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Effect of exogenous abscisic acid on cold acclimation in two *Magnolia* species

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Abstract

In northern China, freezing injury is observed frequently in the rare species *Magnolia wufengensis* but not in the more common species *Magnolia denudata*. To investigate the role of the phytohormone abscisic acid (ABA) on frost tolerance in these two species, exogenous ABA was applied to the seedlings and then physiological and biochemical responses were measured during cold acclimation. Shoot growth cessation was stimulated by ABA in *M. wufengensis* but not in *M. denudata*. Abscisic acid inhibited shoot growth in *M. wufengensis* but not in *M. denudata*. Treatment with ABA stimulated leaf senescence in both species, and this effect was greater in *M. denudata*. For both species, ABA-treated plants exhibited bud dormancy sooner and had an increased tolerance to freezing, decreased water content and increased accumulation of proline, glucose, and fructose in shoots. These effects were generally greater for *M. denudata*. Freezing tolerance was significantly correlated with content of water, proline, glucose, and fructose for both species, but freezing tolerance was significantly correlated with raffinose content only in *M. wufengensis*. We conclude that exogenous ABA could increase cold acclimation and improve cold hardiness of both *Magnolia* species, although *M. denudata* was more responsive to ABA than *M. wufengensis*, which might result from a greater dehydration and accumulation of proline and certain soluble sugars.

Additional key words: freezing tolerance, *Magnolia denudata*, *M. wufengensis,* proline, soluble sugar.

Introduction

Freezing injury affects growth, productivity, and geographical distribution of plants (Weiser 1970, Pearce 2001). The susceptibility of plants to freezing injury may be due not only to insufficient freezing tolerance, but also to the rate of cold acclimation (Suojala and Lindén 1997). Cold acclimation, the process of transition from a cold-sensitive to a cold-hardy state (Zabadal *et al.* 2007), is essential for the survival of woody plants in temperate regions (Teets *et al.* 1989). Physiological and biochemical responses associated with increasing freezing tolerance during cold acclimation include decreased tissue water content, accumulation of proline and soluble sugars, and hormone regulation.

A phytohormone abscisic acid (ABA) plays an important role in cold acclimation, as rapid cold acclimation or higher freezing tolerance are often associated with an elevated endogenous ABA content (Li *et al.* 2005a,b). Similarly to exposure to low temperatures, ABA application can initiate cold acclimation (Mohapatra *et al.* 1988). Exogenous ABA and cold acclimation have a similar effect on cell wall changes including decreasing cell-wall pore size and increasing cell-wall strength (Rajashekar and Lafta 1996). In some plant species, the application of ABA improves freezing tolerance (Dallaire *et al.* 1994, Churchill *et al.* 1998, Mora-Herrera and Lopez-Delgado 2007).

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Abbreviations: ABA - abscisic acid; D50B - number of days to 50 % budburst; LT_{50} - low temperature representing 50 % relative electrolyte leakage; P_N - net photosynthetic rate.

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Magnolia wufengensis is a rare, newly discovered species in southern China (Ma *et al.* 2006a,b). It has colorful flowers (pure, dark, or pale red) and varied flower petal numbers (9-25, 32, or 46) which contribute to its considerable ornamental and economical value. *M. wufengensis* introduced to northern China frequently suffers from freezing injury in winter, whereas the more common species *Magnolia denudata* rarely suffers from freezing injury. In this study, physiological and biochemical changes of field-grown *M. denudata* and *M. wufengensis* were investigated in response to exogenous ABA and compared during cold acclimation. We predicted application of ABA can induce cold acclimation in these two species and we further predicted that the more frost-tolerant *M. denudata* would respond to ABA application with a greater cold acclimation than the less frost-tolerant species *M. wufengensis*.

