTC
	Reverse	TCTGCCTGTTTCCTCACCA
Wlip19	Forward	GACCGAGCTGACCAAGGTG
	Reverse	TTGGCTCAGAACTGGAACG
Wrab17	Forward	GAAAAGCGAGGCTGTCACGA
	Reverse	CTGTAGCGGCACCCACCATA
Wrab19	Forward	CCGCACCGAGGAGAAGACC
	Reverse	ACCCATGCCCAGCGTGTT
Wrab18	Forward	AGGCCCGCACTGAGGAGAA
	Reverse	GGCGGTGTTGGTGTTGTCG
Wrab15	Forward	CTGCTGTATCCTCTGTATGCGTC
	Reverse	CTTCCTGAGCTGCTCCCTGA
Wcbf2-1	Forward	GGGCGGACCAAGTTTAAGGA
	Reverse	TCTCGGCGGTGGTGAAGGT
TaCBF	Forward	GGACCAAGTTCAGGGAGACGC
	Reverse	GTCCATGCCGCCAAACCA
Wcor14	Forward	GGCTTCTTCTTCCGTGCTG
	Reverse	CCCCTTCCGAGACCTTGTC
Wcs19	Forward	CGAGTTGAAGAAGGGCGTG
	Reverse	GGCGACTTTGTCCGTGATG
Wcs120	Forward	GCCACGGAGATCACCAGC
	Reverse	GTGTCCCAGTGCCAGTCG
Wcor15	Forward	ACGACGCTGCGGATGCTAC
	Reverse	CCTTGTCCGTGATGCCCTGT
actin	Forward	ACTGGGATGACATGGGGAA
	Reverse	ACCGCTGGCATACAAGGAC

cDNA synthesis and real-time PCR

Total RNA samples were treated with DNase and then reverse transcribed with a First-Strand cDNA synthesis Kit (Shinegene, Shanghai, China). The cDNA was used as a template for the real-time PCR reaction with an FTC2000 fluorescent quantitative PCR detection system (Funglyn, Toronto, Canada) using SYBR green for detection of the product at the end of each amplification cycle (Karsai *et al.*, 2002). Real time-PCR was carried out according to Van Riet *et al.* (2006) using a one-step RT-PCR kit (Shinegene). The thermal profile was as follows: 4 min denaturation at 94 °C, and then 35 cycles of 20 s at 94 °C, 25 s at 60 °C and 30 s at 72 °C. The gene-specific forward and reverse primers and a 1 : 3 dilution of the cDNA were added to the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Primers for the genes of interest were designed by Primer 5 with wheat gene sequences from GenBank. The wheat *actin* gene was used as a reference for all genes of interest. Primers designed for the genes of interest and reference genes are detailed in Table 2. A dissociation curve was also set up at the end of the 35 cycles in order to ensure that only one product was amplified for each gene. Real-time PCR experiments were conducted on the three biological replicates, with two technical replicates for each sample.

ABA determination

ABA content was measured using high-performance liquid chromatography (HPLC) as follows. The methods for extraction and purification of ABA were as described by Nayyar *et al.* (2005) with some modifications. Leaf samples [2·0 ± 0·01 g fresh weight (f. wt)] were homogenized in ice-cold extraction medium (80 % methanol, 10 mg L^{-1} butylated hydroxytoluene) at a 1 : 10 ratio of sample (g f. wt) to extraction medium (mL). The homogenate was then further extracted in the dark at 4 °C for 15 h. After centrifugation at 5000 *g* for 10 min, the supernatant was removed, the pellet was re-extracted twice more and the supernatants were pooled, dried in vacuum and dissolved in 8·0 mL 0·1 mol L^{-1} ammonium acetate (pH 9·0). After thawing, the extract was centrifuged at 27 000 *g* for 20 min. For purification, the supernatant from the centrifugation step was applied to a preconditioned column combination of PVPP (Sigma, St Louis, MO, USA), DEAE-Sephadex G-25 (Whatman, Maidstone, UK) and ChromosepC18 column (C18 Sep-Pak cartridge, Waters, Milford, MA, USA). Finally, the hormone fraction was collected in 50 % methanol for HPLC (Agilent Technologies, Waldborn, Germany) analysis (Wang *et al.*, 2008).

Determination of plant Mo concentration

Plant samples were ground, carbonized and ashed. Mo was determined by using polarographic catalytic wave analysis with a JP-2 oscilloscope polarograph according to Wan *et al.* (1988).

Determination of freezing tolerance

The freezing tolerance of leaves was estimated using the electrolyte leakage method as described by Gray *et al.* (1997) and Ndong *et al.* (2002) with slight modifications. Leaves from −Mo and +Mo treatments were thoroughly washed with deionized water and cut into 1-cm sections. Leaf segments were wrapped in moist cheesecloth. Each sample was first covered with an ice chip and equilibrated at –1 °C for 1 h to initiate freezing, then placed in a series of controlled-freezing baths. Temperature in the baths was adjusted by use of freezing-alcohol. The temperature inside the bath boxes was, respectively, –3, –6, –9, –12, –15, –18 and –21 °C. At various temperatures, samples were kept for 2 h, and slowly thawed before adding 10 mL of ice-cold deionized water. Samples were then vacuum-infiltrated, shaken and warmed to room temperature, and the conductivity of the leachate was measured with a conductivity meter. Percentage ion leakage was calculated as the ratio of the conductivity before and after boiling. Percentage ion leakage was plotted versus temperature, and a classic logistic function was fitted to the data using the SPSS v13·0 software non-linear regression package (SPSS, Chicago, IL, USA). Freezing tolerance (LT_{50}) was expressed as the temperature which caused 50 % ion leakage.

Statistical analysis

Data are presented as the averages of three or four replicates. Results were analysed by GLM with Duncan multiple comparison using SAS v6·12 (SAS Institute, Cary, NC, USA). All statistically significant differences were tested at $P < 0·05$.

