Development of freezing tolerance in different altitudinal ecotypes of *Salix paraplesia*

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Abstract

Salix paraplesia was used as an experimental model to investigate the effect of short day photoperiod (SD) and low temperature (LT) on development of freezing tolerance and on endogenous abscisic acid (ABA) contents. We characterized differences in SD and LT-induced cold acclimation in three ecotypes from different altitudes. The results demonstrated that cold acclimation could be triggered by exposing the plants to SD or LT alone, and that a combination of the different treatments had an additive effect on freezing tolerance in all ecotypes studied. However, the high altitudinal ecotype was more responsive to SD and LT than the low altitudinal ecotype. Development of freezing tolerance induced by SD and LT was accompanied by changes in ABA contents which were ecotype-dependent. Although the stem had higher initial freezing tolerance, the leaves developed freezing tolerance more quickly than the stem and thus leaves may provide an interesting experimental system for physiological and molecular studies of cold acclimation in woody plants.

Additional key words: abscisic acid, cold acclimation, low temperature, short-day photoperiod.

Introduction

Many plant species, have the ability to cold acclimate, for example, exposure to nonfreezing low temperature (LT) will increase tolerance to freezing stress both in short term as in annual herbaceous plants and seasonally as in overwintering herbaceous and woody plants. Cold acclimation is a complex process involving a number of biochemical and physiological changes (Palva and Heino 1998, Thomashow 1999, Pinedo *et al*. 2000, Szalai *et al*. 2000, Atici *et al*. 2003). There is an increasing body of evidence to show that many of these biochemical and physiological alterations are regulated by changes in gene expression (Palva 1994, Lee *et al*. 1999, Xin and Browse 2000, Nuotio *et al*. 2001). The ability to cold acclimate is under genetic control, and development of freezing tolerance is generally triggered by LT, but can also be induced by mild water stress or exogenous abscisic acid (Lång *et al*. 1989, 1994, Mäntylä *et al*. 1995, Tamminen *et al*. 2001, Li *et al*. 2003).

Cold acclimation in trees is a two-stage process, where the first stage is triggered by short day photoperiod (SD) and the second by LT required to achieve full winter hardiness (Weiser 1970). Photoperiod has a central role as the primary signal to induce growth cessation and formation of terminal buds, resulting in bud dormancy and initiation of cold acclimation (Irving and Lanphear 1967, Fuchigami *et al*. 1971, Junttila and Kaurin 1990, Welling *et al*. 1997). Subsequent exposure to LT appears to be the main factor required for freezing tolerance increase (Stushnoff and Junttila 1986, Rinne *et al*. 1999, Li *et al*. 2002).

Abscisic acid (ABA), a well-known stress-inducible plant hormone and growth inhibitor (Davies and Jones 1991), has long been studied as a potential mediator for development of freezing tolerance in plants. Studies

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Abbreviations: ABA - abscisic acid; LD - long day photoperiod; LT - low temperature; SD - short day photoperiod.
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with herbaceous plants strongly indicate an important role for ABA in cold acclimation as well as in drought stress responses (Skriver and Mundy 1990, Giraudat *et al*. 1994, Lee *et al*. 1999, Tamminen *et al*. 2001, Faltusová-Kadlecová *et al*. 2002). ABA seems to play a predominant role in the conversion of environmental signals into changes in plant gene expression (Rock 2000, Browse and Xin 2001, Zhu 2002). In woody plants, it has been shown to be involved in promoting cold hardiness, both from the use of applied exogenous ABA to intact plants and from measurement of the endogenous ABA contents (Welling *et al*. 1997, Baldwin *et al*. 1998, Li *et al*. 2002, 2003).

We wanted to elucidate the roles and interactions of the different environmental cues, such as SD and LT, on development of freezing tolerance and on endogenous ABA contents in woody plant. To this aim we used *Salix paraplesia* ecotypes, which were from different altitudinal gradients, as plant materials to characterize the cold acclimation process in different tissues of *Salix paraplesia* ecotypes by short term acclimation under controlled conditions. These studies suggested that there were different responses to SD and LT in different ecotypes. Subsequent analysis of ABA contents also showed that increase in ABA contents could be related to cold acclimation in an ecotype-dependent manner.