Materials and methods

Plants and experimental design: In early April 2012, two-year-old seedlings of *Magnolia denudata* Desr. from the Jiufeng Experimental Station of the Beijing Forestry University in Beijing (39° 48′ N, 116° 28′ E), and of *M. wufengensis* L.Y. Ma from the Rare Plant Institute of Forestry Bureau of Wufeng County in Wufeng, Hubei Province (29° 56′ N, 110° 15′ E) were transplanted to the study site, the Puzhaoyuan Nursery of the Jiufeng Experimental Station of the Beijing Forestry University in Beijing, China (39° 48′ N, 116° 28′ E). The seedlings of both species were randomly planted within each of 10 plots in the study site. Each plot had an area of 10 × 3 m and contained 20 plants. Between April and August 2012, the plants were kept well-irrigated and protected from pathogens and weed competition. Supplemental irrigation ended on 31 August. In a previous experiment, ABA concentrations between 600 and 900 mg·dm^{-3} were observed as optimum for the two *Magnolia* species (data not shown). Based on this range, two concentrations of ABA (provided by *Lomon Bio Corporation*, China) were selected: 0 mg dm^{-3} (CK treatment) and 600 mg dm^{-3} (ABA treatment). All seedlings from a half of the plots were sprayed with the ABA solution to runoff using a 5-dm^3 handheld sprayer averaging a spray volume of 0.5 dm^3 per seedling.

Determination of shoot growth, leaf senescence, and bud dormancy: Shoot length was measured every 3 d between 1 September and 1 October. Two seedlings in each plot were selected randomly, for a total of 10 replicate seedlings from each treatment. A healthy upper-crown shoot facing the sun was selected on each seedling to measure shoot length. Growth cessation was calculated as the number of days from 1 September until shoot growth stopped.

Leaf senescence, assessed as decrease in leaf net photosynthetic rate (P_N), was determined every 10 d from 1 September to 31 October. One healthy upper-crown leaf facing the sun from the same shoot as used for growth measurement was selected for leaf P_N measurement using a *LI-6400* portable photosynthesis system (*Li-COR, Biosciences*, Lincoln, NE, USA). Data were recorded between 9:00 - 11:00. Air cuvette irradiance, temperature, and carbon dioxide (CO_2) concentration were maintained at 1000 μmol m^{-2} s^{-1}, 25 °C, and 400 μmol mol^{-1}, respectively.

Bud dormancy, assessed as days to 50 % budburst (D50B), was determined monthly from 30 September 2012 to 30 January 2013. Two seedlings in each plot were selected randomly, for a total of 10 replicate seedlings from each treatment and one healthy upper-crown shoot facing the sun on each representative seedling was excised into a foam medium and then placed in plastic trays filled with water. The trays were then placed into a growth chamber with the following settings: a 24-h photoperiod with an irradiance of 500 μmol m^{-2} s^{-1}, a temperature of 22 °C, and a relative humidity of 80 %. The budburst was observed daily. The greater D50B values, the higher dormancy.

Determination of shoot freezing tolerance: Freezing tolerance, assessed as low temperature representing a 50 % relative electrolyte leakage (LT_{50}), was determined monthly from 30 September 2012 to 30 January 2013. The LT_{50} was determined by using the method described by Jun *et al.* (2012) with slight modifiations. Two seedlings in each plot were selected randomly, for a total of 10 replicate seedlings from each treatment, and wrapped in moist paper inside a plastic bag to prevent dehydration. Shoot segments were rinsed under running cold deionized water for 15 s and then placed in a conical tube. The tubes were incubated in a bath circulator (*RW-1040W*, *Jeio Tech*, Seoul, Korea) equipped with a temperature controller (*UP351E*, *Yokogawa Electric Korea*, Seoul, Korea) and cooled at a rate of 2 °C h^{-1} until they reached 0 °C. After being maintained at 0 °C for 1 h, the tubes were cooled at a rate of 5 °C·h^{-1} until the target temperatures (-5, -10, -15, -20, -25, and -30 °C) were reached, and then maintained for 2 h at each target temperature. Then the tubes were withdrawn and thawed overnight at 4 °C. After the freezing treatment, the shoots were cut into 1-cm-long sections, placed in a 15-cm^3 conical tube containing 8 cm^3 of deionized water, and vacuum-infiltrated for 3 min. The tubes were then incubated at room temperature for 20 h with a gentle agitation, and the electrical conductivity of the aliquots was measured using a conductivity meter (*Model 1461-81*, *Cole Parmer*, Vernon Hills, IL, USA).