RESULTS

Mo concentrations in leaves of winter wheat

In wheat leaves of the +Mo treatment, Mo concentrations were significantly higher than those in the −Mo treatment

TABLE 3. *Mo contents in leaves of Mo-deficient (−Mo) and Mo-fertilized (+Mo) winter wheat during low temperature stress* ($\mu g\ g^{-1}$)

		Period of low-temperature stress (h)			
Cultivar	Treatment	0	3	6	48
'97003'	−Mo	$0{\cdot}035 \pm 0{\cdot}02^{b}$	$0{\cdot}033 \pm 0{\cdot}02^{b}$	$0{\cdot}037 \pm 0{\cdot}03^{b}$	$0{\cdot}035 \pm 0{\cdot}01^{b}$
	+Mo	$0{\cdot}049 \pm 0{\cdot}04^{a}$	$0{\cdot}053 \pm 0{\cdot}03^{a}$	$0{\cdot}052 \pm 0{\cdot}04^{a}$	$0{\cdot}048 \pm 0{\cdot}03^{a}$
'97014'	−Mo	$0{\cdot}025 \pm 0{\cdot}02^{c}$	$0{\cdot}027 \pm 0{\cdot}03^{c}$	$0{\cdot}024 \pm 0{\cdot}03^{c}$	$0{\cdot}028 \pm 0{\cdot}04^{c}$
	+Mo	$0{\cdot}047 \pm 0{\cdot}05^{a}$	$0{\cdot}049 \pm 0{\cdot}02^{a}$	$0{\cdot}051 \pm 0{\cdot}05^{a}$	$0{\cdot}050 \pm 0{\cdot}02^{a}$

−Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Different letters in a column indicate significant differences at $P < 0{\cdot}01$ as determined by ANOVA followed by Duncan's test.

TABLE 4. *LT_{50} (°C) in leaves of Mo-efficient and Mo-inefficient winter wheat cultivars under low-temperature stress*

		Period of low-temperature stress (h)			
Cultivar	Treatment	0	3	6	48
'97003'	−Mo	$-5{\cdot}8 \pm 0{\cdot}1^{b}$	$-6{\cdot}4 \pm 0{\cdot}3^{b}$	$-7{\cdot}5 \pm 0{\cdot}2^{b}$	$-8{\cdot}2 \pm 0{\cdot}3^{b}$
	+Mo	$-6{\cdot}5 \pm 0{\cdot}3^{c}$	$-7{\cdot}6 \pm 0{\cdot}2^{c}$	$-8{\cdot}7 \pm 0{\cdot}3^{c}$	$-9{\cdot}5 \pm 0{\cdot}4^{c}$
'97014'	−Mo	$-5{\cdot}0 \pm 0{\cdot}1^{a}$	$-5{\cdot}4 \pm 0{\cdot}1^{a}$	$-6{\cdot}5 \pm 0.\ 2^{a}$	$-7{\cdot}2 \pm 0{\cdot}2^{a}$
	+Mo	$-6{\cdot}8 \pm 0{\cdot}2^{c}$	$-7{\cdot}5 \pm 0{\cdot}3^{c}$	$-9{\cdot}1 \pm 0{\cdot}7^{c}$	$-9{\cdot}9 \pm 0{\cdot}3^{c}$

−Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Different letters in a column indicate significant differences at $P < 0{\cdot}01$ as determined by ANOVA followed by Duncan's test.

across all the time courses of low-temperature stress in two cultivars (Table 3). In the −Mo treatment, Mo concentrations in the Mo-efficient cultivar '97003' were significantly higher than those in the Mo-inefficient cultivar '97014' (Table 3), showing the greater ability of cultivar '97003' to take up Mo from Mo-deficient soil. Mo concentration was not affected by the different time courses of low-temperature stress.

Effects of Mo on freezing tolerance in winter wheat

Freezing tolerance was determined by measuring the lethal temperature of 50 % of the leaf tissues (LT_{50}, also called index of freezing injury) by ion leakage (Flint *et al.*, 1967). LT_{50} values in +Mo treatments were lower than those in −Mo treatments at all times in the two cultivars (Table 4), suggesting that Mo application increased the freezing tolerance of winter wheat. In −Mo treatments, the LT_{50} values in cultivar '97014' were all higher than those in cultivar '97003' (Table 4), indicating a greater decrease of the freezing tolerance of cultivar '97014' under Mo-deficient conditions.

Effects of Mo on AO activities

Application of Mo resulted in a substantial increase of AO activity in the two cultivars (Fig. 1). With sufficient Mo supply, AO activity significantly increased at 3 h post low-temperature stress, and then decreased sharply until 6 h post stress. Under Mo-deficiency conditions, however, AO activity decreased continuously (Fig. 1). Compared with those in control plants, AO activities in the leaves of Mo-treated plants in cultivar '97003' increased by about 32, 49, 49 and 74 % after 0, 3, 6 and 48 h of low-temperature stress, respectively; the increases seen in cultivar '97014' were even higher at 88, 173, 140 and 232 %, respectively. The results suggested that the AO activity of the Mo-inefficient cultivar was more dependent on Mo supply than that of the Mo-efficient cultivar (Fig. 1).

Effects of Mo on ABA levels

Mo application also significantly increased the ABA content in the leaves of the two winter wheat cultivars after low-temperature stress (Fig. 2). ABA concentrations in leaves of wheat increased rapidly and reached a maximum after 3 h of exposure to chilling, and then decreased slightly with the continuation of low-temperature stress. As compared with controls, +Mo treatment induced a clear increase of ABA content in the leaves of cultivars '97003' and '97014', with rates of increase being several times higher in the latter (Fig. 2A, B), which also indicated that application of Mo fertilizer caused cultivar '97014' to produce more endogenous ABA in its leaf tissue than cultivar '97003'.