Materials and methods

Plants and growth conditions: Three ecotypes of *Salix paraplesia* C.K. Schneider, which were from different altitudes, *i.e.*, 1550, 2680 and 3720 m, in southwest China (32º25' - 32º53' N, 104º20' - 104º41' E), were used in our study. Plants were initially propagated by tissue culture, and then transferred to plastic pots containing fertilized peat as growth medium. 90 pots of each ecotype were watered to 100 % of field capacity by supplying an amount of water equal to transpiration losses every other day. The plants were grown in a phytotron at 18 ºC with a 20-h photoperiod as long day photoperiod (LD), fluorescent lamps (*TL 65W/83* fluorescent tubes, *Philips*, Eindhoven, The Netherlands) gave a photon flux density of 90 - 100 μmol m^{-2} s^{-1} at 400-740 nm. After 12 weeks the plants were about 45 cm in height. In each ecotype, two thirds of the plants were still grown under LD for 2 weeks, then half of the plants were grown at 18 ºC with a 10-h photoperiod condition as SD for 2 weeks, another half of the plants were grown at 4 ºC with a 20-h photoperiod condition as LT for 2 weeks. Another one third of the plants were grown under SD for 2 weeks, then transferred to 4 ºC with a 10-h photoperiod condition as SD + LT for 2 weeks.

Determination of freezing tolerance: Freezing tolerance was determined from five replicate samples. The different tissues, the intermediate leaves and the uppermost part of the stem (10 cm) were measured after exposure to the respective treatment for 0, 1, 2, 4, 7 and 14 d. Leaf pieces and stem pieces were wrapped in *Miracloth* (*Calbiochem*, La Jolla, CA, USA) and placed in test tubes in a controlled freezing bath. Extracellular ice formation was initiated at -1.5 ºC by touching the samples with a wire frozen in liquid nitrogen. After a 1 h equilibration period the bath temperature was decreased by 2 ºC h^{-1}. Samples were withdrawn at 5 ºC intervals and thawed on ice overnight. The extent of freezing injury was determined by the ion-leakage method (Sukumaran and Weiser 1972). Ions were extracted with deionized water (40 cm^3) by shaking the samples for 2 h at room temperature and the conductivity was measured. The samples were put into boiling water 30 min, re-extracted with the original solution by shaking for 2 h at room temperature, and the conductivity was measured again. LT_{50} was calculated according to the temperature giving 50 % ion leakage.

Quantitative analysis of ABA: Three replicate samples of leaves were weighed, frozen in liquid nitrogen and freeze-dried, of which samples of 30 - 50 mg dry mass was homogenized in 5 cm^3 of 50 mM sodium phosphate buffer, pH 7.0 with 0.02 % sodium diethyldithio-carbamate as antioxidant and 30 ng [$^2H_4$]ABA as internal standard. The samples were extracted for 2 h at 4 ºC under shaking, then centrifuged at 9 000 *g*. The supernatant was adjusted to pH 2.7, shaken with 150 mg *Amberlite XAD-7* (*Sigma Chemical Company*, St Louis, MO, USA) for 30 min at 4 ºC, the buffer removed by pipetting carefully, and the *Amberlite* washed with 2 × 4 cm^3 1 % acetic acid. The *Amberlite* was dried in *SpeedVac* (*DuPont SORVALL Products*, Newtown, CN, USA), ABA was eluted from *Amberlite* with 2 × 2.5 cm^3 dichloromethane with shaking for 30 min each time at 4 ºC, and evaporated to dryness in *SpeedVac*. After that, the samples were methylated with ethereal diazomethane and dryness, dissolved in 0.1 cm^3 50 % methanol and subjected to HPLC using standard commercially available instruments from *Waters* (Milford, MA, USA). A 100 × 8 mm i.d. *C18 Water Novapak* column (4 μm particle size) was operated at 25 ºC. The mobile phase was a 20-min linear gradient of 30 to 70 % methanol in water. The flow rate was 2 cm^3 min^{-1} and a 2-min fraction (retention time 16.2 - 18.2, ABA eluted at 17.2 min) was collected and reduced to dryness (*SpeedVac*). ABA was measured by gas chromatography-mass spectrometry as described by Jensen *et al.* (1986) with selective ion monitoring (SIM). Ions at 190.1 and 194.1 were monitored and amount of ABA in the sample was calculated using a standard curve drawn from the area ratios of known amounts of ABA and [$^2H_4$]ABA.