Electrical conductivity was measured again after autoclaving at 120 °C for 30 min. The LT_{50} was estimated from an asymmetric sigmoid curve constructed with the Gompertz function fit to percentage injury as calculated by analyzing electrolyte leakage using the method of Lim and Arora (1998).

Shoot physiological and biochemical parameters were measured monthly from 30 September 2012 to 30 January 2013. For each parameter, 10 representative seedlings from each treatment (two seedlings from each plot) were selected and one healthy upper-crown shoot facing the sun on each representative seedling was selected. Water content was calculated by the following formula: water content = (fresh mass - dry mass) / fresh mass. Proline content was measured according to the method of Bates *et al.* (1973); glucose, fructose, and raffinose content was measured according to the method of Liu *et al.* (2004).

Statistical analysis: All data were analyzed using *SPSS Statistics v. 18.0* (*SPSS Inc.*, Chicago, IL, USA), including one-way analysis of variance for main effects of different treatments and a correlation analysis within cold hardiness and other physiological and biochemical parameters.

Results and discussion

Exogenous ABA had no significant effect on shoot growth in *M. denudata* (Fig. 1*A*) but the ABA-treated seedlings of *M. wufengensis* had a significantly ($P < 0.05$) slower shoot growth compared with the control seedlings (Fig. 1*B*). Growth cessation under both the control treatment and the ABA treatment occurred at 12 d for *M. denudata*, whereas growth cessation for *M. wufengensis* was at 24 and 15 d under the control and ABA treatments, respectively. Although vegetative growth in autumn has been shown to negatively correlate with winter hardiness (Dhont 2006), our results seemingly suggest that shoot growth was inhibited by ABA only in the cold-sensitive species *M. wufengensis* but not in the cold-tolerant species *M. denudata*. This might be because the shoots of *M. denudata* already elongated very slowly in early September, implying interspecific difference in the timing of initiating cold acclimation, as growth cessation is an early physiological step in dormancy and cold acclimation processes (Williams *et al.* 1972).

