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A 25-kDa dehydrin associated with genotype- and age-dependent leaf freezing-tolerance in *Rhododendron*: a genetic marker for cold hardiness?

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Abstract Dehydrins are plant proteins that may play a critical role in stabilizing cell functions during freezing and other dehydrative stresses. This study examines whether dehydrin expression in leaves is associated with varying levels of freezing-tolerance among F_2 segregants, species, and cultivars of evergreen *Rhododendron*. Experiments were also conducted to determine whether physiological and chronological aging affects freezing-tolerance and dehydrin accumulation in *Rhododendron* leaf tissues. Our results indicate that in cold-acclimated F_2 populations, levels of a 25-kDa dehydrin were closely associated with differences in leaf freezing-tolerance (LFT) among segregants. Studies of wild and cultivated plants indicated that LFT increased with both chronological age and developmental phase-change (juvenile to mature plants) and that this trend was accompanied by increased accumulation of the 25-kDa dehydrin. It is suggested that presence or absence of the 25-kDa dehydrin could serve as a genetic marker to distinguish between super cold-hardy and less cold-hardy rhododendron genotypes. Similarly, the relative level of this protein within a genotype can serve as a physiological indicator of freezing-tolerance status under a range of phenological (acclimation) or developmental (age) conditions.

Key words Cold acclimation · Dehydrins · Juvenility · Genetic marker · Woody perennials

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Introduction

Dehydrins (also known as group 2 LEA family of proteins) are hydrophilic, heat-stable proteins that are induced in response to plant stresses possessing a dehydration component, such as salt, water, or freezing stress (Close 1996). They comprise a gene family which encodes dehydrins varying in molecular weight and p*I* but sharing one or more copies of a consensus sequence – a 15 residue, amphipathic α-helix-forming domain (K-segment) – that is highly conserved in higher and lower plants (Close et al. 1993; Close 1997). During the cold acclimation (CA) process, dehydrins and dehydrin-like proteins and/or their transcripts accumulate in a wide array of plant tissues (Lång et al. 1989; Neven et al. 1993; Welin et al. 1994; Wisniewski et al. 1996) and have been cellularly localized in the cytosol and nucleus (Neven et al. 1993; Egerton-Warburton et al. 1997; Danyluk et al. 1998; Wisniewski et al. 1999).

A functional role for dehydrins in freezing-tolerance (FT) is suggested, in part, by their *in vitro* cryoprotectant properties (Lin and Thomashow 1992; Wisniewski et al. 1999). Recently, a direct relationship between dehydrins and FT was demonstrated by the ability of constitutively regulated *cor* proteins (some of which are dehydrins) in *Arabidopsis* to confer FT without prior acclimation (Jaglo-Ottosen et al. 1998). It has also been postulated that dehydrins may act as ion-sequesters (Palva and Heino 1998) or as molecular chaperones (Campbell and Close 1997; Close 1997) under stressful conditions – thereby stabilizing proteins and membranes via hydrophobic interactions.

Accumulation of dehydrin protein and transcripts during CA has been amply documented in a number of herbaceous species (Close 1997), where the FT of cold-acclimated tissues typically does not exceed –15°C. Investigations of dehydrin expression and its association with CA in woody perennials, which exhibit significantly higher CA ability and FT than herbaceous plants, are comparatively scarce. Thus far, studies of woody perennials have used deciduous species to document CA and

dehydrin profiles in overwintering tissues such as xylem, bark, and floral buds (Arora and Wisniewski 1994; Muthalif and Rowland 1994; Salzman et al. 1996; Artlip et al. 1997; Welling et al. 1997). The results of these studies are in general agreement with findings from herbaceous plants, indicating that dehydrins accumulate during the acclimation period.

Woody plants have several physiological traits that confound cold hardiness (CH) research. A juvenile period in woody perennials raises the possibility of differences in FT between the juvenile versus mature (flowering) phases of development. In addition, tissues within an overwintering plant can exhibit different FT mechanisms; for example, supercooling in xylem parenchyma and bud tissues in contrast to equilibrium freezing in leaf and bark tissues (Wisniewski and Arora 1993). Furthermore, some woody plant tissues (e.g. buds) undergo dormancy and CA transitions simultaneously, making it difficult to associate physiological changes with one or the other phenological event (Arora et al. 1992; Arora et al. 1997).

