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ORIGINAL ARTICLE

Altitude variation in chilling tolerance among natural populations of *Elymus nutans* in the Tibetan Plateau

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Abstract Low temperatures limit plant growth, development, and reproductive success. A series of complex adaptive responses in plants evolved to withstand this environmental challenge. Here, eight accessions of *Elymus nutans*, which originated in Tibet at altitudes between 3720 and 5012 m above sea level, were used to identify heritable adaptations to chilling stress. Dynamic responses of phytohormone, sugar, and gene expression levels related to chilling tolerance were analyzed. During the initial stage of chilling stress (0–24 h), some high-altitude *E. nutans* accessions exhibited rapid increases in abscisic acid (ABA), jasmonic acid (JA), and zeatin content. This coordinated with decreases in the levels of auxin (IAA), salicylic acid (SA), gibberellins (GA), and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). *EnCBF9* and *EnCBF14* expression in the high-altitude accessions, Baqing, Xainza, Damxung, and Ali, increased within 1 h of chilling exposure, while chilling induction of *EnCOR14a* was detected after 3 h of chilling stress. Accessions from high altitudes displayed an increased sucrose and raffinose accumulation and a reduced degradation of chlorophyll under chilling stress. After 24–120 h of chilling exposure, plant adaptation to the chilling treatment was associated with a lower accumulation of ABA and moderate rise of zeatin, IAA, GA, ACC, SA, and JA. *EnCBF9*, *EnCBF14*, and *EnCOR14a* genes were down-regulated during the late stage of chilling stress. Taken together, the dynamic responses of phytohormones and sugars, and the higher expression of the *EnCBFs* and *EnCOR* genes play critical roles in the acclimation to chilling in high-altitude accessions of *E. nutans,* thereby allowing them to achieve higher chilling tolerance.

Keywords Altitude variation · Chilling stress · Phytohormone · Regulatory gene · Sugars

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Introduction

Plants are sessile organisms and hence inevitably undergo adverse environmental interference, such as cold. To overcome these limitations, plants have the ability to adapt to low temperatures by activating a cascade of defense responses through the regulation of gene expression and subsequent to physiochemical modifications that enhance their cold tolerance. Phytohormones are involved in modulating the response to various biotic and abiotic stresses, including cold stress (Jibran et al. 2013; O'Brien and Benková 2013). Abscisic acid (ABA) acts as a stress hormone and has been implicated in plant responses to cold stress (Kosová et al. 2012). The cytokinin receptors *Arabidopsis* histidine kinase 2/3 and type-A *Arabidopsis* response regulators (ARRs) function as negative regulators in cold stress, by inhibiting the ABA-dependent pathway (Jeon et al. 2010). A previous report has also provided evidence that jasmonic acid (JA) exerts an important role in multiple physiological processes, including: development, secondary metabolism, senescence,

and ripening. It has also been involved in the response to various environmental stresses, such as wounding, water deficit, ozone exposure, pest infestation, and pathogen infection (Zhao et al. 2013). The participation of ethylene in a wide range of cellular and developmental processes has been confirmed and it also plays a central role in the response of plants to multiple abiotic and biotic stresses (Shi et al. 2012).

Leaf sugar content typically increases during chilling (Zeng et al. 2011), where sugars act as osmoprotectants to stabilize cellular membranes. Sucrose also functions as an important signaling molecule to regulate the cold acclimation pathway (Palma et al. 2014). Evidence suggests that crosstalk between sugar and phytohormone signalling pathways exists in plants, including interactions between ABA, ethylene, cytokinin, and auxin (Moore et al. 2003; Gibson 2004). A recent study has also reported sucrose interaction with JA in response to chilling stress (Wingler et al. 2015).

The metabolic changes that occur when plants adapt to cold stress are mainly the result of changes in the expression of cold-responsive (COR) genes (Thomashow 1999). Until now, the most well-known cold signaling pathway in plants is the C-repeat-Binding Factor/DRE-Binding Factor (CBF/DREB) transcriptional regulatory cascade (Achard et al. 2008). Many studies indicate that cold stress induces *CBF* expression (Shi et al. 2012; Kidokoro et al. 2015) and triggers the cascade of a large subset of *COR* genes (Vogel et al. 2005; Zhao et al. 2013). These studies also indicate that the overexpression of *CBFs* results in constitutively enhanced tolerance to freezing in Arabidopsis (Gilmour et al. 2000; Zhen and Ungerer 2008). It has been shown that *CBF2* exhibits significant population differentiation between higher and lower altitude regions in *Solanum chilense* (Mboup et al. 2012). However, transcriptional variation of *CBF* genes in the regulation of chilling stress in the perennial alpine *Elymus nutans* Griseb. originating from a wide range of altitudes remains to be identified.

Low temperatures restrict the growth and reproduction of plant inhabiting high-latitude regions, but intraspecific genetic variation in the ability of plants to overcome chilling remains to be determined (Oakley et al. 2014). Natural intra\specific variation can be used to investigate the relationship between traits in plants. A few pieces of evidence point out the correlation between sugar, phytohormones metabolism and chilling stress response in *Arabis alpina* (Wingler et al. 2015), and the molecular characteristics in *Populus balsamifera* (Menon et al. 2015). For alpine plants, however, the contrary reports have been revealed about genetic adaptation along altitudinal gradients. *E. nutans*, an important alpine perennial forage, is distributed in north, northwest, and southwest regions. It is especially distributed in the Qinghai–Tibetan Plateau in China from 3000 to 5000 m. Owing to its high adaptability, good nutrition, high yield, and good resistance to cold, drought, and biotic stress, it is often used for ecological restoration and the construction of cultivated pastures in alpine areas (Dong and Liu 2007; Chen et al. 2009). We exploited the natural variation in *E. nutans* to determine the variations in sugar accumulation, phytohormone content, and *EnCBF* and *EnCOR* gene expression patterns in response to chilling stress. Physiological adaptations of accessions from different altitudes were found, such as transient changes in phytohormone concentrations in accessions from higher altitudes at the early stage of chilling stress and accumulation of more sucrose during the late phase of chilling. Moreover, higher expression of *EnCBF9*, *EnCBF14*, and *EnCOR14a* genes was detected in accessions originating from high altitudes.

Materials and methods

Plant materials

Elymus nutans Griseb. seeds were harvested from different populations in different regions in Tibet, China (Table 1). Seeds were cleaned and stored at 4 °C until the experiments began.