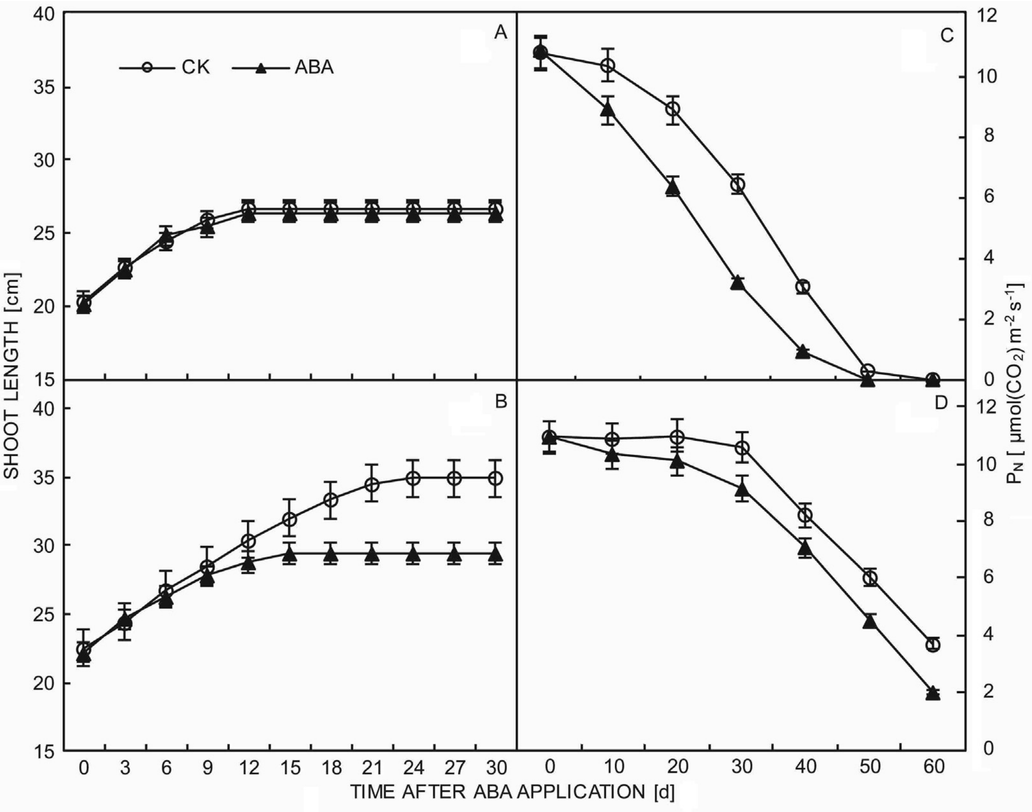

Fig. 1. The effect of abscisic acid (ABA; 600 mg dm^{-3}) on shoot growth and net photosynthetic rate (P_N) of *Magnolia denudata* (*A*,*C*) and *Magnolia wufengensis* (*B*,*D*). CK - control without ABA. Means ± SDs (n = 10).

Leaf senescence is another early step in dormancy and cold acclimation (Kozlowski and Pallardy 2002). Leaf P_N significantly ($P < 0.05$) decreased due to ABA treatment in both *Magnolia* species (Fig. 1*C*,*D*). The effect of ABA on leaf senescence has been demonstrated previously in *Arabidopsis thaliana* with an up-regulation of two senescence-associated mRNAs (pSEN4 and pSEN5; Park *et al.* 1998). In the present study, the rates of ABA-induced P_N declines were different between the species (Fig. 1*C*,*D*). By day 10 after ABA application, a