Measurement of growth and some leaf characteristics: When the seedlings were exposed to SD and LT, height growth was measured every other day during the experimental period. For sampling of leaves, they were tagged at the beginning of the experiment and these leaves were used for analysis and freezing test. Thus the studied leaves in various treatments were at similar stage of development when exposed to the experimental conditions. Leaf water content was calculated (as % of fresh mass) after drying the leaf samples at 80 ºC for 24 h. The occurrence of open stomata on the abaxial surface was examined. A piece of leaf about 0.5 cm^2 in area was excised from the middle of the leaf close to the central vein and between two sub-veins. The leaf specimens were gold-plated to fix the stomata and the abaxial surfaces were observed at 400 × magnification with a *JSM-840* scanning electronic microscope (*JEOL*, Tokyo, Japan).

Statistical analysis: Data were subjected to analyses of variance using the *SYSTAT* statistical software package.

Results

Growth cessation and leaf structural changes: The LD-grown seedlings of the three altitudinal ecotypes of *Salix paraplesia* used ceased elongation growth when transferred to SD or LT condition, but the accurate timing of growth cessation was affected both by the ecotype and the treatment (Fig. 1). LT induced an earlier growth cessation in the high altitudinal (after 6 d) than in the low altitudinal (after 10 d) ecotype. This is in contrast to SD exposure, which stopped stem elongation almost synchronously (after 8 d) in all these ecotypes. SD or LT condition also resulted in decreased leaf water content and number of open stomata, and increased leaf mass to area ratio (Table 1). Compared with the control plants, these structural changes were significant ($P < 0.05$) after 2 weeks exposure of plants to SD or LT alone as well as after exposure to the combination of SD and LT. However, the extent of these leaf structural changes was dependent of the ecotype and the treatment. LT had larger effect than SD, and the combination of SD and LT had the largest effect. In addition, SD and LT affected leaf structural changes more in the high altitudinal ecotype than in the low altitudinal ecotype.

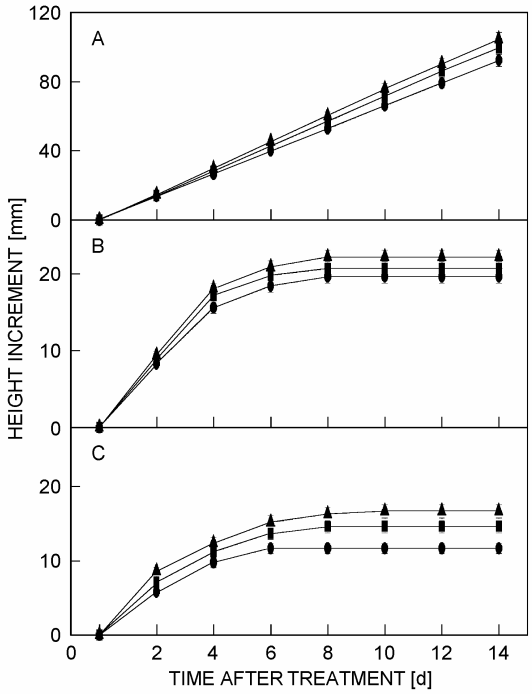

Fig. 1. Apical shoot elongation in seedlings of three ecotypes of *Salix paraplesia* (*circles* - the high altitudinal ecotype, *squares* - the middle altitudinal ecotype, *triagles* - the low altitudinal ecotype) as affected by the different treatments (*A* - 18 ºC with a 20-h photoperiod; *B* - 18 ºC with a 10-h photoperiod; *C* - 4 ºC with a 20-h photoperiod). Means ± SE of 10 replicates.

Table 1. Leaf water content, leaf mass to area ratio and number of open stomata (means ± SE) of three altitudinal ecotypes of *Salix paraplesia* seedlings after exposure for 2 weeks to short day photoperiod (SD), low temperature (LT) or a combination of SD and LT. Control, non-acclimated plants.