All seeds were surface sterilized in 0.1 % (w/v) sodium hypochlorite and germinated on moistened filter paper for 7 days with 14-h photoperiod at a photon flux density of 350 μmol m^{-2} s^{-1}. Morphologically, uniform seedlings were cultured in silica sand by irrigation in Hoagland nutrient solution once every 5 days. Plants were germinated and grown in a growth chamber at 14-h-light/10-h-dark at 25 °C with a photon flux density of 350 μmol m^{-2} s^{-1}, 70/60 % relative humidity. A light source was provided by fluorescent tubes (Philips Electronics N. V. Holland, Nanjing, China). The 28-day-old seedlings were then shifted to chilling-stress conditions at 4 °C, the rest of the growth conditions was identical. Leaf samples were taken after 0 (control), 1, 3, 6, 12, 24, 48, 72, and 120 h of chilling treatment.

Plants were subjected to chilling stress at 4 °C for up to 120 h to detect changes in the plants during chilling stress, including: phytohormone content, carbohydrate metabolites, and transcription of genes related to chilling stress. Chilling stress responses were classified into two stages: early (changes occurred from 1 to 24 h) and chilling stress acclimation (changes occurred over 24–120 h).

Determination of electrolyte leakage

The electrolyte leakage of eight *E. nutans* accessions was measured according to the method of Song et al. (2006), with

Table 1 Locations of eight wild *E. nutans* accessions used in this study

Tibet	GPS coordinates		Altitude (m.a.s.l.)
Site			
Dazi (DZ)	N: 9°45.417′	E: 91°27.726′	3720
Maizhokunggar (MZG)	N: 29°45.201′	E: 91°56.472′	3991
Nanmulin (NML)	N: 29°49.603′	E: 89°13.688′	4125
Kangmar (KM)	N: 28°49′21.7″	E: 89°59′54.9″	4402
Ali (AL)	N: 32°064′	E: 80°013′	4532
Damxung (DX)	N: 30°28.535′	E: 91°06.246′	4618
Xainza (XZ)	N: 31°06′187″	E: 91°41.960′	4763
Baqing (BQ)	N: 31°915′	E: 94°056′	5012

some modifications. Briefly, 0.5 g of fresh leaves were washed in deionized water and placed in Petri dishes-containing 5 mL deionized water at 25 °C. After 2 h, the electrical conductivity (EC_1) was measured using a conductivity meter (INESA Scientific Instruments Inc. Shanghai, China). The leaf samples were then boiled for 20 min and the conductivity was measured again (EC_2). The electrolyte leakage (%) was calculated as (EC_1/EC_2) × 100.

Measurement of chlorophyll content and maximal photochemical efficiency of PSII

Chlorophyll content was measured in the same position of leaves for each accession. Chlorophyll content was determined spectrophotometrically using 80 % acetone as an extractive solvent (Lichtenthaler 1987). Extract absorbance was measured at 645 and 663 nm with Optizen 5100 UV spectrophotometer (Shanghai, China). Maximal photochemical efficiency of PSII (F_v/F_m) was measured using a PAM-2100 fluorometer (Walz, Effeltrich, Germany) as described by Oliveira and Peñuelas (2004). All measurements were carried out after maintaining leaves in darkness for at least 25 min.

Quantification of phytohormone contents

Leaf samples from eight accessions were frozen in liquid nitrogen and stored at −80 °C prior to phytohormone analysis. The extraction and analysis of the endogenous phytohormone content of these samples were performed using ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem spectrometry (UHPLC/ESI–MS/MS) as described by Müller and Munné-Bosch (2011). The phytohormone contents of these samples were expressed on a leaf fresh weight basis.

Assay of soluble sugar contents

Approximately 1 g of leaves was frozen in liquid nitrogen for determination of sugar contents. Contents of glucose, fructose, sucrose, and raffinose were assayed using high-performance liquid chromatography (Waters Corporation, America) according to the methods of Liu et al. (2004).

RT-qPCR analysis

Total RNA was extracted from leaves of 28-d-old plants treated at 4 °C for 0, 1, 3, 6, 12, 24, 48, 72, and 120 h using RNAiso Reagent (TaKaRa, Dalian, China). Gel electrophoresis was performed and absorbance measured at 260 and 280 nm to confirm RNA integrity. First-strand cDNA was synthesized from total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The cDNA was subjected to polymerase chain reaction (PCR) for 40 cycles using primers as described in supplemental Table 1. The primers were designed with the help of Premier 5 software (Version 5.0 for Windows and Power Macintosh, Palo Alto, CA, USA). Each gene sequence was obtained from the transcriptome data, and corresponding gene sequences were listed in supplemental material. Each primer used in the qRCP experiment produces approximately 100 bp-amplicon. Gene specificity of each primer was confirmed through BLAST searches of public databases (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The threshold cycles (C_t) of each target gene were averaged for triplicate reactions, and the values were normalized according to the C_t of reference gene (*En18S rRNA*). The relative expression levels of target gene were calculated using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Statistical analysis

Each experiment was designed to three biological replicates. Data were represented as mean ± SD. Statistical analyses were performed by the analysis of variance (ANOVA) using the SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Means were compared by Tukey's test and differences at $P < 0.05$ were considered significant.

Results

Altitude variation in chilling tolerance in *E. nutans*

A steep altitude cline in chilling tolerance among natural populations of perennial plant *Arabis alpine* has recently been investigated (Wingler et al. 2015). Here, we report on eight accessions of *E. nutans* (Table 1) that showed a different type of chilling tolerance. An increase in relative electrolyte leakage under chilling stress indicates significant damage to cell membrane integrity (Lyons 1973). Genotypes of *E. nutans* originating from higher altitudes displayed lower relative electrolyte leakage and lost less chlorophyll in response to chilling (Fig. 1). Wild *E. nutans* from higher altitudes had lower electrolyte leakage after 72 h at 4 °C in comparison with lower altitude accessions. Chilling inhibited photosynthesis in the investigated *E. nutans* accessions to different degrees, especially in those from lower altitudes: NML, MZG, and DZ. Chlorophyll declined more rapidly at the chilling temperature in some of the accessions (DZ, MZG, NML, KM, and AL), resulting in remarkably lower chlorophyll content after 120 of chilling stress (Fig. 1b). Assessment of chlorophyll fluorescence revealed reductions in F_v/F_m in all accessions, suggesting down-regulation of photosynthesis or photoinhibition during chilling stress. After 120 h of chilling stress, a higher F_v/F_m was observed in the three accessions from higher altitudes, BQ, XZ, and DX (Fig. 1c). These results indicate that accessions from higher altitudes showed an attenuated stress response to chilling temperature.

