Effects of acclimation and pretreatment with abscisic acid or salicylic acid on tolerance of *Trigonobalanus doichangensis* to extreme temperatures

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Abstract

The effects of acclimation to cold (4 °C) and heat (36/38/40 °C) on corresponding freezing and heat tolerances of one-year-old *Trigonobalanus doichangensis* seedlings were studied. In addition, the effects of abscisic acid (ABA) and salicylic acid (SA) pretreatments on the tolerance of this species to temperature extremes were tested. The results show that the content of soluble sugars increased with the duration of acclimation to cold (4 °C), and the relative electrical conductivity and malondialdehyde content increased significantly after 7 d; however, the content of proline did not vary significantly. After acclimation to cold for 3 and 7 d, the semilethal low temperature (LLT_{50}) was 0.8 and 1.1 °C lower, respectively, compared with that of the control. The maximum quantum yield of photosystem II (measured as variable to maximum fluorescence ratio, F_v/F_m) decreased significantly after freezing treatments (-4 to -8 °C), however, less when the plants were pretreated with 1 - 100 mg dm^{-3} ABA. Acclimation to heat did not increase the semilethal high temperature (LHT_{50}). A low concentration (1 mg dm^{-3}) of SA increased LHT_{50}, but medium and high concentrations (10 and 100 mg dm^{-3}) decreased it. F_v/F_m decreased significantly after a heat shock (45 - 54 °C). The pretreatment with 1 - 50 mg dm^{-3} SA ameliorated a subsequent heat (48 °C) stress.

Additional key words: cold stress, heat stress, plant hormones, semilethal temperature.

Introduction

The genus *Trigonobalanus* shares morphological characteristics with both *Fagus* and *Quercus* (Forman 1964, Crepet and Nixon 1989, Nixon and Crepet 1989) and is considered to be an important taxon for understanding the phylogeny and biogeography of *Fagaceae* (Zhou 1992). However, due to habitat degradation, over-exploitation, and reproductive barriers, *T. doichangensis* is restricted to five locations scattered across north Thailand, and south and southwest Yunnan Province, China. Therefore, there is an urgent need for its conservation.

Abiotic stresses are major threats to plants, and membranes are the first sites of responses (Levitt 1980). Electrolyte leakage and malondialdehyde (MDA) content are often used to assess stress-induced damage (Nautiyal *et al*. 2008, Zhang *et al*. 2012). The ratio of variable to maximal chlorophyll *a* fluorescence (F_v/F_m) of dark-adapted leaves is also used widely to represent the photosynthetic response of plants to stresses (Krause and Weis 1991, Fracheboud *et al*. 1999).

Among the various environmental stresses, temperature is a main factor that limits the distribution of plants. However, during the course of evolution, many plant species have developed mechanisms that enable

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them to minimize the negative effects of cold or heat stress. The ability to acclimate to cold or heat, which means to increase tolerance to severe cold (freezing) or heat stress as the result of previous exposure to moderately sub- or supra-optimal temperatures, is one of adaptive mechanisms (Thomashow 1999). Some molecular and physiological changes that occur during cold or heat acclimation are established (Hsieh *et al.* 2002, Wahid *et al.* 2007, Zhu *et al.* 2007). In addition, abscisic acid (ABA) and salicylic acid (SA) act as signals to trigger and mediate a diverse array of defense responses (Spoel and Dong 2008). Some researchers have shown that ABA and SA could improve plant cold/heat tolerance (Gusta *et al.* 2005, Shi *et al.* 2006, Mutlu *et al.* 2013).

The main objective of the study was to determine the effects of cold or heat acclimation on a subsequent tolerance to a severe cold or heat stress, and the effects of an ABA pretreatment for cold-treated plants, and an SA pretreatment for heat-treated plants on the tolerance of *T. doichangensis*.