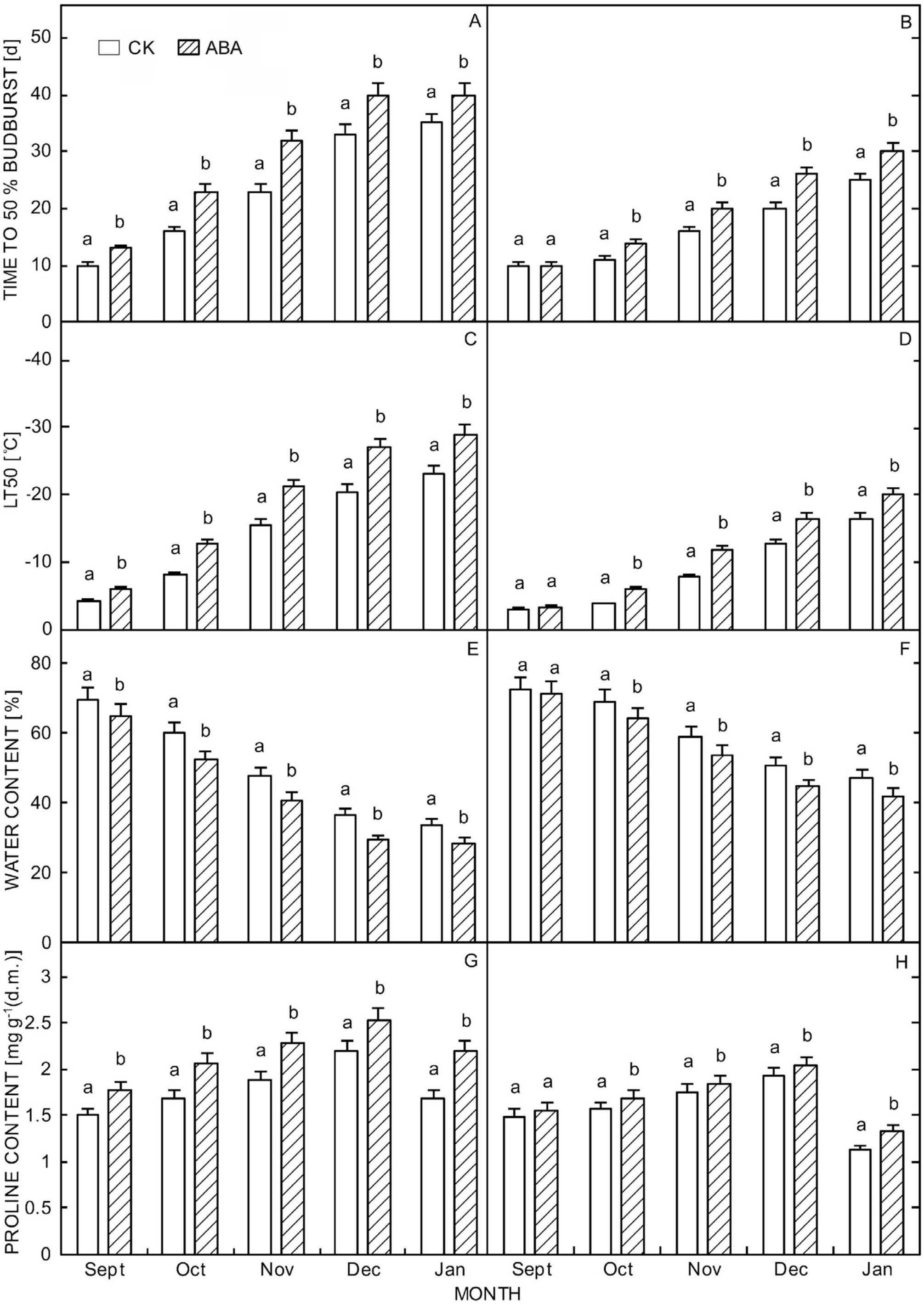

Fig. 2. The effect of ABA on bud dormancy (estimated as number of days to a 50 % budburst), shoot freezing tolerance (estimated as LT_{50}), and water and proline content in the shoots of *Magnolia denudata* (*A*,*C*,*E*,*F*) and *M. wufengensis* (*B*,*D*,*F*,*H*). CK and ABA mean ABA application of 0 and 600 mg·dm^{-3}, respectively. Means ± SDs (n = 10). Different letters mean significant differences ($P < 0.05$) between treatments.

significant ($P < 0.05$) decrease of P_N was detected only in *M. denudata* (Fig. 1*C*) but not in *M. wufengensis* (Fig. 1*D*). Compared with the CK treatment, the P_N values of *M. denudata* treated with ABA decreased by 1.46, 2.53, 3.25, and 2.11 μmol(CO_2)·m^{-2}·s^{-1} on days 10, 20, 30, and 40, respectively, after ABA application; the corresponding values for *M. wufengensis* were 0.54, 0.89, 1.41, and 1.13 μmol(CO_2) m^{-2} s^{-1}, respectively. These results suggest that ABA accelerated leaf senescence more in *M. denudata* than in *M. wufengensis*.

Although there was lack of direct data, ABA application may have resulted in an increase in endogenous ABA content in the two *Magnolia* species. An nncreased endogenous ABA content has been associated with dormancy (Alvim *et al.* 1976, Kucera *et al.* 2005), and in this study, D50B values significantly ($P < 0.05$) increased under ABA application in both *M. denudata* and *M. wufengensis* (Fig. 2*A*,*B*). The rates of ABA-induced dormancy differed between species: by late September, a significant increase ($P < 0.05$) of D50B was detected in the ABA treated *M. denudata* (Fig. 2*A*) but not in *M. wufengensis* (Fig. 2*B*). Compared with the CK treatment, the D50B of *M. denudata* increased 3, 7, 9, 7, and 5 d after ABA application in September, October, November, December, and January, respectively; the corresponding values for *M. wufengensis* were 0, 3, 4, 6, and 5 d. Thus, dormancy development under the ABA treatment was faster in *M. denudata* than in *M. wufengensis*.