Treatment	Altitude	Water content [%]	Leaf mass to area ratio [g dm^{-2}]	Open stomata [No. mm^{-2}]
Control	high	83.9 ± 2.5	0.647 ± 0.018	172 ± 15
	middle	85.2 ± 3.1	0.638 ± 0.016	169 ± 13
	low	84.5 ± 2.9	0.642 ± 0.023	163 ± 12
SD	high	76.3 ± 2.2	0.706 ± 0.021	135 ± 7
	middle	79.8 ± 1.8	0.677 ± 0.019	148 ± 10
	low	82.7 ± 1.5	0.659 ± 0.025	151 ± 9
LT	high	70.2 ± 1.1	0.758 ± 0.032	74 ± 8
	middle	74.7 ± 1.5	0.739 ± 0.029	89 ± 9
	low	78.1 ± 1.6	0.705 ± 0.025	96 ± 7
SD + LT	high	63.7 ± 1.9	0.817 ± 0.039	56 ± 5
	middle	67.8 ± 2.1	0.776 ± 0.032	73 ± 7
	low	71.4 ± 2.4	0.741 ± 0.027	87 ± 8

Development of freezing tolerance: The development of freezing tolerance in leaves was most clearly triggered by LT, but could also be induced by SD in all ecotypes tested (Fig. 2). The rates and degrees of freezing tolerance increase in leaves were different. LT was the most potent inducer of freezing tolerance, while SD only induced a moderate increase in freezing tolerance. However, the effect of LT was enhanced by preceding and simultaneous exposure to SD (Fig. 2). The LT induced tolerance was clearly enhanced by SD.

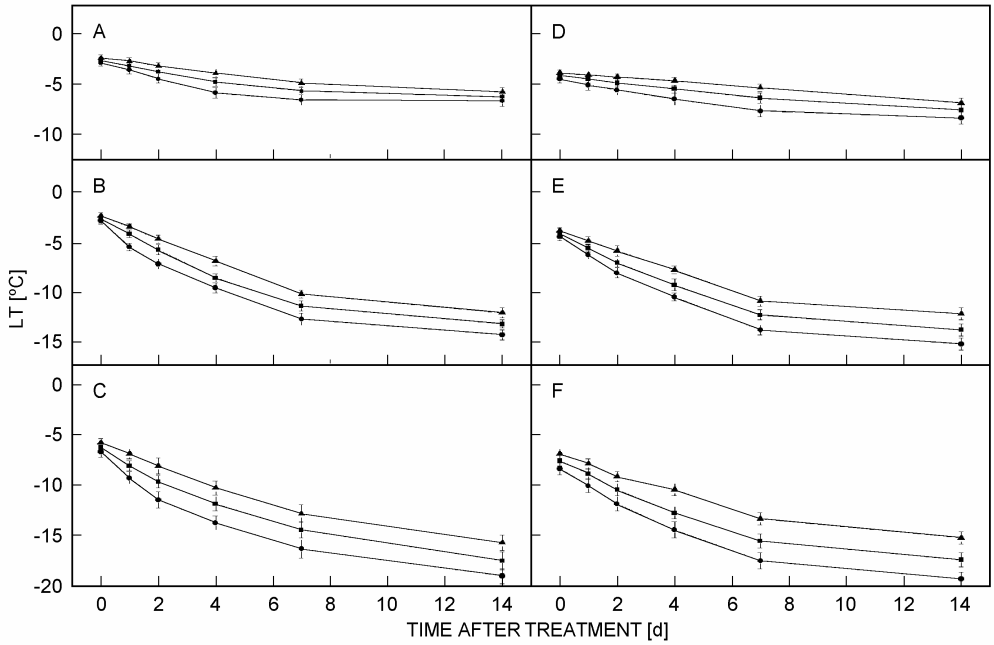

Fig. 2. Freezing tolerance (LT_{50}) of leaves (*A*, *B* and *C*) and stem (*D*, *E* and *F*) in seedlings of three ecotypes of *Salix paraplesia* (*circles* - the high altitudinal ecotype, *squares* - the middle altitudinal ecotype, *triagles* - the low altitudinal ecotype) as affected by the different treatments (*A* and *D* - 18 ºC with a 10-h photoperiod; *B* and *E* - 4 ºC and a 20-h photoperiod; *C* and *F* - 4 ºC with a 10-h photoperiod). Means ± SE of 5 replicates.