Materials and methods

One-year-old seedlings (about 30 cm high) of *Trigonobalanus doichangensis* (A. Camus) Forman grown at the Kunming Botanical Garden (the latitude of 25° 01′ N, the longitude of 102° 41′ E, and the elevation of 1 900 m) were used in this study. Each seedling was cultivated in a plastic bag filled with laterite soil. To test the effects of cold/heat acclimation on cold/heat tolerance of *T. doichangensis*, the seedlings were put in incubators set at 4 °C for 1, 3, 5, or 7 d for cold acclimation, and at 36, 38, or 40 °C for 1.5 h for heat acclimation. To test the effects of exogenous SA on heat tolerance, the seedlings and leaf discs were saturated with SA (1, 10, 50 or 100 mg dm^{-3}) 24 h before the assessment of heat tolerance. To test the effect of ABA on cold tolerance, the leaf discs were incubated in ABA (1, 10, 50 or 100 mg dm^{-3}) for 24 h before being subjected to low temperatures.

Electrical conductivity (EC) was measured as described by Nautiyal *et al.* (2008) with slight modifications. Leaf discs of 8 mm in diameter were punched from leaves of seedlings that were subjected to the cold/heat acclimation and SA pretreatment. Nonacclimated seedlings were used as control. The leaf discs were washed thoroughly with distilled water to remove electrolytes adhering to their leaf surfaces. To assay cold tolerance, three leaf discs were placed in individual test tubes containing 3 cm^3 of pre-cooled distilled water. These tubes were put in a programmable temperature incubator and, after being maintained at 0 °C for 2 h, they were cooled at a rate of 2 °C h^{-1} until the target temperature (0, -2, -4, -6, or -8 °C) was reached. They were then maintained at the target temperature for 2 h. The tubes were then withdrawn from the incubator and thawed overnight at 4 °C, after which they were incubated at room temperature for 24 h and EC_1 was measured. To assay heat tolerance, three leaf discs were placed in individual test tubes containing 3 cm^3 of distilled water. These tubes were capped with aluminium foil, exposed to 42, 45, 48, 51, or 54 °C in water baths for 1 h and then allowed to cool at room temperature, after which EC_1 was recorded. The samples were subsequently autoclaved at 120 °C for 30 min to ensure the complete disruption of the cell membrane and then cooled at room temperature. After this, the final electrical conductivity (EC_2) was recorded. Relative electrical conductivity (REC) was calculated as follows: REC [%] = $EC_1/EC_2 \times 100$. The relationship between REC and temperature (T) was described by a sigmoid curve, which was fitted using the following logistic function: REC = $k/(1+ae^{-bT})$, where k is the maximum percentage of cell damage, a and b are equation parameters. According to the regression analysis, the inflection point of temperature (ln a/b) in the equation was the semi-lethal temperature (LT_{50}) (Xia *et al.* 2012).

MDA content was measured as described by Wang (2006). For each treatment, 0.2 g of fresh leaves was homogenized with 5 cm^3 of trichloroacetic acid in an ice bath. Then, 2 cm^3 of the homogenate was added to 2 cm^3 of 0.6 % (m/v) thiobarbituric acid. Each sample was placed in a boiling water bath for 30 min and then placed in cold water for rapid cooling. Absorbances were read at 450, 532 and 600 nm using a spectrophotometer (*UV-2450*, *Shimadzu*, Kyoto, Japan).

Proline accumulation was determined by the method described by Roy *et al.* (2009). For each treatment, 0.5 g of fresh leaves was homogenized with 10 cm^3 of 3 % (m/v) sulphosalicylic acid and 2 cm^3 of the homogenate was added to 2 cm^3 of glacial acetic acid and 2 cm^3 of acid ninhydrin. The samples were kept at 100 °C in a water bath for 1 h followed by an ice bath. The reaction mixture was then vortexed with 4 cm^3 of toluene. The toluene layer was separated and an absorbance was read at 520 nm using the spectrophotometer *UV-2450*.

Soluble sugar content was measured by the method described by Farhad *et al.* (2011) with slight modifications. For each treatment, 0.5 g of fresh leaves was homogenized with 5 cm^3 of 75 % (v/v) ethanol, and 0.1 cm^3 of the alcoholic homogenate was mixed with 3 cm^3 of anthrone. The samples were then placed in a boiling water bath for 30 min. An absorbance was estimated at 625 nm using the spectrophotometer *UV-2450*. The content of soluble sugars was determined using a glucose standard.