Our results suggest that freezing tolerance of shoots, based on the LT_{50} values, was significantly improved ($P < 0.05$) by exogenous ABA in both *Magnolia* species, but the rates differed between species (Fig. 2*C*,*D*). By late September, a significant decrease ($P < 0.05$) of LT_{50} after the ABA treatment was detected only in *M. denudata* (Fig. 2*C*) but not in *M. wufengensis* (Fig. 2*D*). Compared with the CK treatment, the LT_{50} of *M. denudata* under ABA application decreased by 1.68, 4.46, 5.62, 6.58, and 5.90 °C in September, October, November, December, and January, respectively; the corresponding values for *M. wufengensis* were 0.31, 2.18, 4.20, 3.59, and 3.64 °C. This suggests that the cold acclimation process in *M. denudata* was more responsive to exogenous ABA than in *M. wufengensis*. It has been reported that the northern ecotype of *Betula pendula* is more responsive to ABA application than the southern ecotype under controlled conditions (Li *et al.* 2003). Our results supported this view under field conditions.

An increased freezing tolerance is commonly associated with a reduced water content (Guy 2003), which was well exhibited in both *Magnolia* species as the water content in shoots was significantly decreased ($P < 0.05$) under the ABA treatment (Fig. 2*E*,*F*), and there was a significant positive correlation ($P < 0.01$) between the water content and the LT_{50} (Table 1). Thus, the decreased water content may at least partially explain the better freezing tolerance under ABA application for the two *Magnolia* species. It has been demonstrated previously in grapevine that water stress in late autumn can help plants acquire an early cold acclimation and dormancy (Keller 2010). The rates of ABA-induced dehydration differed between the species (Fig. 2*E*,*F*). By late September, a significant decrease ($P < 0.05$) in water content induced after the ABA treatment was found in *M. denudata* (Fig. 2*E*) but not in *M. wufengensis* (Fig. 2*F*). Compared with the CK treatment, the water content of *M. denudata* under ABA application decreased by 4.66, 7.89, 6.99, 7.05, and 5.35 % in September, October, November, December, and January, respectively; the corresponding values for *M. wufengensis* were 0.94, 5.20, 5.30, 5.86, and 5.41 %, respectively. Thus, the water content in shoots of *M. denudata* decreased faster than that of *M. wufengensis* under ABA application.

A significant increase ($P < 0.05$) in proline content was also induced by exogenous ABA (Fig. 2*G*,*H*), and a significant negative correlation between the proline content and the LT_{50} (Table 1) was found in both *Magnolia* species. Thus, a higher accumulation of proline under ABA application might also account for an increased freezing tolerance. As an amphiphilic molecule, proline can bind to hydrophobic surfaces using its hydrophobic moieties, converting them to hydrophilic ones. Such conversions enable a cell to preserve the structural integrity of cytoplasmic proteins under dehydration conditions that develop under drought, salinity, and freezing (Papageorgiou and Murata 1995). Moreover, there is evidence that proline functions as a free radical scavenger (Matysik *et al.* 2002) and a redox potential buffer (Hare and Cress 1997). The rates of ABA-induced accumulation of proline differed between the two species. By late September, a significant increase ($P < 0.05$) of proline content was detected after the ABA treatment only in *M. denudata* (Fig. 2*G*) but not in *M. wufengensis* (Fig. 2*H*). Compared with the CK treatment, the proline content in *M. denudata* under ABA application increased by 0.27, 0.38, 0.39, 0.34, and 0.50 mg·g^{-1}(d.m.) in September, October, November, December, and January, respectively; the corresponding values in *M. wufengensis* were 0.06, 0.11, 0.08, 0.11, and 0.21 mg·g^{-1}(d.m.). Thus, proline accumulated faster in the shoots of *M. denudata* than in those of *M. wufengensis* under ABA application.