A combination of SD and LT showed that the different treatments had additive effects on the development of freezing tolerance in leaves. Compared with the low altitudinal ecotype, the high altitudinal ecotype was more responsive to SD and LT, resulting in an earlier cold acclimation and higher freezing tolerance. Similar results were also shown in the stem (Fig. 2), although the stem had higher initial levels of freezing tolerance, the leaves developed freezing tolerance more quickly than the stem in short term.

Endogenous ABA alterations: To elucidate the possible correlation of freezing tolerance development to ABA contents, we measured ABA content in leaves (Fig. 3). SD and LT exposure affected ABA contents, but the rates and extent of endogenous ABA alterations were different. LT exposure triggered a transient increase in ABA contents. In contrast, SD exposure resulted in a gradual increase in ABA contents for the first 7-d period and remained at a high level during subsequent days. Exposure of plants subjected to 2 weeks SD treatment to LT resulted in a further transient increase in ABA (Fig. 3). Under LT, ABA content increased rapidly in the high altitudinal ecotype, followed by the middle altitudinal ecotype, and the low altitudinal ecotype ABA content increased gradually during the whole 14-d experimental period (Fig. 3). In contrast, SD induced a gradual increase in ABA content in all ecotypes (Fig. 3). Compared with separate exposure to SD and LT, combined SD and LT treatment resulted in a transient increase and decrease in ABA content in all three ecotypes, with the most prominent effect seen in the high altitudinal ecotype.

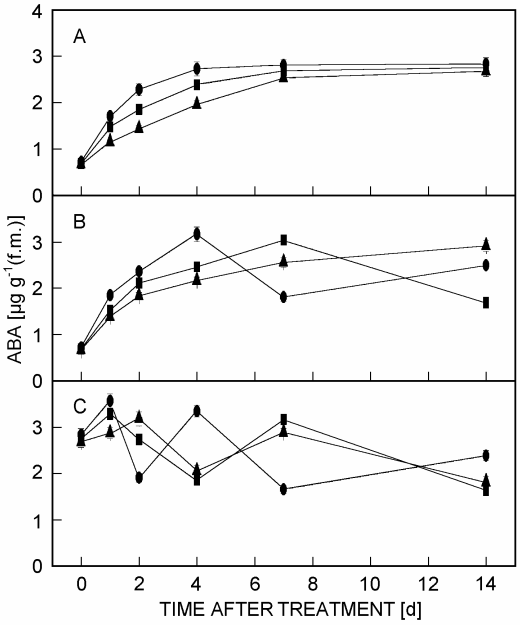

Fig. 3. ABA contents of leaves in seedlings of three ecotypes of *Salix paraplesia* (*circles* - the high altitudinal ecotype, *squares* - the middle altitudinal ecotype, *triagles* - the low altitudinal ecotype) as affected by the different treatments (*A* - 18 ºC with a 10-h photoperiod; *B* - 4 ºC with a 20-h photoperiod; *C* - 4 ºC with a 10-h photoperiod). Means ± SE of 3 replicates.

Discussion

Our results demonstrated that cold acclimation could be triggered by exposing the plants to SD or LT alone, and that a combination of SD and LT had an additive effect on freezing tolerance in all ecotypes of *Salix paraplesia*. Development of freezing tolerance was dependent on the ecotypes, the high altitudinal ecotype was clearly more responsive to SD and LT than the low altitudinal ecotype. Interestingly, development of freezing tolerance induced by SD or LT was accompanied by changes in ABA contents, these alterations in ABA contents were ecotype-dependent, the high altitudinal ecotype reacting more strongly to the environmental cues. The kinetics of acclimation and the degree of freezing tolerance achieved also depended on the environmental cues triggering the response, LT being the most efficient inducer of short-term acclimation. Although the stem had higher initial levels of freezing tolerance, the leaves developed freezing tolerance more quickly than the stem in short term, these data clearly suggest that leaves are more responsive to the environmental cues triggering acclimation than the stem tissue. In addition, acclimation response in leaves was accompanied by changes in structural characters, *i.e.*, increased leaf mass to area ratio, decreased leaf water content and number of open stomata.