The parameters of chlorophyll *a* fluorescence were measured at room temperature with a fluorometer *PAM-2500* (Walz, Germany). Leaf discs of 8 mm in diameter were punched out from leaves of cold-treated

(0 to -8 °C) or heat treated (42 to 54 °C) seedlings acclimated or pretreated with SA and ABA. They were adapted in the dark for 30 min before measurements. Then the F_v/F_m ratio was determined.

The experimental design was completely randomized with 10 replicates for the electrolyte leakage assay and 5 replicates for the MDA, proline, soluble sugars, and F_v/F_m assays. Statistical analyses were performed using the *SPSS 15.0* software. The data were subjected to the one-way analysis of variance. Significant differences between means were tested by the Fisher's least significant difference (LSD) test at 0.05 and 0.01 levels of probability.

Results

The seedlings of *T. doichangensis* were acclimated to cold at 4 °C for 1, 3, 5 or 7 d. REC and the MDA content of the seedlings that were acclimated for 1, 3 or 5 d did not differ significantly compared with those of the control (Fig. 1). However, both the parameters increased significantly after 7 d of the acclimation ($P < 0.01$), by 38 and 20 %, respectively. The proline content did not vary significantly between the control and acclimated seedlings, being in average 0.06 mg g^{-1}(f.m.). The content of soluble sugars increased significantly with the acclimation time ($P < 0.01$) by 45 and 104 % after 3 and 7 d of the acclimation, respectively. However, there were no significant differences between 1 and 3 d of the acclimation and between 5 and 7 d of the acclimation.

The values of LLT$_{50}$ of the control and the seedlings acclimated for 3 and 7 d were estimated. The changes of REC of the seedlings at the low temperatures (Fig. 2*A*) were described by sigmoid curves (correlation coefficients $\geq$ 0.95) whose inflection points predicted LLT$_{50}$. When the treatment temperature was above -2 °C, REC increased slowly as the temperature decreased. When the temperature reached -4 °C, REC increased sharply, and when the temperature was lower than -6 °C, REC was stable. LLT$_{50}$ of the control was -3.9 °C, and those of the seedlings that were acclimated for 3 and 7 d decreased by 0.8 and 1.1 °C, respectively (Table 1).

The F_v/F_m ratio of leaves treated at 0 and -2 °C did not change significantly compared with that of the control (0.8016). However, F_v/F_m decreased significantly in leaves treated at -4 to -8 °C (Fig. 2*B*). As F_v/F_m of leaves began to decrease significantly from -4 °C, the effect of the pretreatments with ABA (1, 10, 50 or 100 mg dm^{-3}) on this variable for leaves treated at -4 °C was measured. The F_v/F_m ratios of leaves pretreated with ABA were higher compared with those of the control (without ABA), but there was no significant variation among different concentrations of ABA (Fig. 3*A*).

LHT$_{50}$ of nonacclimated, heat-acclimated and SA-pretreated seedlings were also estimated. The changes in REC (Fig. 4*A,B*) were described by sigmoid curves (correlation coefficients > 0.91.) whose turning points
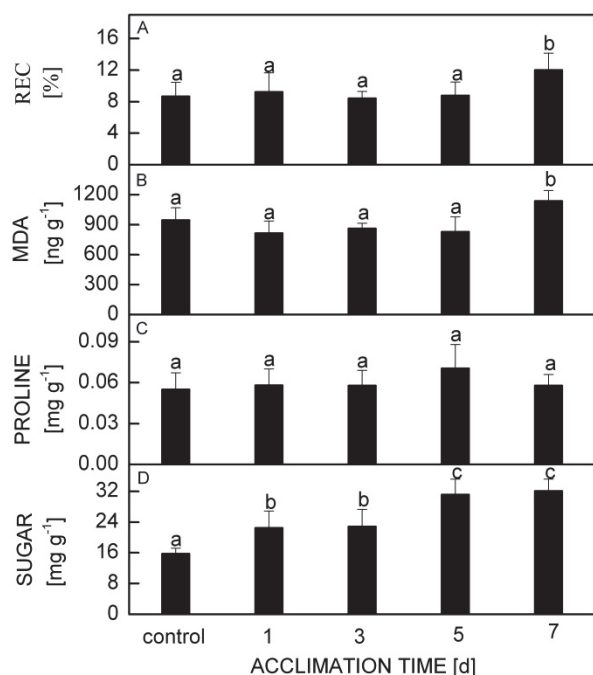