Effects of Mo on the expression of the bZIP genes

Both *Wlip19* and *Wabi5* are bZIP-type TF genes (Kobayashi *et al.*, 2006; Ishibashi *et al.*, 2007). Mo application significantly increased the mRNA levels of *Wabi5* (Fig. 3A, B) and *Wlip9* (Fig. 3C, D) in the leaves of both wheat cultivars after 0, 3, 6 and 48 h of low-temperature stress. The expression of *Wabi5* and *Wlip9* reached a maximum after 3 h exposure to low temperature, after which a further temperature decrease resulted in a reduction in expression of these genes. There was a clear difference in gene expression between cultivar '97003' and '97014'. This difference was similar to the results seen for AO activity and ABA concentration; the

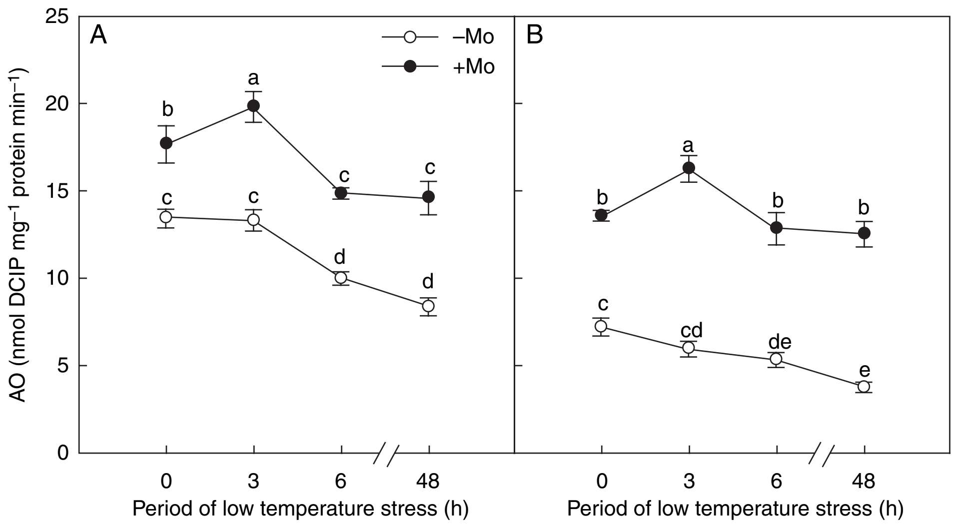

FIG. 1. Effects of molybdenum on aldehyde oxidase (AO) activities in Mo-efficient winter wheat cultivar '97003' (A) and Mo-inefficient winter wheat cultivar '97014' (B) under low-temperature stress. −Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Error bars represent s.e. from $n = 4$ experiments. Different letters indicate significant differences at $P < 0·05$ as determined by ANOVA followed by Duncan's test.

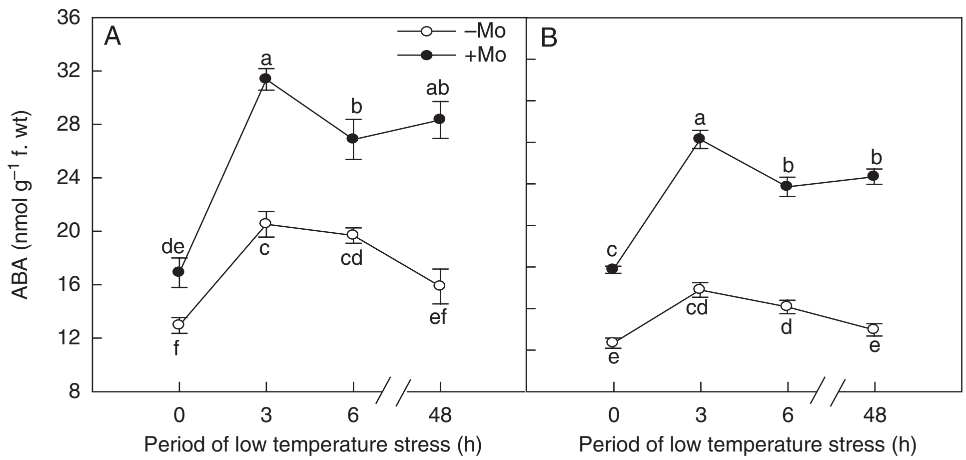

FIG. 2. Effects of molybdenum on ABA contents in Mo-efficient winter wheat cultivar '97003' (A) and Mo-inefficient winter wheat cultivar '97014' (B) under low-temperature stress. −Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Error bars represent s.e. from $n = 4$ experiments. Different letters indicate significant differences at $P < 0·05$ as determined by ANOVA followed by Duncan's test.

average rates of increase of the transcripts of *Wlip19* and *Wabi5* (at 0, 3, 6, 48 h of low-temperature stress) in +Mo treatment in cultivar '97003' were 50 and 224 %, and those in cultivar '97014' were 84 and 464 %, respectively (Fig. 3). The results also indicated that Mo fertilizer induced a greater increase of *Wlip19* and *Wabi5* transcripts in cultivar '97014' than that in cultivar '97003'.

Effects of Mo on expression of ABA-dependent COR genes

Wrab15, *Wrab17*, *Wrab18* and *Wrab19* belong to the ABA-dependent COR genes. Before low-temperature stress, expression levels of *Wrab17* (Fig. 4C, D), *Wrab18* (Fig. 4E, F) and *Wrab19* (Fig. 4G, H) in the leaves of Mo-treated plants were significantly higher than those in control plants; the expression level of *Wrab15* in Mo-treated plants was not significantly different from that in control plants (Fig. 4A, B). After low-temperature stress, expression of the four genes (*Wrab15*, *Wrab17*, *Wrab18* and *Wrab19*) in Mo-treated plants was significantly higher than that in control plants. With the prolongation of low-temperature stress, the expression level of ABA-dependent COR genes for both Mo-deficient and Mo-fertilized treatments increased rapidly in the first 3 h of exposure, and then decreased gradually. The average rates of increase of the transcripts of *Wrab15*, *Wrab17*, *Wrab18* and *Wrab19* (at 0, 3, 6, 48 h of low-temperature stress) exhibited a similar tendency as the expression levels of bZIP TFs for each cultivar: 256, 77, 124 and 186 % in cultivar '97003', and 660, 340, 186 and 212 % in cultivar '97014', respectively. The increased rates of ABA-dependent COR gene expression in cultivar '97014' were significantly higher than those in cultivar '97003' with Mo application under low-temperature stress.