Variation of phytohormone levels in response to chilling stress

The dynamic variation in phytohormone content was measured in all 8 *E. nutans* accessions over the time course of chilling stress. The ABA content increased transiently within the first days of chilling treatment and reached a maximum at 3 h of stress. After this, the levels declined to a value that was larger than what was observed prior to the chilling exposure. This effect was significant for the accessions BQ, XZ, DX, and KM (Fig. 2a).

ACC production rapidly declined in plants exposed to chilling stress for 1 h in accessions BQ, XZ, and DX, exhibiting a minimum after 3 h of chilling stress and remaining at this low level during the time course. The rapid increase in ACC production was observed in low-altitude accessions, DZ, MZG, and NML during 1–3 h of chilling exposure (Fig. 2b).

The contents of the cytokinin zeatin remained low in NML, MZG, and DZ accessions in response to chilling.

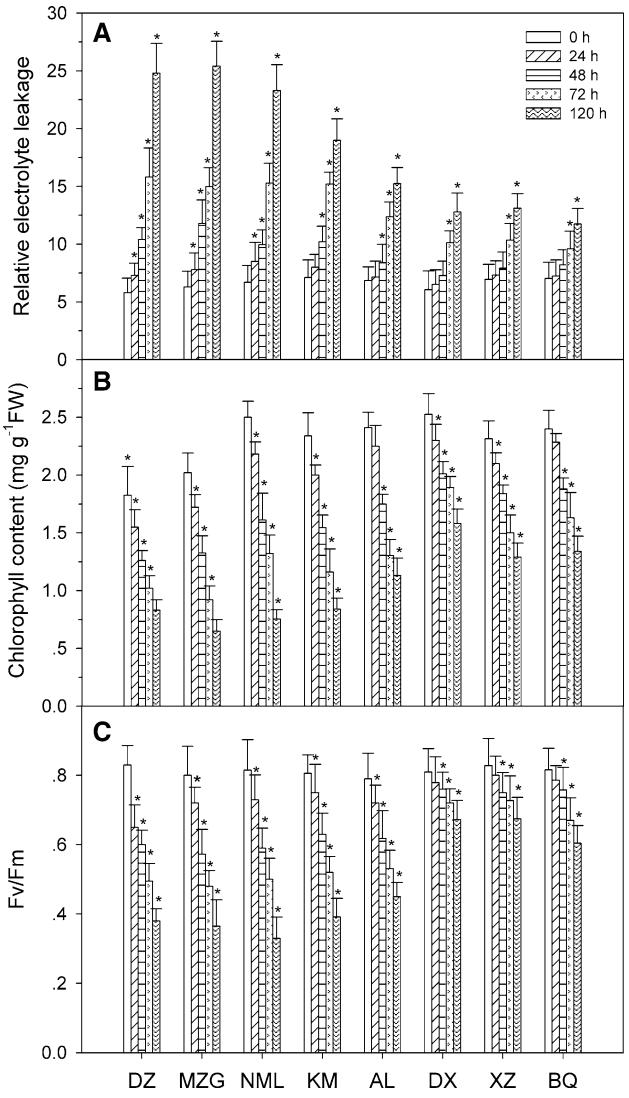

Fig. 1 Changes in relative electrolyte leakage (**a**), chlorophyll content (**b**), and F_v/F_m (**c**) in the leaves of *E. nutans* accessions during the chilling stress time course. Each value represents the mean ± SD. Each experiment was repeated three times. An ANOVA test followed by Tukey's test was performed. Asterisks indicate significant differences at $P < 0.05$ from 0 h of chilling stress

However, zeatin was induced in high-altitude accessions after 1 h of chilling exposure, reaching a maximum at 12 h in BQ, XZ, and DX accessions (Fig. 2c).

Bioactive GA3 levels were reduced immediately after exposure to 1 h of chilling treatment, and maintained the declining trend until 24 h. After that time, it increased until 120 h of chilling exposure. All accessions showed the similar trends, but the highest altitude accessions had lower bioactive GA contents during early chilling stress (Fig. 2d).

IAA levels of BQ, XZ, DX, AL, and KM decreased significantly after 6 h of chilling treatment, and continued to decrease for up to 48 h, before increasing thereafter. A significant increase of IAA content in low accessions DZ,