Among the major soluble sugars, glucose and fructose have been shown to act in water replacement to maintain membrane phospholipids in the liquid-crystalline phase and to prevent structural changes in soluble proteins when cells suffer from frost-induced water deficit (Bravo *et al.* 1998). In our study, both glucose content and fructose content increased after the ABA application in the two *Magnolia* species (Fig. 3*A-D*). Moreover, the glucose and fructose content were highly correlated with the LT_{50} (Table 1), suggesting a putative role for these two soluble sugars in the freezing tolerance of both species. Indeed, the accumulation patterns of glucose and fructose have frequently been shown to correlate with freezing tolerance in herbaceous plants (Guy *et al.* 1992, Gusta *et al.* 2004), whereas in woody plants, the majority of studies have found no implication of hexoses in cold acclimation (Cox and Stushnoff 2001, Kasuga *et al.* 2007). Thus, it is noticeable that the alterations in content of glucose and fructose paralleled the alterations in freezing tolerance in the *Magnolia* plants. However, the rates of ABA-induced accumulation of these two sugars differed between the two *Magnolia* species. A significant increase ($P < 0.05$) of glucose and fructose content induced by ABA was detected only in *M. denudata* (Fig. 3*A*,*C*) but not in *M. wufengensis* (Fig. 3*B*,*D*) by late October. Compared with the CK treatment, the glucose content in *M. denudata* under ABA application increased by 0.24, 4.44, 6.64, 5.85, and 5.69 mg·g^{-1}(d.m.) in September, October, November, December, and January, respectively, whereas the corresponding values in *M. wufengensis* were 0.07, 0.40, 2.00, 3.82, and 1.90 mg·g^{-1}(d.m.). Fructose content in *M. denudata* under ABA application increased by 0.53, 2.98, 6.40, 6.05, and 5.56 mg·g^{-1}(d.m.) in September, October, November, December, and January, respectively, whereas the corresponding values in *M. wufengensis* were 0.06, 0.97, 4.55, 2.33, and 2.30 mg·g^{-1}(d.m.). Shoot glucose and fructose in *M. denudata* accumulated faster than those in