Increased freezing tolerance induced by SD has been reported in some previous studies (Arora *et al*. 1992, Welling *et al*. 1997, Repo *et al*. 2000, Rinne *et al*. 2001). To test whether this is a general phenomenon in woody plants, we determined freezing tolerance of *Salix paraplesia* seedlings in response to SD treatment. The data showed that SD did indeed result in increased freezing tolerance. Furthermore, SD appeared to enhance the effect of LT on freezing tolerance. Compared with LT induced tolerance, the effect of SD was relatively moderate. This could depend on the degree of stress and the time of exposure to stress. Similarly, differences in SD and LT-induced freezing tolerance were also shown in previous studies (Olsen *et al*. 1997, Welling *et al*. 2002, Li *et al*. 2002).

The accurate timing of growth cessation and cold acclimation was different between the ecotypes when the plants were exposed to LT, the high altitudinal ecotype exhibited an earlier growth cessation and freezing tolerance development than the low altitudinal ecotype. These responses of *Salix paraplesia* ecotypes might also reflect a particular adaptation, at the low altitudinal gradient, where temperature drops slowly in autumn, and the slow response of the low altitudinal ecotype could be beneficial. It may delay the cessation of height growth and cold acclimation and allow the greater use of favourable growing temperatures in autumn. In contrast, at the high altitudinal gradient, where temperature drops rapidly in autumn, and the rapid responses of the high altitudinal ecotype to LT could be an important adaptation for survival.

The present results give some further support to the hypothesis that ABA is involved in cold acclimation. In general, there were parallel changes between the ABA contents and freezing tolerance development. Several types of evidence have suggested that ABA-controlled processes are central to cold acclimation and development of freezing tolerance in herbaceous plants, *i.e.*, the low temperature stimulus can be substituted by exogenous ABA, resulting in an increase in freezing tolerance at normal growth temperatures (Chen *et al*. 1983, Lång *et al*. 1989, Mäntylä *et al*. 1995, Tamminen *et al*. 2001); endogenous ABA contents can be enhanced by LT both in chilling-sensitive (Daie and Campbell 1981, Pan 1990), chilling-tolerant annual (Chen *et al*. 1983, Guy and Haskell 1988), and overwintering (Lalk and Dörffling 1985) species. In our study, there were corresponding differences between the ecotypes in ABA alterations during cold acclimation: compared with the low altitudinal ecotype, ABA contents of the high altitudinal ecotype was more responsive to SD and LT. Accordingly, ABA may be important for controlling the rate and degree of cold acclimation in *Salix paraplesia*. In addition, the distinct patterns in ABA alterations and freezing tolerance development observed in plants exposed to SD or LT suggested that SD and LT may have separate pathways for induction of ABA increase.

Most studies with deciduous woody plants have focused on investigation cold acclimation in bark and xylem tissues as well as overwintering buds (Arora *et al.* 1992, Rinne *et al*. 1999). Arora *et al*. (1996) have reported that cold acclimation in leaves of deciduous peach can be induced under natural conditions. In *Salix paraplesia*, cold acclimation of deciduous leaves was induced under controlled conditions. Such responses are known for needles of conifers, *e.g.*, *Picea abies* (Qamaruddin *et al*. 1993) and *Pinus sylvestris* (Repo *et al*. 2000), but in these cases the needles are perennial and their ability for acclimation is critical for survival of the trees. Ecological function of cold acclimation in *Salix paraplesia* leaves is unclear, but it could provide some protection against frost periods during early spring and fall prolonging the period for photosynthesis.

Moreover, although the general pattern was similar for apical growth cessation and leaf cold acclimation, significant level of acclimation was obtained before cessation of elongation growth. Cold acclimation in leaves may take place independently of changes in the apical meristem and stem tissue. In such case, leaves may provide an interesting and useful system for physiological and molecular studies in early phases of freezing tolerance induction.

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