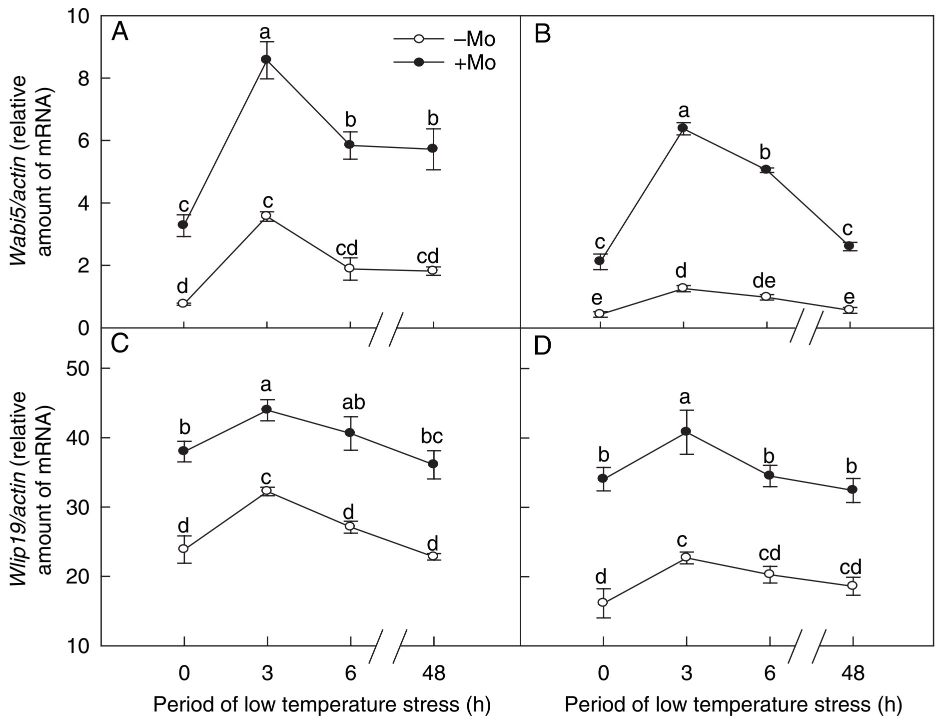

FIG. 3. Effects of molybdenum on expression of the bZIP genes (*Wlip19* and *Wabi5*) in Mo-efficient winter wheat cultivar '97003' (A, C) and Mo-inefficient winter wheat cultivar '97014' (B, D) under low temperature stress. −Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Error bars represent s.e. from $n = 6$ experiments. Different letters indicate significant differences at $P < 0{\cdot}05$ as determined by ANOVA followed by Duncan's test.

Effects of Mo on expression of the CBF/DREB TF genes

TaCBF (Fig. 5A, B) and *Wcbf2-1* (Fig. 5C, D) belong to CBF/DREB TF genes (Kume *et al.*, 2005). There was no significant difference between the −Mo and +Mo treatments in the expression of CBF/DREB TF genes (*TaCBF* and *Wcbf2-1*) before low-temperature stress (0 h). However, 3–48 h of low-temperature stress significantly up-regulated the expression of CBF/DREB TF genes (*TaCBF* and *Wcbf2-1*) in the leaves from the +Mo treatment. With extended exposure to low-temperature stress, the expression levels of CBF/DREB TF genes in both Mo-deficient and Mo-fertilized treatments increased rapidly and reached a maximum at 6 h of low-temperature stress, but then decreased at 48 h (Fig. 5A–D).

Effects of Mo on expression of the ABA-independent COR genes under low-temperature stress

Wcs120 (Ouellet *et al.*, 1998), *Wcs19* (Fowler *et al.*, 2001), *Wcor14* (Tsvetanov *et al.*, 2000) and *Wcor15* (Takumi *et al.*, 2003) belong to the ABA-independent COR family of genes. There was no significant difference between the control and Mo-fertilized treatments in the expression of these genes at 0 and 3 h of exposure to low temperature. However, at 6 and 48 h of low-temperature stress, ABA-independent COR gene expression levels in winter wheat were significantly up-regulated in the Mo-fertilized treatment (Fig. 6A–H). Similarly, with continued low-temperature stress, ABA-independent COR gens expression levels for both Mo-deficient and Mo-fertilized plants reached a maximum at 6 h of exposure and then decreased at 48 h (Fig. 6A–H).

DISCUSSION

Mo might regulate the ABA-dependent pathway of COR gene expression in winter wheat under low-temperature stress

As previously mentioned, AO catalyses the last step of ABA, and IAA synthesis has been verified in many plants such as *Arabidopsis thaliana* (Akaba *et al.*, 1999; Seo *et al.*, 2000), maize (Katalin Barabas *et al.*, 2000), tomato (Min *et al.*, 2000) and pea (Zdunek-Zastocka, 2008); however, few data have been reported in wheat. In the present study, Mo application significantly increased AO activities and ABA concentrations of leaves of winter wheat. This indicates that Mo has a close relationship with ABA biosynthesis. ABA plays a critical role in the ABA-dependent signal pathway. Many reports have shown that ABA might activate bZIP transcription factors, and then regulate ABA-dependent COR genes through ABREs in *Arabidopsis* (Uno *et al.*, 2000; Xiong *et al.*, 2002). Recently, a number of bZIP-type genes and ABA-dependent COR genes have been isolated and characterized in wheat and related species (Kobayashi *et al.*, 2004; Ishibashi *et al.*, 2007). ABA regulated ABA-dependent COR genes via expression of bZIP genes, which has also been reported in wheat (Kobayashi *et al.*, 2008*a*, *b*). *Wlip19* and *Wabi5* are bZIP-type TF genes in wheat (Kobayashi *et al.*, 2006). *Wrab15*, *Wrab17*, *Wrab18* and *Wrab19* all belong to the ABA-dependent COR genes of wheat. ABA levels, expression