Fig. 1. Effects of acclimation to cold at 4 °C for different numbers of days on REC (*A*), and MDA (*B*), proline (*C*), and soluble sugar (*D*) content per fresh mass unit in *T. doichangensis* leaves. Means ± SD, *n* = 5, The *bars* indicated by different lowercase letters are significantly different at $P < 0.01$ (the LSD test).
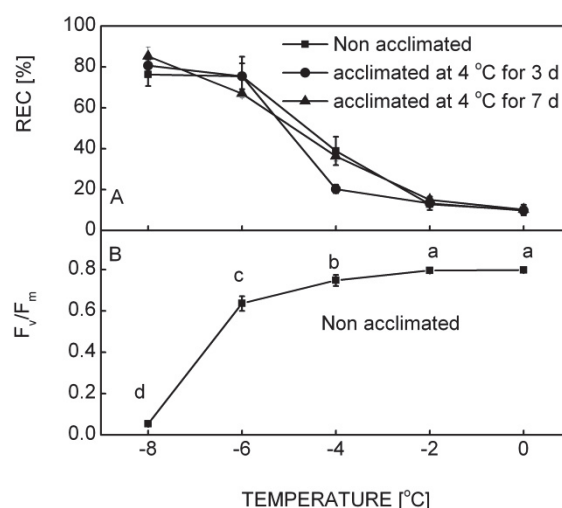

Fig. 2. Changes of REC (*A*) and F_v/F_m (*B*) in *T. doichangensis* leaves at low temperatures. Means ± SD, *n* = 10 for REC, *n* = 5 for F_v/F_m. Different letters mark significant differences at $P < 0.01$.

predicted LHT_{50}. When the treatment temperature was below 45 °C, REC increased slowly as the temperature increased. When the temperature reached 45 °C, REC increased sharply, and when the temperature was higher than 51 °C, REC was stable (Fig. 4*A*,*B*). The LHT_{50} of

Table 1. The correlation coefficients (R^2) between REC and temperature, semilethal low temperature (LLT_{50}), and semi-lethal high temperature (LHT_{50}) of *T. doichangensis*. * and ** indicate that the correlation is significant at 0.05 and 0.01 levels, respectively.

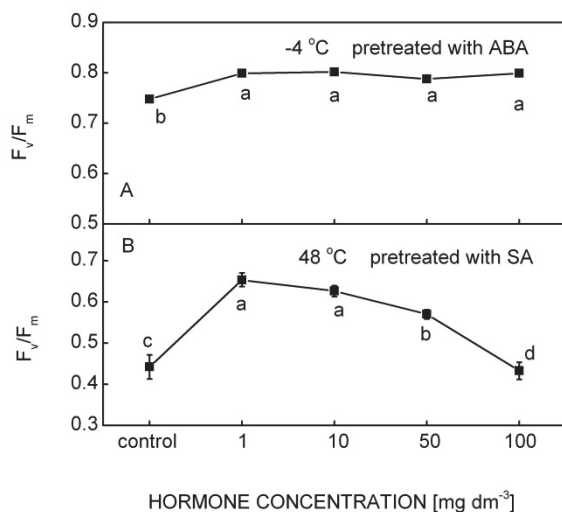| Treatment	R^2	LLT_{50} [°C]	LHT_{50} [°C]
Control	0.976**	-3.9	
Acclimation for 3 d at 4 °C	0.950**	-4.7	
Acclimation for 7 d at 4 °C	0.994**	-5	
Control	0.991**		48
Acclimation at 36 °C for 1.5 h	0.990**		47.4
Acclimation at 38 °C for 1.5 h	0.989**		47.3
Acclimation at 40 °C for 1.5 h	0.998**		48.3
Pretreatment with 1 mg dm^{-3} SA	0.916*		58.2
Pretreatment with 10 mg dm^{-3} SA	0.978**		47.1
Pretreatment with 100 mg dm^{-3} SA	0.978**		45.3

Fig. 3. Effects of ABA or SA pretreatments on F_v/F_m of *T. doichangensis* leaves treated at -4 °C (*A*) and 48 °C (*B*), respectively. Means ± SD, n = 5. Different letters mark significant differences at $P < 0.01$.