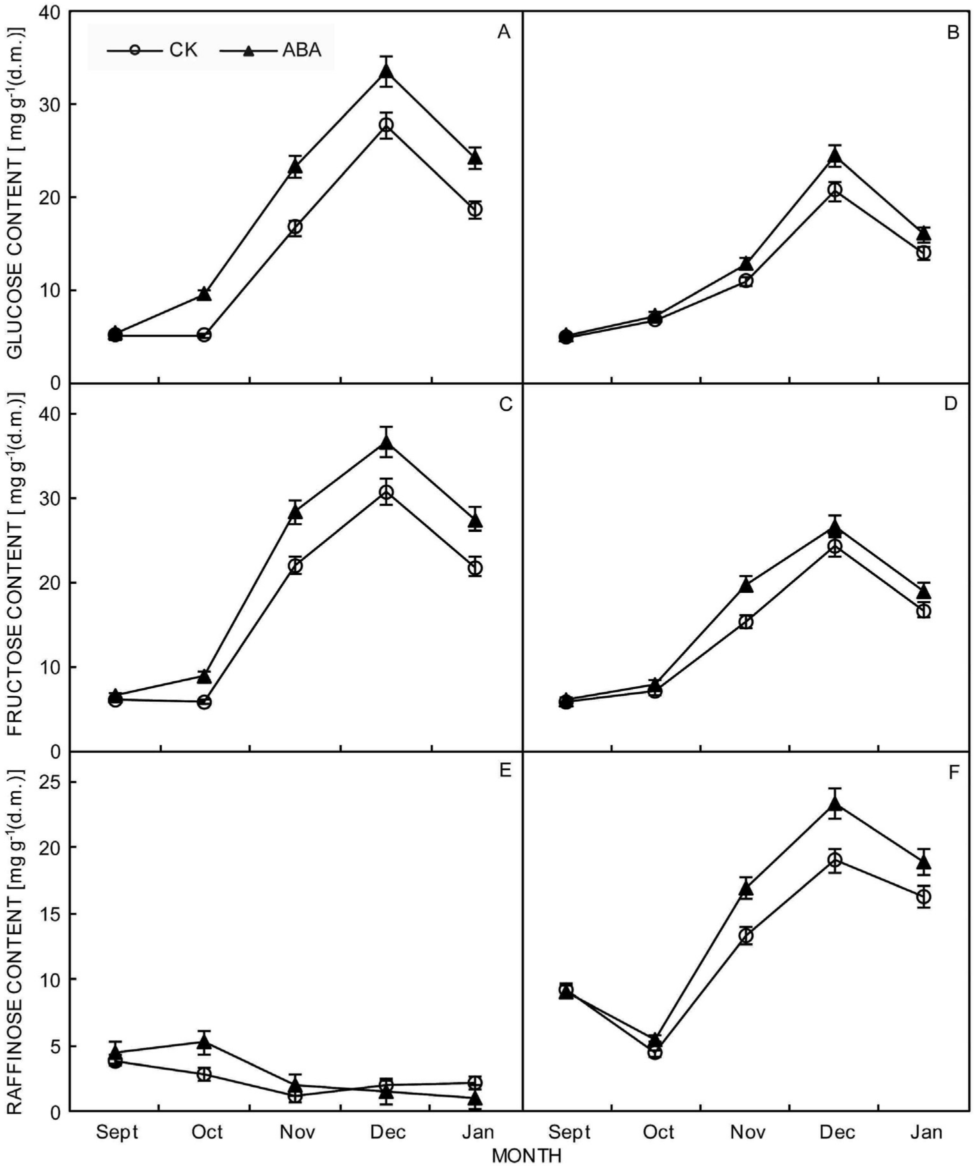

Fig. 3. The effect of ABA on glucose, fructose, and raffinose content in the shoots of *Magnolia denudata* (*A*,*C*,*E*) and *M. wufengensis* (*B*,*D*,*F*). CK and ABA mean ABA application of 0 and 600 mg dm^{-3}, respectively. Means ± SDs ($n = 10$).

M. wufengensis in the ABA treatment.

Another marked soluble sugar raffinose has been shown to play a cryoprotective role by protecting cell membranes, stabilizing proteins, and retaining enzyme activities (Anchordoguy *et al.* 1987, Hincha *et al.* 1993, Stushnoff *et al.* 1997). Raffinose content after the ABA application significantly ($P < 0.05$) increased only during early cold acclimation in *M. denudata* (Fig. 3*E*) but throughout cold acclimation in *M. wufengensis* (Fig. 3*F*). Additionally, a significant correlation between the raffinose content and the LT_{50} was detected in *M. wufengensis* but not in *M. denudata* (Table 1). These results suggest the function of raffinose in freezing tolerance might be species-dependent. Previously, an inconsistent relationship between raffinose content and freezing tolerance has been reported. For example, the maximum freezing tolerance of grape buds is not always associated with the highest raffinose content in their tissues (Koussa *et al.* 1998, Jones *et al.* 1999), and a raffinose-deficient mutant can develop cold acclimation without raffinose accumulation within the tissues (Zuther *et al.* 2004). However, we cannot provide a convincing explanation for the underlying mechanism of this observation. A special experiment should be designed for researching this in the future.

Table 1. Correlation coefficients between freezing tolerance (estimated as LT_{50}) and water, proline, glucose, fructose, and raffinose content in the shoots of *Magnolia denudata* and *Magnolia wufengensis* under control (CK) and abscisic acid (ABA) treatments. NS, *, and ** mean not significant, significant at $P < 0.05$, and significant at $P < 0.01$, respectively.

Variable	*M. denudata*		*M. wufengensis*	
	CK	ABA	CK	ABA
Water	0.97**	0.98**	0.98**	0.99**
Proline	-0.89**	-0.92**	-0.80*	-0.91**
Glucose	-0.88**	-0.90**	-0.78*	-0.88**
Fructose	-0.85*	-0.91**	-0.94**	-0.86**
Raffinose	NS	NS	-0.80*	-0.87**

In conclusion, our results suggest that exogenous ABA application can contribute to increased cold acclimation and cold hardiness of both *M. denudata* and *M. wufengensis*. Distinct differences were found in the physiological and biochemical responses to exogenous ABA between these two *Magnolia* species, and *M. denudata* was more responsive to the applied ABA than *M. wufengensis*, possibly as result of species-specific differences in dehydration rate and the accumulation of proline and soluble sugars.

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