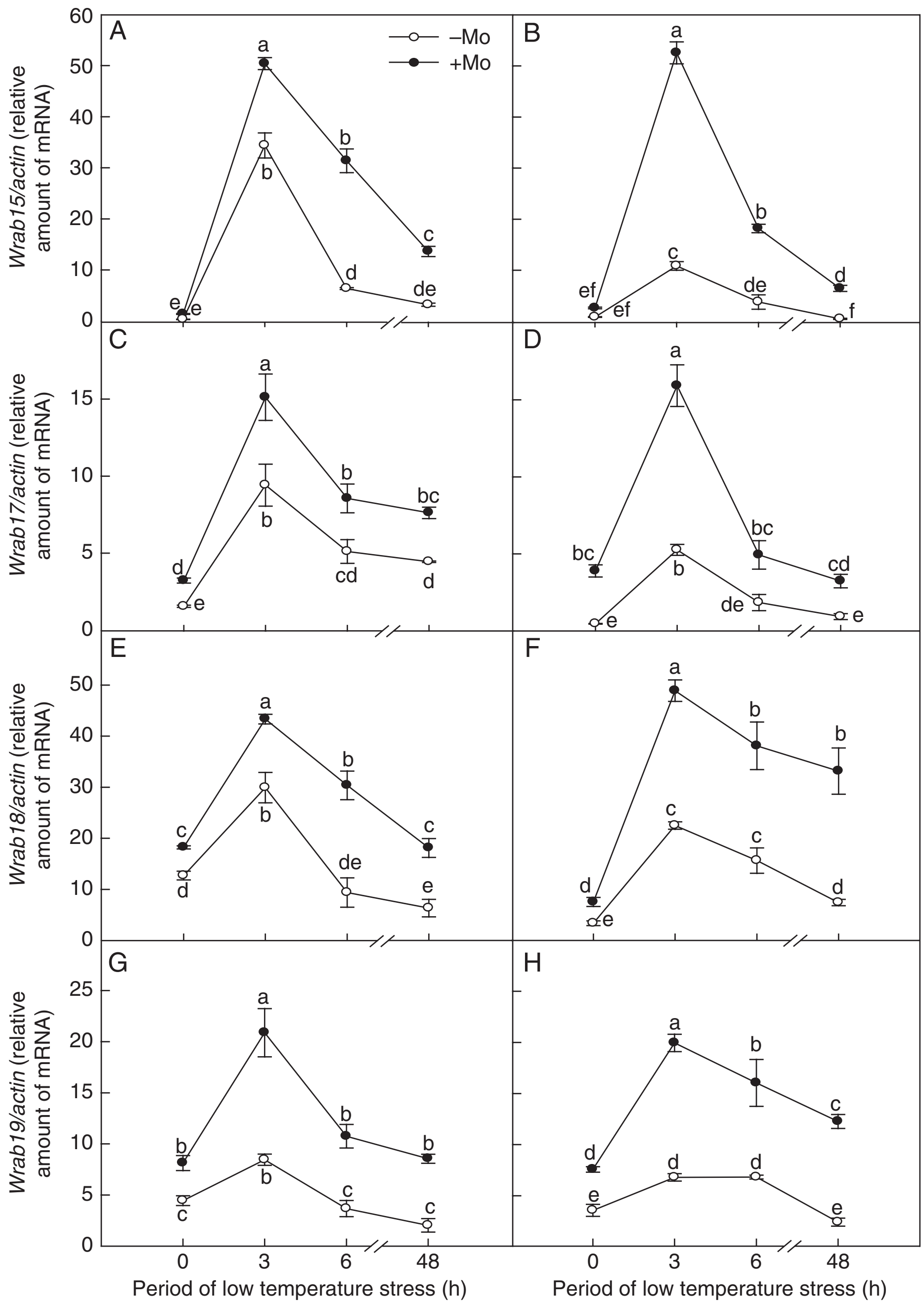

FIG. 4. Effects of molybdenum on expression of the ABA-dependent COR genes (*Wrab15*, *Wrab17*, *Wrab18* and *Wrab19*) in Mo-efficient winter wheat cultivar '97003' (A, C, E, G) and Mo-inefficient winter wheat cultivar '97014' (B, D, F, H) under low-temperature stress. −Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Error bars represent s.e. from $n = 6$ experiments. Different letters indicate significant differences at $P < 0{\cdot}05$ as determined by ANOVA followed by Duncan's test.

levels of bZIP-type TF genes (*Wlip19* and *Wabi5*) and ABA-dependent COR genes (*Wrab15*, *Wrab17*, *Wrab18*, and *Wrab19*) were all significantly increased in Mo-fertilized winter wheat at 0, 3, 6, 48 h of low-temperature stress. The results also showed that AO activity, ABA concentration, expression levels of bZIP TFs and ABA-dependent COR genes changed in a synchronous manner. They reached a maximum at 3 h of low-temperature stress, and then decreased slightly both in Mo-deficient and in Mo-fertilized winter wheat after prolonged exposure to low-temperature stress (Fig. 4). These results suggest that Mo regulates COR gene expression in winter wheat from the ABA-dependent signal pathway: Mo → AO → ABA → bZIP → ABA-dependent COR genes.