Fig. 4. Changes of REC (*A*,*B*) and F_v/F_m (*C*) in *T. doichangensis* leaves at high temperatures. The effect of heat acclimation (*A*) or SA (*B*) on REC and effect of high temperature on F_v/F_m (*C*). Means ± SD, n = 10 for REC, n = 5 for F_v/F_m. Different letters mark significant differences at $P < 0.01$.

the control was 48 °C, and LHT_{50} of seedlings acclimated at 36, 38, or 40 °C for 1.5 h were 47.4, 47.3, and 48.3 °C, respectively (Table 1). LHT_{50} of seedlings pretreated with 1 mg dm^{-3} SA increased markedly, reaching 58.2 °C. However, LHT_{50} of seedlings pretreated with 10 and 100 mg dm^{-3} SA decreased by 0.9 and 2.7 °C, respectively, compared with that of the control (Table 1).

The F_v/F_m ratio of leaves treated at 42 °C did not differ significantly from the control (0.8016), but it decreased significantly with an increase of temperature and reached zero in leaves treated at 54 °C (Fig. 4*C*). The effects of the pretreatments with SA (1, 10, 50 or 100 mg dm^{-3}) on F_v/F_m of leaves treated at 48 °C were also measured. The results show that the F_v/F_m ratios of leaves pretreated with 1, 10, or 50 mg dm^{-3} SA were significantly higher compared with those of the control (without SA); however, they were lower in leaves pretreated with 100 mg dm^{-3} SA (Fig. 3*B*).

Discussion

Plants are exposed continually to a variety of biotic and abiotic stresses. This has led to the evolution of adaptive mechanisms that enable plant cells to sense changes in the environment and to activate responses that increase their tolerance to subsequent stresses (Heino and Palva 2003, Naliwajski and Skłodowska 2014). Temperature
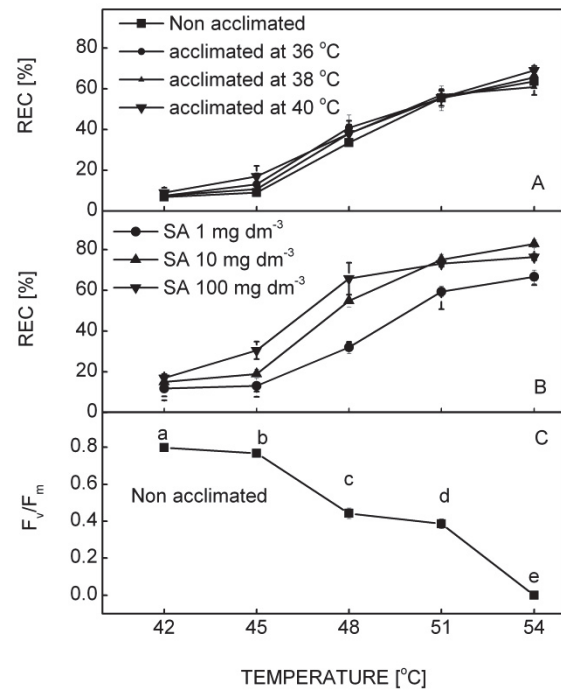
acclimation is one such mechanism. Cold/freezing tolerance of many plants can be improved through cold acclimation (Heino and Palva 2003, Minami *et al.* 2005). It has been reported that acclimation to cold causes various biochemical and physiological changes in plant cells including the expression of numerous genes, the