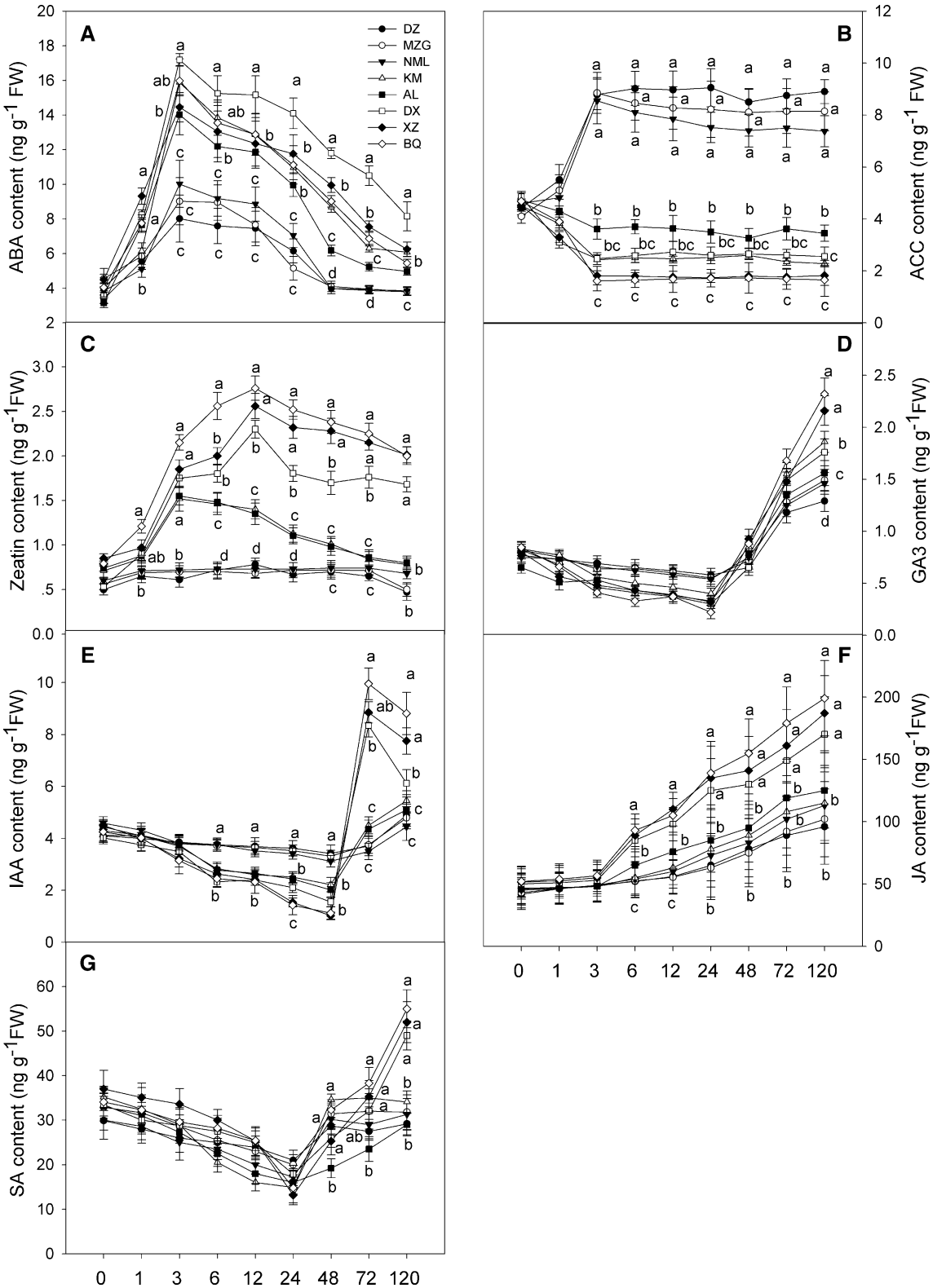

Fig. 2 Dynamic variation of abscisic acid (ABA, **a**), ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, **b**), zeatin (**c**), gibberellins (GA3, **d**), auxin (IAA, **e**), jasmonic acid (JA, **f**), and salicylic acid (SA, **g**) content in the leaves of *E. nutans* accessions during the chilling stress time course. Each value represents the mean ± SD. Each experiment was repeated three times. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different ($P < 0.05$)

MZG, NML, and KM was observed at 72 h of chilling. Apparent increases ($P < 0.5$) in JA content were observed in accessions BQ, XZ, and DX after 6 h of chilling stress, and progressively increased until 120 h of chilling exposure, reaching a maximum at 120 h (Fig. 2e, f). Chilling significantly decreased SA content in accessions BQ, XZ, and DX during the period between 12–24 h and reached the lowest level at 24 h. The levels then increased until 120 h. Notable exceptions to the coordinate increases were in our measure of SA content, which remained unchanged at time-zero levels in some low-altitude accessions during the period between 48–120-h chilling stress (Fig. 2g).

Effects of chilling stress on sugar accumulation

Sugar response lagged behind phytohormone levels in all accessions exposed to chilling stress (Fig. 3). Sucrose and raffinose levels in a large range of accessions were higher than fructose and glucose during a chilling stress period of 120 h. This was especially prevalent in high-altitude accessions, demonstrating that sucrose and raffinose were the main carbohydrates involved in chilling tolerance.

There were a variety of responses for other sugars in response to chilling stress. Glucose content in high-altitude accessions BQ, XZ, DX, AL, and KM declined during the 120-h period of chilling stress. Similar effects were also observed in low-altitude accessions NML, MZG, and DZ. Fructose content was found at a low level in all accessions, but some low-altitude accessions had enhanced levels and reached a maximum at 72 h of chilling treatment. Following this time, the levels declined to a point that was higher than non-stressed accessions. Sucrose content in high-altitude accessions, BQ, XZ, DX, and AL, increased immediately at 24 h of chilling stress, and increased continuously until 120 h. In contrast, sucrose content in low-altitude accessions increased after 72 h of chilling stress. Raffinose levels generally increased ($P < 0.05$) in response to chilling stress, except for DZ and MZG. High-altitude accessions, BQ, XZ, and DX showed higher raffinose content than low-altitude accessions.

EnCBFs and *EnCOR* gene expression in plants exposed to chilling stress

According to the previous transcriptome data, *EnCBF9*, *EnCBF14*, and *EnCOR14a* were significantly induced by cold in *E. nutans* accessions (data not shown). As shown in Figs. 4 and 5, chilling induced diverse patterns of expression in *EnCBF9*, *EnCBF14*, and *EnCOR14a* genes in eight broad altitude ranges of *E. nutans* accessions. The data showed that the *CBFs* and *COR* transcriptional patterns were dynamic during the 120-h time course of chilling stress. *EnCBF9* and *EnCBF14a* gene transcriptional levels in high-altitude accessions BQ, XZ, DX and AL increased ($P < 0.05$) immediately within 1 h of chilling exposure, peaked at 3 h, and then decreased until 120 h (Fig. 4a, b). While expression patterns of the two *CBFs* exhibited a small amount of variation in low-altitude accessions, indicating that other *CBF* genes and specific transcriptional pathways in response to chilling stress may have occured in low-altitude accessions.

Upon transfer to low temperature, *EnCOR14a* expression levels in accessions BQ, XZ, DX and AL increased ($P < 0.05$) at 3 h, reaching a maximum at 48 h, and then decreased until 120 h of chilling stress. In low-altitude accessions, expression of these genes was induced after 6 h. This was a low level compared with the high-altitude species (Fig. 5).

Discussion

Accessions from higher altitudes have a greater chilling tolerance

Accessions of *E. nutans* originating from higher altitudes exhibited lighter stress responses to chilling stress. This was evidenced by their lower relative electrolyte leakage and lower reduction in F_v/F_m in response to chilling exposure.

We next explored the acclimation mechanisms with respect to *E. nutans* altitude variations in response to chilling stress. To clearly elucidate dynamic changes in physiological parameters and key genes related to chilling, two phases of chilling stress were classified: early phase (1–24 h) and acclimation phase (24–120 h).