The four ABA-dependent COR genes encoded different stress-inducible polypeptides or proteins. *Wrab15* putatively encoded a polypeptide of 130-amino-acid residues, which showed a high level of identity with a stress-inducible protein, HVA22, in barley (Shen *et al.*, 1993, 2001). *Wrab17*

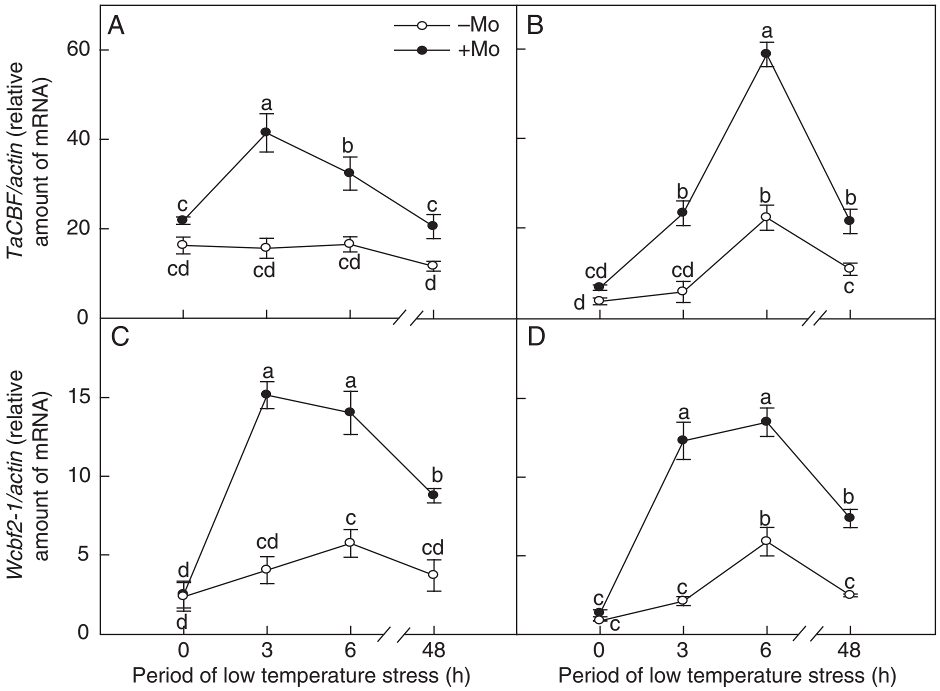

FIG. 5. Effects of molybdenum on the expression of the CBF/DREB transcription factor genes (*TaCBF* and *Wcbf2-1*) in Mo-efficient winter wheat cultivar '97003' (A, C) and Mo-inefficient winter wheat cultivar '97014' (B, D) under low-temperature stress. −Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Error bars represent s.e. from $n = 6$ experiments. Different letters indicate significant differences at $P < 0{\cdot}05$ as determined by ANOVA followed by Duncan's test.

encoded an acidic and hydrophobic protein, which showed high homology (a mean of 84 % identity) with a barley gibberellic acid (GA_3)-inducible protein, ES2A, and several other group-3 LEA/RAB proteins (Tsuda *et al.*, 2000). WRAB18 was hydrophilic and showed 80·4 % amino-acid identity with WRAB19 (Kobayashi *et al.*, 2004). Wrab17, WRAB18 and WRAB19 were homologous to other cereal RAB proteins, which belongs to the group 3 LEA protein family (Dure, 1993). Group 3 LEA proteins generally correlated well with desiccation tolerance in young seedlings (Baker *et al.*, 1988), as well as salt tolerance (Moons *et al.*, 1997) and freezing tolerance (Ndong *et al.*, 2002). Transcripts of *Wrab15*, *Wrab17*, *Wrab18* and *Wrab19* in Mo-fertilized winter wheat increased significantly under low-temperature stress (Fig. 4), which may increase the expression of stress-inducible proteins (e.g. a homologue of HVA22) or group 3 LEA proteins, and then enhance the cold resistance of winter wheat.

Mo affects the ABA-independent pathway of COR gene expression in winter wheat under low-temperature stress

In the ABA-independent pathway, low temperature triggers the expression of the CBF family of TFs, which in turn activate many downstream COR genes that confer or enhance the freezing tolerance of plants (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). *TaCBF* and *Wcbf2-1* belong to the CBF/DREB TF genes in wheat. The deduced polypeptides of the wheat TaCBF showed high degrees of identity to *Arabidopsis* CBF1/DREB1B within AP2/EREBP DNA-binding domains. The amino-acid sequence of WCBF2-1 AP2 domain showed perfect identity with those of TaCBF. *Wcs120* (Ouellet *et al.*, 1998), *Wcs19* (Fowler *et al.*, 2001), *Wcor14* (Tsvetanov *et al.*, 2000) and *Wcor15* (Takumi *et al.*, 2003) are ABA-independent COR genes, which contain the CRT/DRE sequence. Here no significant difference was found between Mo-deficient and Mo-fertilized treatment in the expression of CBF/DREB TF genes (*TaCBF* and *Wcbf2-1*) and ABA-independent COR genes (*Wcs120*, *Wcs19*, *Wcor14* and *Wcor15*) before low-temperature stress. Expression of CBF/DREB TF genes (*TaCBF* and *Wcbf2-1*) in Mo-fertilized winter wheat was significantly up-regulated as compared with the Mo-deficient treatment, after 3 h of low-temperature stress, and the transcripts of ABA-independent COR genes (*Wcs120*, *Wcs19*, *Wcor14* and *Wcor15*) in Mo-fertilized winter wheat was significantly increased after 6 h of low-temperature stress (Figs 5 and 6). This suggests that after low-temperature stress Mo first affects expression of CBF/DREB TF genes (*TaCBF* and *Wcbf2-1*), which in turn activate the expression of ABA-independent COR genes (*Wcs120*, *Wcs19*, *Wcor14* and *Wcor15*).