accumulation of proteins, soluble sugars, and proline, and changes in membrane lipid composition (Guy 1990, Thomashow 1999, Iba 2002, Wahid *et al.* 2007, Theocharis *et al.* 2012). However, some of these changes are not observed in all species (Kayihan *et al.* 2012). To test the effect of cold acclimation on the freezing tolerance of *T. doichangensis*, seedlings were acclimated to cold at 4 °C. REC and the MDA content of these seedlings increased significantly when they were acclimated for 7 d, which suggest that the exposure to this temperature for a longer period could damage *T. doichangensis*. Proline is considered to be a compatible solute. It protects folded protein structures against denaturation, stabilises cell membranes by interacting with phospholipids, functions as hydroxyl radical scavenger, and serves as source of energy and nitrogen (Aspinall and Paleg 1981, Samaras *et al.* 1995). Although proline accumulates robustly under cold stress in some plants (Koster and Lynch 1992, Kaplan *et al.* 2007), its contribution to freezing tolerance remains a matter of debate (Ruelland *et al.* 2009). It has been reported that the temporal patterns of proline accumulation at a cold temperature vary among different plant species (Koster and Lynch 1992, Kaplan *et al.* 2007). The proline content of *T. doichangensis* that was acclimated to cold for 1 - 7 d did not change significantly, which might be related to the short duration of this treatment. In the process of cold acclimation, the soluble sugars content of *T. doichangensis* increased significantly with increasing duration of the acclimation. Many other studies have also shown that the content of soluble sugars increases dramatically during acclimation to cold (Kaplan *et al.* 2004, Theocharis *et al.* 2012). Although the precise function of soluble sugars remains to be determined, its accumulation in plants that are acclimated to cold might be related to its roles as osmoregulators, cryoprotectants, signaling molecules, or reactive oxygen species (ROS) scavenger (Bohnert and Sheveleva 1998, Welling and Palva 2006).

Due to cold acclimation, many woody plants can seasonally increase their freezing tolerance down to -50 °C, despite otherwise being killed at about -10 °C (Sakai and Larcher 1987). Depending on the plant species, it may take a few days to several weeks to reach the maximum level of freezing tolerance (Webb *et al.* 1994). LLT_{50} of *T. doichangensis* seedlings that were acclimated to the cold for 3 and 7 d decreased by 0.8 °C and 1.1 °C, respectively. However, it remains to be seen in future whether the freezing tolerance of *T. doichangensis* will be increased further by an increased duration of acclimation to cold.

Some evidence suggests that ABA can substitute for a low-temperature stimulus, whereas other studies have shown either little or no effect of ABA on the development of freezing tolerance (Gusta *et al.* 2005). Because of these conflicting results, several reseachers have suggested that there may be ABA-dependent and ABA-independent pathways associated with acclimation to cold (Theocharis *et al.* 2006). The F_v/F_m ratio was reduced significantly by the cold stress, but less when leaves were pretreated with ABA. Therefore, ABA might be involved in the freezing tolerance of *T. doichangensis*.

It is well-documented that acclimation to heat can improve the heat tolerance of some plants (Wahid *et al.* 2007); however, the acclimation did not improve the heat tolerance of *T. doichangensis*. Some studies have shown that the induction of heat shock proteins (HSPs) during acclimation to heat is associated with the development of heat tolerance (Marmiroli *et al.* 1997, Sridevi *et al.* 1999). In our preliminary study, HSP70 of *T. doichangensis* seedlings that were acclimated to heat was measured, but none was detected. The expression of HSPs should be tested further during acclimation to heat to determine whether HSPs affect the development of heat tolerance in *T. doichangensis*.

SA is long known to be a signal molecule that is involved in the induction of defense mechanisms in plants. Pretreatment of plants with a low concentration of SA might have an effect similar to that of acclimation, causing increased tolerance to most kinds of abiotic stresses (Horváth *et al.* 2007b). In *T. doichangensis*, the F_v/F_m ratio decreased significantly by the heat stress, however, less after the pretreatment with low concentrations (1 - 50 mg dm^{-3}) of SA, and more after the pretreatment with a high SA concentration (100 mg dm^{-3}). In addition, the semilethal high temperature of *T. doichangensis* increased by the pretreatment with SA at 1 mg dm^{-3}, but decreased by the pretreatments at 10 and 100 mg dm^{-3}. It may be concluded that SA affected the heat tolerance of *T. doichangensis*, but the effect depended on its concentration. It has also been suggested elsewhere that the effect of exogenous SA depends on numerous factors, such as species and developmental stage, the mode of application, and the concentration of SA and its endogenous content in the given plant (Dat *et al.* 2000, Shi *et al.* 2006, Horváth *et al.* 2007a,b).

In summary, the acclimation to the cold and the pretreatment with ABA could increase the freezing tolerance of *T. doichangensis*. The acclimation to the heat did not increase the heat tolerance of the species; SA affected the heat tolerance of *T. doichangensis*, but its effect depended on its concentration.

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