Early phase of chilling stress

Phytohormones serve as crucial signaling molecules that are implicated in the regulation of plant growth, development, and various abiotic stress responses (Peleg and Blumwald 2011). This early response to chilling stress was consistent with an elevation in ABA, JA, and bioactive cytokinin zeatin content in high-altitude BQ, XZ, and DX. This was coordinated with a reduction in IAA, SA, GA3, and ACC contents in these *E. nutans* accessions (Fig. 2). In addition to changes in phytohormone content, we also found that the transcriptional levels of *CBFs* and *CORs* in high-altitude accessions were dramatically induced during early chilling stress (Figs. 4, 5). Natural variation analyses in *Arabidopsis* and *Arabis alpine* accessions with wide range of freezing tolerance further confirm the idea that phytohormone signaling and up-regulation of *CBF* genes contribute to low-temperature tolerance (Hannah et al. 2006; Kang et al. 2013; Wingler et al. 2015).

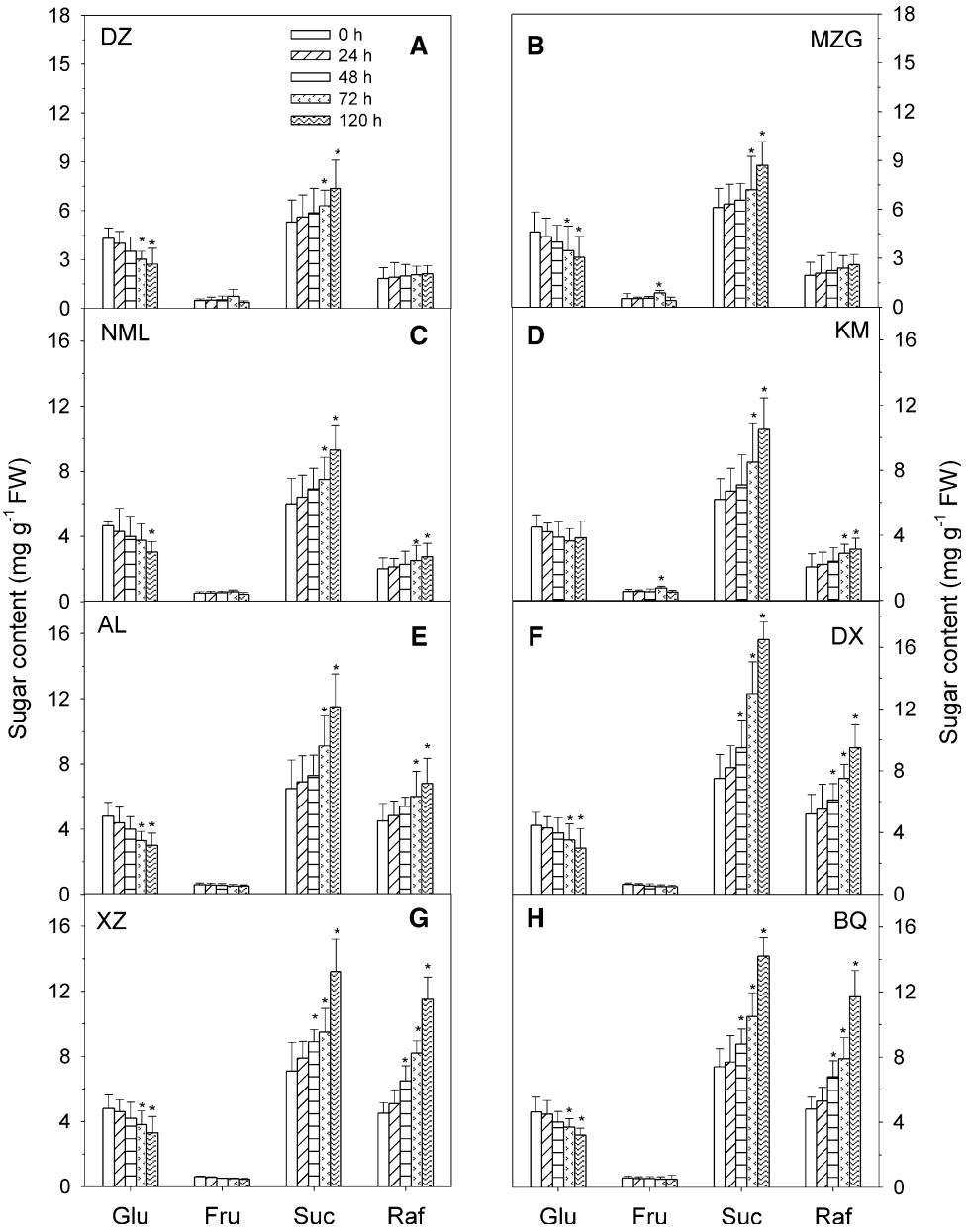

Fig. 3 Changes in glucose (Glu), fructose (Fru), sucrose (Suc), and raffinose (Raf) contents in the leaves of *E. nutans* accessions during the chilling stress time course. Each value represents the mean ± SD. Each experiment was repeated three times. An ANOVA test followed by Tukey's test was performed. Asterisks indicate significant differences at $P < 0.05$ from 0 h of chilling stress

We also found that the ACC content declined during 1–3 h course of chilling stress in AL, DX, XZ, and BQ populations. This seems to have coincided with the observation that the transcript of *EnCBF9* and *EnCBF14* genes reaches a peak at the initial 1–3 h of chilling and decreases after 6 h of chilling treatment (Figs. 2, 4). Therefore, we propose that during the onset of chilling stress, a reduction in ethylene content in several accessions may inactivate the transcriptional repression of *CBFs* through ethylene signaling pathways and trigger *CBF*-dependent chilling acclimation. This response is also observed in *Arabidopsis* exposed to chilling stress (Shi et al. 2012).

It is noteworthy that low-altitude accessions showed higher ACC production during 1–6 h of chilling exposure. This finding is supported by prior studies in maize demonstrating that increased ACC levels showed a high positive linear relationship with the sensitivity to chilling in maize genotypes. These results suggest that the ACC content is a physiological index of cold injury (Janowiak and Dörffling 1996). Shi et al. (2012) also pointed out that ethylene biosynthesis and signaling play negative roles in the regulation of freezing tolerance in *Arabidopsis*. A previous report demonstrated that an antagonistic interplay between ethylene and cytokinin signaling existed in *Arabidopsis* exposed to cold stress (Shi et al. 2012). Similarly, ACC production sharply decreased with the increase in bioactive cytokinin zeatin contents in the investigated *E. nutans* accessions.