The wheat gene *Wcs120* was shown to be cold-inducible in both monocotyledonous and dicotyledonous transgenic plants (Ouellet *et al.*, 1998). Wheat possesses a small family of Cor genes including *Wcs19* (Fowler *et al.*, 2001), *Wcor14* (Tsvetanov *et al.*, 2000) and *Wcor15* (Takumi *et al.*, 2003), all of which encode chloroplast-targeted COR proteins analogous to the *Arabidopsis* protein COR15a (Lin and Thomashow, 1992; Thomashow, 1999). Constitutive expression of COR15a enhances the freezing tolerance of chloroplasts and reduces freezing-induced damage to photosystem II in non-acclimated plants (Artus *et al.*, 1996). Recent research showed that the WCOR15 and WCOR14 proteins, which were induced by

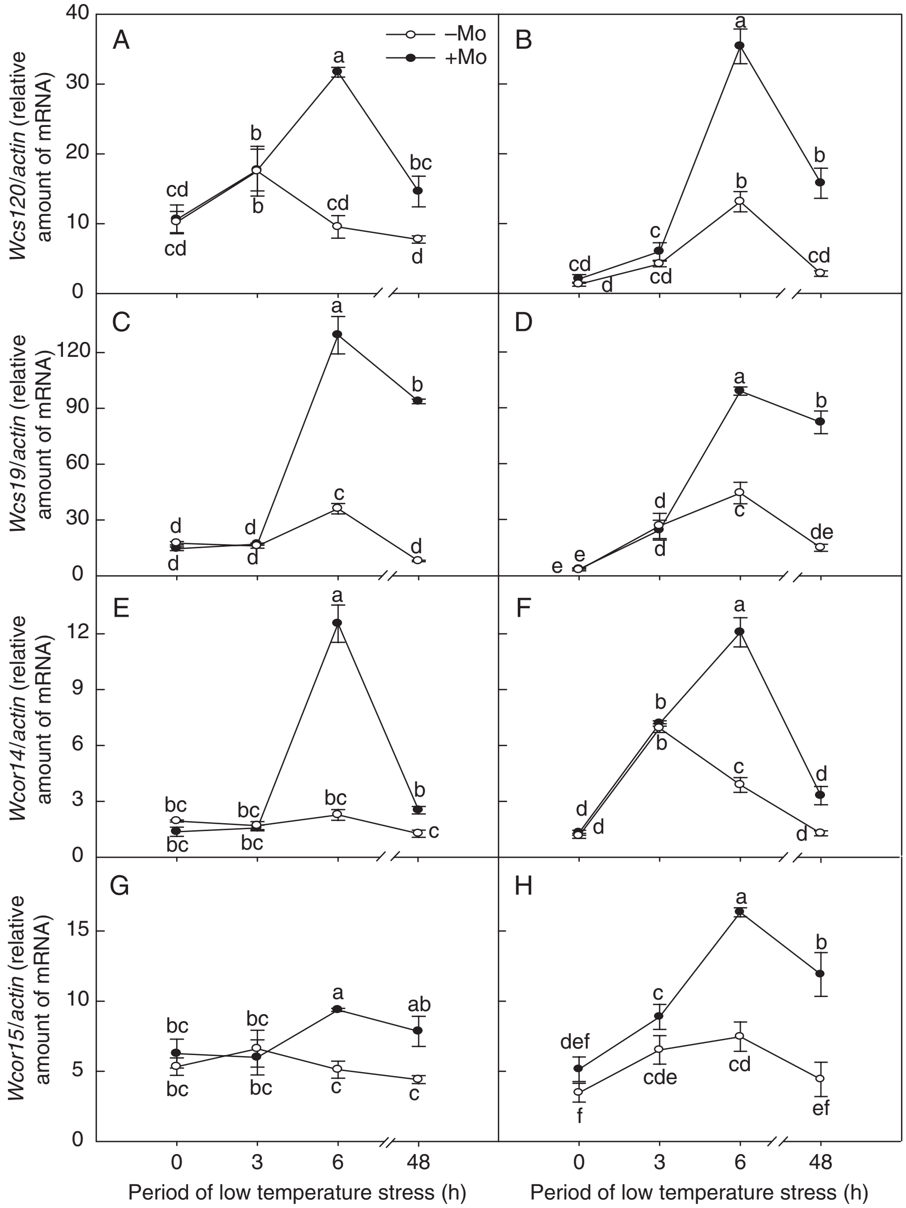

FIG. 6. Effects of molybdenum on the expression of the ABA-independent COR genes (*Wcs120*, *Wcs19*, *Wcor14* and *Wcor15*) in Mo-efficient winter wheat cultivar '97003' (A, C, E, G) and Mo-inefficient winter wheat cultivar '97014' (B, D, F, H) under low-temperature stress. −Mo and +Mo treatments represent plants fertilized with 0 and 0·15 mg Mo [$(NH_4)_6Mo_7O_{24}.4H_2O$] per kg soil, respectively. Error bars represent s.e. from $n = 6$ experiments. Different letters indicate significant differences at $P < 0{\cdot}05$ as determined by ANOVA followed by Duncan's test.

low-temperature stress, were transported into chloroplasts and accumulated in the chloroplast stromal compartments (Shimamura *et al.*, 2006). The deduced proteins of WCS19 possess chloroplast leader peptides that are highly homologous to wheat WCOR14 and WCOR15 (Takumi *et al.*, 2003). It appears that these proteins encoded by ABA-independent COR genes have similar function. It is thus suggested that expression of the wheat COR genes, whose protein products are transported into chloroplasts, are regulated through the same or at least partly overlapped signal transduction pathways (Takumi *et al.*, 2003). The present experiments show that the transcripts of ABA-independent COR genes (*Wcs120*, *Wcs19*, *Wcor14* and *Wcor15*) in Mo-fertilized winter wheat were several times greater than those in −Mo treatment after 6 h of low-temperature stress (Fig. 6). Rapid increases in the expression level of these COR genes and accumulation of the COR proteins in Mo-fertilized winter wheat may help to maintain the activity of the photosynthetic apparatus through regulation of the redox state in chloroplasts (Takumi *et al.*, 2003). Recent research also found that the biosynthesis of chlorophyll was inhibited and the net photosynthetic rate (P_n) decreased in Mo-deficient winter wheat under low-temperature stress (Sun *et al.*, 2006*a*; Yu *et al.*, 2006). These results suggest that Mo enhances freezing tolerance and photosynthesis through regulation of ABA-independent COR gene expression.