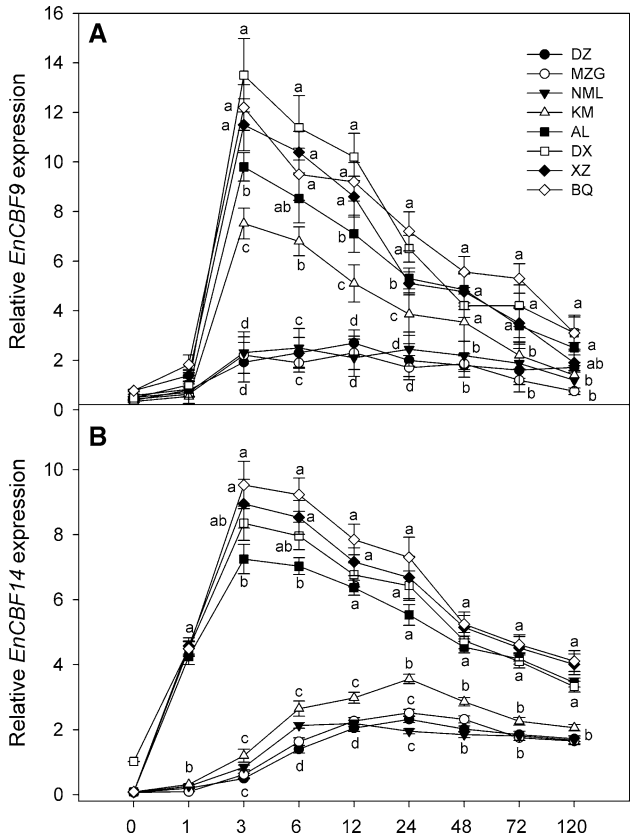

Fig. 4 Normalized expression of *EnCBF9* (**a**) and *EnCBF14* (**b**) genes in eight accessions of *E. nutans* following 0, 1, 3, 6, 12, 24, 48, 72, and 120 h of chilling stress at 4 °C. Each value represents the mean ± SD. Each experiment was repeated three times. The data for each sample were normalized to the level of the house-keeping gene *18S rRNA*. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different ($P < 0.05$)

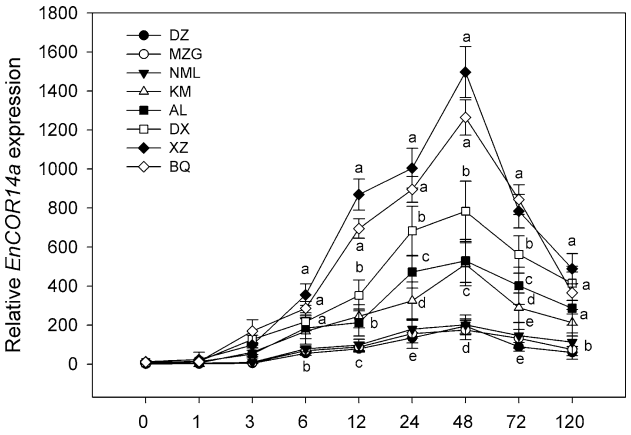

Fig. 5 Normalized expression of *EnCOR14a* gene in eight accessions of *E. nutans* following 0, 1, 3, 6, 12, 24, 48, 72, and 120 h of chilling stress at 4 °C. Each value represents the mean ± SD. Each experiment was repeated three times. The data for each sample were normalized to the level of the house-keeping gene *18S rRNA*. An ANOVA test followed by Tukey's test was performed. Different letters are used to indicate means that are significantly different ($P < 0.05$)

In *A. thaliana*, cold-induced *CBF* gene expression has been shown to be involved in the accumulation of DELLA proteins. These proteins repress the GA signalling pathway, and thus inhibit plant growth (Achard et al. 2008). *CBF1* activated the expression of genes encoding the GA 2-oxidases enzymes in *Arabidopsis*. We found a similar decrease in GA3 in *E. nutans* accessions during the early stage of chilling stress. In addition, the *CBF1*-induced DELLA accumulation also provides positive synergies with the *CBF1*-induced *COR* pathway to enhance cold tolerance (Achard et al. 2008). It is well established that the COR proteins play important roles in protecting the cell from dehydration and freezing injury during the acclimation process (Thomashow 1999). Here, we have shown that *EnCOR14a* gene expression significantly increased in *E. nutans*, especially in high-altitude accessions, after 3 h of chilling exposure.

Accumulating evidence demonstrates that JAs trigger an important transcriptional regulatory pathway in cells allowing them to transform their basal developmental programs into necessary stress responses (Mandaokar et al. 2006; Pauwels et al. 2008). A continuous increase in JA content exposed to chilling treatment was observed in winter wheat (Kosová et al. 2012). A JA-induced signaling pathway in *Arabidopsis* was also found by Hu et al. (2013) who pointed out that jasmonate was shown to enhance freezing tolerance by inducing the *ICE CBF/DREB1* cold-response pathway. Our investigations further support a role for JA in chilling response in *E. nutans*. High-altitude accessions exhibited a transient accumulation of JA content, demonstrating that adaptation to higher altitude accompanied by an enhanced capacity to accumulate JA during the early stages of chilling.

IAA is one of the hormones involved in the regulation of plant growth and development and it also plays pivotal roles in the adaptive responses to abiotic stresses (Du et al. 2013; Wingler et al. 2015). An opposing trend between IAA and JA concentrations in BQ, XZ, DX, AL, and KM was found in the early phase of chilling stress. This observation in *E. nutans* is supported by the prior work of Du et al. (2013) who pointed out that JA and IAA antagonistically regulate each other's metabolism and signaling, which are critical for plant development and stress responses.

The ABA-dependent cold signaling pathway has widely been studied for many years. A previous study showed that about 10 % of ABA responsive genes are responsive to cold stress in *Arabidopsis* (Kreps et al. 2002). In this study, ABA content increased immediately within the 24 h of chilling treatment and peaked at 3 h of chilling stress,

indicating that ABA signaling exerts an important positive effect on the initial chilling response process. The up-regulation of ABA content together with rapid down-regulation of SA content was observed in the leaves of accessions BQ, XZ, and DX. This finding confirmed a report by Pociecha et al. (2009), who found that ABA and SA contents show contrasting effects in frost resistant genotypes of *Festulolium* during the first 54 h of the cold stress process. Similarly, ABA treatment also inhibited SA signaling pathway by reducing low-temperature-induced SA production in maize leaves (Pal et al. 2011). These observations reveal the antagonistic regulation of the two stress hormones in the chilling response.

The upward regulation and downward regulation of the content and/or signaling pathways of hormones involved in cell division, growth, and abiotic stress responses indicates that growth inhibition is an important component of the early chilling stress response in *E. nutans*. In addition, this seems to be associated with the reallocating of energy sources for the synthesis cellular protective metabolites under unfavorable conditions.

Acclimation phase of chilling stress

Plants acclimate to the cold stress within a 24-h period, and after this time course, the cold signaling mechanism becomes insensitive to low temperatures. This leads to the down-regulation in the expression of *CBF* and *COR* genes (Zarka et al. 2003). Consistent with this finding, we found that *EnCBF9*, *EnCBF14*, and *EnCOR14a* genes were down-regulated after 24 h at 4 °C (Figs. 4, 5). The available evidence has shown that cold-induced *CBF* genes are involved in transcriptional regulation of the accumulation of soluble sugars, including sucrose, glucose, and fructose, via the modulation of the *CBF* regulon (Gilmour et al. 2004). Carbohydrate accumulation is common in freeze-resistant plants (Hoffman et al. 2010). In this study, a higher accumulation of sugars in high-altitude *E. nutans* during chilling stress demonstrated the tight relationship between sugar metabolism and chilling tolerance. This is in agreement with results reported in perennial ryegrass (Bhowmik et al. 2006). In particular, the sucrose response appears to have a key role during chilling treatment. Zeng et al. (2011) also observed a higher accumulation of sucrose and fructose during the process of freezing tolerance in winter wheat.

The interplay of different phytohormones and multiple sugars forms a complex network of overlapping signals to coordinate overall plant growth, development, and various stress responses (Loreti et al. 2008; Wingler et al. 2015). Phytohormone alterations that are characteristic for this response phase were: slight accumulation of ABA levels and significant increases in bioactive CKs, GA3, IAA, SA, and JA contents. This indicated plant acclimation to low temperatures and a rebalance of metabolic processes under the unfavorable conditions. As CKs display a positive role in plant photosynthesis, their enhancement during the acclimation stage might contribute to energy generation (Guo et al. 2010; Kosová et al. 2012). Similar findings have been indicated by Hu et al. (2005) who confirmed improved maintenance of chlorophyll levels as well as enhanced cold tolerance in calluses of *Festuca arundinacea* that constitutively expressed the CK biosynthetic gene. No significant variation in ABA content was observed and this may indicate that ABA-independent signaling pathway exerts a key effect on chilling tolerance of the investigated *E. nutans* during a later stage of chilling stress. Interestingly, a substantial overlap between glucose and ABA was detected in *Arabidopsis* in the regulation of cold stress tolerance (Li et al. 2006; Dekkers et al. 2008). Increase in SA content might be associated with SA functions in the regulation of ROS evolution, which is an important component of defensive responses under stress conditions. JA levels showed an elevation with some delay and increased at 6 h of chilling treatment in all accessions. Synergistic effects between sucrose and jasmonates were obtained in *Arabis alpina* (Wingler et al. 2015). Our results suggest that the sugar-response pathway and phytohormone-response pathway play crucial roles in the chilling tolerance of *E. nutans*, especially in high-altitude accessions.

Conclusions

Our work demonstrates that natural accessions of *E. nutans* that originate from a wide range of altitudes show clinal variation in chilling tolerance. Physiological adaptations and stimulation of key genes occur over an altitudinal gradient in this plant. The induction of *EnCBFs* and *EnCOR* genes may exert crucial roles in the regulation of the early chilling stress response. Natural variations in the accumulation of sugars and dynamic changes in phytohormone levels at two stages of chilling stress showed different acclimation mechanisms to that stress. The difference between natural accessions in these physiological parameters and the genetic responses contributes to the exploration of the genetic basis of the chilling response in plants.

Author contribution statement Xu YF and Hu TM conceived and designed research. Fu JJ conducted the experiments, performed statistical analysis of the data, and wrote the manuscript. Miao YJ provided the experimental materials. All authors read and approved the final manuscript.

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References

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible *CBF1* factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 20:2117–2129

Bhowmik PK, Tamura K, Sanada Y, Tase K, Yamada T (2006) Sucrose metabolism of perennial ryegrass in relation to clod acclimation. Z Naturforsch C 61:99–104

Chen SY, Ma X, Zhang XQ, Chen ZH (2009) Genetic variation and geographical divergence in *Elymus nutans* Griseb. (Poaceae: Triticeae) from West China. Biochem Syst Ecol 37:716–722

Dekkers BJW, Schuurmans JAMJ, Smeekens SCM (2008) Interaction between sugar and abscisic acid signalling during early seedling development in Arabidopsis. Plant Mol Biol 67:151–167

Dong YC, Liu X (2007) Crops and their wild relatives in China. China Agriculture Press, Beijing

Du H, Liu HB, Xiong LZ (2013) Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. Front Plant Sci 4:397

Gibson SI (2004) Sugar and phytohormone response pathways: navigating a signalling network. J Exp Bot 55:253–264

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124:1854–1865

Gilmour SJ, Fowler SG, Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol 54:767–781

Guo JC, Duan RJ, Hu XW, Li KM, Fu SP (2010) *Isopentenyl transferase gene* (*ipt*) downstream transcriptionally fused with gene expression improves the growth of transgenic plants. Transgenic Res 18:197–209

Hannah MA, Wiese D, Freund S, Fiehn O, Heyer AG, Hincha DK (2006) Natural genetic variation of freezing tolerance in Arabidopsis. Plant Physiol 142:98–112

Hoffman L, DaCosta M, Ebdon JS, Watkin E (2010) Physiological changes during cold acclimation of perennial ryegrass accessions differing in freeze tolerance. Crop Sci 50:1037–1047

Hu YL, Jia WL, Wang JD, Zhang YQ, Yang LL, Lin ZP (2005) Transgenic tall fescue containing the *Agrobacterium tumefaciens ipt* gene shows enhanced cold tolerance. Plant Cell Rep 23:705–709

Hu Y, Jiang L, Wang F, Yu D (2013) Jasmonate regulates the INDUER OF CBF EXPRESSION-C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 cascade and freezing tolerance in *Arabidopsis*. Plant Cell 25:2907–2924

Janowiak F, Dörffling K (1996) Chilling tolerance of 10 maize genotypes as related to chilling-induced changes in ACC and MACC contents. J Agron Crop Sci 177:175–184