Response of the ABA-dependent pathway to Mo was prior to that of the ABA-independent pathway

Prior to low-temperature stress, Mo application increased AO activity, ABA concentration, and expression levels of all the bZIP genes and the majority of the ABA-dependent COR genes. After low-temperature stress, AO activity, ABA concentration, expression levels of all the bZIP genes and the ABA-dependent COR genes in Mo-treated plants were all higher than those in Mo-deficient plants (Figs 1–4). The results indicated that Mo regulated the ABA-dependent pathway and increased expression levels of ABA-dependent COR genes at all times. However, results regarding the ABA-independent pathway were quite different; no significant difference in the expression levels of CBF genes and ABA-independent COR genes was observed between control and Mo-treated plants for each cultivar before low-temperature stress. Mo application increased the expression levels of CBF genes and ABA-independent COR genes until 3 and 6 h of low-temperature stress, respectively (Figs 5 and 6). This suggests that the ABA-dependent pathway in COR gene expression was more sensitive to Mo deficiency as compared with the ABA-independent pathway. This may be due to the fact that Mo can directly regulate ABA biosynthesis through AO, and then trigger the ABA $\rightarrow$bZIP $\rightarrow$ABA-dependent COR gene expression pathway (Milborrow, 2001; Seo and Koshiba, 2002). We can infer that the response of the ABA-dependent pathway to Mo occurs prior to that of the ABA-independent pathway.

Recent genetic evidence suggests that the ABA-independent and ABA-dependent pathways are not completely independent, but instead have extensive interactions in controlling gene expression under abiotic stress. Thus, it is not yet certain whether ABA-independent COR gene expression is completely independent of ABA (Thomashow, 1999; Xiong *et al.*, 2002). Whether Mo affects ABA-independent COR gene expression through interactions of the two pathways needs further investigation.

Similarity and differences of the Mo-efficient and Mo-inefficient wheat cultivars in response to Mo under cold stress

The response of these two wheat cultivars ('97003' and '97014') to Mo was similar under low-temperature stress with a few exceptions. Mo application increased AO activity, ABA content, expression levels of bZIP TFs and ABA-dependent COR genes in both cultivars under low-temperature stress. Mo fertilizer also significantly up-regulated expression levels of CBF/DREB TF genes and ABA-independent COR genes after 3 and 6 h of low-temperature stress, respectively, for both cultivars. The similarity of the responses of these two wheat cultivars verified that the ABA-dependent and ABA-independent pathway of COR gene expression was affected by Mo under low-temperature stress.

On the other hand, the Mo-efficient cultivar '97003' and Mo-inefficient cultivar '97014' displayed differences in the rates of increase of AO activity, ABA content and CBF/bZIP/COR gene expression. Compared with control plants, AO activities in Mo-treated plants in cultivar '97003' increased by about 32, 49, 49 and 74 % after 0, 3, 6 and 48 h of low-temperature stress, respectively; those in cultivar '97014' were even higher at 88, 173, 140 and 232 %, respectively (Fig. 1). Similarly, the rates of increase of ABA content in cultivar '97003' were 31, 53, 36, 79 at 0, 3, 6, 48 h of low-temperature stress, and those in cultivar '97014' were 151, 148, 142 and 248 %, respectively (Fig. 2). There was a clear difference in bZIP gene expression between cultivar '97003' and '97014'. The average rates of increase of the transcripts of *Wlip19* and *Wabi5* (at 0, 3, 6, 48 h of low-temperature stress) in +Mo treatment in cultivar '97003' were 50 and 224 %, and those in cultivar '97014' were 84 and 464 %, respectively (Fig. 3). The average rates of increase of the transcripts of *Wrab15*, *Wrab17*, *Wrab18* and *Wrab19* (at 0, 3, 6 and 48 h of low-temperature stress) exhibited a similar tendency as the expression levels of bZIP genes for each cultivar: 256, 77, 124 and 186 % in cultivar '97003', and 660, 340, 186 and 212 % in cultivar '97014' (Fig. 4). The rates of increase of AO activity, ABA concentration, and the transcripts of bZIP genes and ABA-dependent COR genes in the leaves of Mo-treated plants in cultivar '97014' were higher than those in cultivar '97003'. Differences in these parameters between the −Mo and +Mo treatment in '97014' was greater than those in '97003'. These differences were also verified by phenotypic characteristics and other physiological parameters. After 48 h of low-temperature stress (see Supplementary Data, Fig. S1, available online), Mo-deficiency symptoms such as leaf etiolation were more obvious in cultivar '97014'. The Mo-efficient winter wheat cultivar '97003' had a yield that was 90 % more and the Mo-inefficient winter wheat cultivar '97014' 50 % less under Mo-deficient conditions when compared with the Mo fertilizer treatment (Yu *et al.*, 1999, 2002). Previous studies also showed that net photosynthetic rate (P_n) and activities of antioxidative enzymes (SOD, CAT, POD, APX) in the leaves of Mo-deficient plants in cultivar '97014' decreased more than those in cultivar '97003' under low-temperature stress (Sun *et al.*, 2006*a*, *b*). The difference between cultivar '97003' and cultivar '97014' was also confirmed by LT_{50} values. Differences in LT_{50} between −Mo and +Mo treatment in cultivar '97014' were greater than that in '97003'(Table 4). These results all revealed a differential response to Mo between these two cultivars.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org, illustrating the phenotypic differentiation of the Mo-efficient cultivar '97003' and Mo-inefficient cultivar '97014' to Mo (Fig. S1).

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