Jeon J, Kim NY, Kim S, Kang NY, Novák O, Ku SJ, Cho C, Lee DJ, Lee EJ, Strnad M, Kim J (2010) A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in Arabidopsis. J Biol Chem 285:23371–23386

Jibran R, Hunter DA, Dijkwel PP (2013) Hormonal regulation of leaf senescence through integration of developmental and stress signals. Plant Mol Biol 82:547–561

Kang J, Zhang H, Sun T, Shi Y, Wang J, Zhang B, Wang Z, Zhou Y, Gu H (2013) Natural variation of *C-repeat-binding factor* (*CBFs*) genes is a major cause of divergence in freezing tolerance among a group of Arabidopsis thaliana populations along the Yangtze River in China. New Phytol 199:1069–1080

Kidokoro S, Watanabe K, Ohori T, Moriwaki T, Maruyama K, Mizoi J, Myint Phyu Sin Htwe N, Fujita Y, Sekita S, Shinozaki K, Yamaguchi-Shinozaki K (2015) Soybean DREB1/CBF-type transcription factors function in heat and drought as well as cold stress-responsive gene expression. Plant J 81:505–518

Kosová K, Prášil IT, Vítámvás P, Dobrev P, Motyka V, Flokovác K, Novákc O, Turečková V, Rolčik J, Pešek B, Trávničková A, Gaudinová A, Galiba G, Janda T, Vlasáková E, Prášilová P, Vanková R (2012) Complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, winter Samanta and spring Sandra. J Plant Physiol 169:567–576

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol 130:2129–2141

Li YH, Lee KK, Walsh S, Smith C, Hadingham S, Sorefan K, Cawley G, Bevan MW (2006) Establishing glucose- and ABA-regulated transcription networks in *Arabidopsis* by microarray analysis and promoter classification using a Relevance Vector Machine. Genome Res 16:414–427

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Method Enzymol 148:350–382

Liu F, Jensen CR, Andersen MN (2004) Drought stress effect on carbohydrate concentration in soybean leaves and pods during early reproductive development: its implication in altering pod set. Field Crop Res 86:1–13

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T) method. Methods 25:402–408

Loreti E, Povero G, Novi G, Solfanelli C, Alpi A, Perata P (2008) Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in *Arabidopsis*. New Phytol 179:1004–1016

Lyons JM (1973) Chilling injury in plants. Annu Rev Plant Physiol 24:445–466

Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Browse J (2006) Transcriptional regulators of stamen development in *Arabidopsis* identified by transcriptional profiling. Plant J 46:984–1008

Mboup M, Fischer I, Lainer H, Stephan W (2012) Trans-species polymorphism and allele-specific expression in the *CBF* gene family of wild tomatoes. Mol Biol Evol 29:3641–3652

Menon M, Barnes WJ, Olson MS (2015) Population genetics of freeze tolerance among natural populations of *Populus balsamifera* across the growing season. New Phytol 207:710–722

Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX, Hwang I, Jones T, Sheen J (2003) Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light and hormonal signaling. Science 300:332–336

Müller M, Munné-Bosch S (2011) Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Plant Methods 7:37

Oakley CG, Agren J, Atchison RA, Schemske DW (2014) QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs. Mol Ecol 23:4304–4315

O'Brien JA, Benková E (2013) Cytokinin cross-talking during biotic and abiotic stress responses. Front Plant Sci 4:451

Oliveira G, Peñuelas J (2004) Effects of winter cold stress on photosynthesis and photochemical efficiency of PSII of the Mediterranean *Cistus albidus* L. and *Quercus ilex* L. Plant Ecol 175:179–191

Pal M, Janda T, Szalai G (2011) Abscisic acid may alter the salicylic acid-related abiotic stress response in maize. J Agron Crop Sci 197:368–377

Palma F, Carvajal F, Lluch C, Jamilena M, Garrido D (2014) Changes in carbohydrate content in zucchini fruit (*Cucurbita pepo* L.) under low temperature stress. Plant Sci 217–218:78–86

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. Proc Natl Acad Sci USA 105:1380–1385

Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol 14:290–295

Pociecha E, Plazek A, Janowiak F, Waligorski P, Zwierzykowski Z (2009) Changes in abscisic acid, salicylic acid and phenylpropanoid concentrations during cold acclimation of androgenic forms of Festulolium (*Festuca pratensis* × *Lolium multiflorum*) in relation to resistance to pink snow mould (*Microdochium nivale*). Plant Breed 128:397–403

Shi YT, Tian SW, Hou LY, Huang XZ, Zhang XY, Guo HW, Yang SH (2012) Ethylene signaling negatively regulates freezing tolerance by repressing expression of *CBF* and type-A *ARR* genes in *Arabidopsis*. Plant Cell 24:2578–2595

Song L, Ding W, Zhao M, Sun B, Zhang L (2006) Nitric oxide protects against oxidative stress under heat stress in the calluses from two ecotypes of reed. Plant Sci 171:449–458

Thomashow MF (1999) PLANT COLD ACCLIMATION: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. Plant J 41:195–211

Wingler A, Juvany M, Cuthbert C, Munné-Bosch S (2015) Adaptation to altitude affects the senescence response to chilling in the perennial plant *Arabis alpine*. J Exp Bot 66:355–367

Zarka DG, Vogel JT, Cook D, Thomashow MF (2003) Cold induction of *Arabidopsis CBF* genes involves multiple ICE (inducer of *CBF* expression) promoter elements and a coldregulatory circuit that is desensitized by low temperatures. Plant Physiol 133:910–918

Zeng Y, Yu J, Cang J, Liu LJ, Mu YC, Wang JH, Zhang D (2011) Detection of sugar accumulation and expression levels of correlation key enzymes in winter wheat (*Triticum aestivum*) at low temperatures. Biosci Biotechnol Biochem (BBB) 75:681–687

Zhao ML, Wang JN, Shan W, Fan JG, Kuang JF, Wu KQ, Li XP, Chen WX, He FY, Chen JY, Lu WJ (2013) Induction of jasmonate signalling regulators MaMYC2s and their physical interactions with MaICE1 in methyl jasmonate-induced chilling tolerance in banana fruit. Plant Cell Environ 36:30–51

Zhen Y, Ungerer MC (2008) Relaxed selection on the CBF/DREB1 regulatory genes and reduced freezing tolerance in the southern range of *Arabidopsis thaliana*. Mol Biol Evol 25:2547–2555