We are using species of *Rhododendron* to study the genetics and physiology of CA and FT in a woody plant. This genus has a number of attributes that make it amenable to CH research. There are over 800 species of *Rhododendron* distributed throughout the Northern Hemisphere, ranging from tropical to polar climates and varying widely in FT (Leach 1961; Sakai et al. 1986). Some species, notably those in section *Ponticum*, are evergreens with leaves that can withstand freezing temperatures as low as −40 to −60°C (Sakai et al. 1986; Uosukainen and Tigerstedt 1988). By using leaves to estimate CH, the problem of dormancy transitions in buds is avoided. In addition, FT is conferred without supercooling in *Rhododendron* leaves (Sakai et al. 1986), enabling the use of freeze-thaw experiments and ion-leakage assays for the determination of leaf freeze-tolerance (LFT). This method of estimating CH provided LFT rankings in an array of evergreen *Rhododendron* cultivars that were consistent with USDA hardiness zone rankings (Lim et al. 1998a).

Our present research was initiated to determine whether dehydrin profiles differ qualitatively and quantitatively among progeny from a population segregating for CH. This line of inquiry was derived from earlier work, which documented that F_2 segregants from a cross between moderately cold-hardy and super cold-hardy *Rhododendron* species varied from −18° to −48°C in LFT (Lim et al. 1998b). Remnant leaf tissue from progeny already evaluated for LFT was used for dehydrin analysis in order to determine the relationship between biochemical phenotypes (dehydrin accumulation) and CH phenotypes in the F_2 population.

A second research objective was to study the effect of plant age on CH. The study of F_2 segregants (Lim et al. 1998b) revealed that 2-to 3-year-old progeny displayed a mean LFT that was 12°C less hardy than the approximately 30-year-old F_1 parent. This parent-offspring discrepancy in CH is difficult to account for genetically, and an alternative explanation was sought in the dependency of FT on physiological age (juvenile progeny vs. mature parents). Age-dependent CH in rhododendrons and parallel changes in dehydrin profiles were studied by comparing juvenile and mature (flowering) plants in natural populations (physiological-age effects) and by comparing of mature cultivars differing widely in age (chronological-age effects).

Materials and methods

Plant materials

Current-year leaves were collected from three groups of naturally cold-acclimated plants.

Group 1

Comparisons of LFT and dehydrin profiles among F_2 segregants included a super cold-hardy parent (*R. catawbiense*, approx. 40 years-old), a moderately-hardy parent (*R. fortunei*, approx. 40 years old), the F_1 hybrid cultivar 'Ceylon' (approx. 30-year old) derived from the cross *R. catawbiense* × *R. fortunei*, and F_2 seedlings resulting from the self-pollination of 'Ceylon'. This group of field-grown plants is maintained at the Holden Arboretum's Leach Research Station in Madison, Ohio. A collection of acclimated leaves from 51 F_2 progeny (3-year-old seedlings) and the parental plants was made in December 1997, and T_{max} values (an estimate of LFT defined as the temperature causing the maximum rate of cell injury) were determined for each individual by the ion-leakage method described below. T_{max} distributions for this population based on a similar collection of acclimated leaves in 1996 (2-year-old seedlings) were previously reported (Lim et al. 1998b).

Remnant leaf tissue from selected progeny in the 1997 study was used for dehydrin analysis. Initially, the progeny were classified by hardiness phenotype into "low", "medium", and "high" LFT groups. Each group differed significantly from the adjacent group by a mean leaf T_{max} of approximately 10°C. Leaves from 5 random progeny in each group were pooled equally on a fresh weight basis, and the three phenotypic "bulks" were extracted and analyzed for differences in dehydrin profiles. Once bulk differences in dehydrin expression were evident, leaves from 3 individuals in each F_2 bulk were extracted and evaluated separately.

Group 2

To examine the effect of physiological aging on LFT and dehydrin levels in rhododendrons, we compared juvenile seedlings and mature plants of *R. maximum* – growing in the wild near Cooper State Rock Park, West Virginia. Leaf collections from 7 seedlings without floral buds (approx. 2 to 3 years old) and 3 large mature plants (approx. 30 years old) were made in mid-January 1996, and individual LFTs were determined. A similar collection from the population was made in February 1997 using different juvenile and mature individuals. Leaves from the 1996 and 1997 collections were used to determine individual T_{max} values for each year. For dehydrin comparisons, remnant leaf tissue from 1997 was pooled equally by fresh weight to form juvenile (n=5) and mature plant (n=3) leaf bulks.

Group 3

Comparisons of LFT and dehydrin profiles were also made among mature plants differing in chronological age. For this study, leaves were field-collected in January 1997 from the 'ortets' (seed-

ling/stock plants) of 'Hawaii', 'Swansdown', and 'Pink Parasol' maintained as 30- to 40-year-old cultivars at the Leach Research Station. At the same time, leaves were collected from 4-year-old 'ramets' (cuttings taken from ortets for vegetative propagation) of these cultivars grown as container plants at Losely Nursery in Perry, Ohio. These propagated plants had been maintained outdoors through mid-November, then placed in cold storage for the winter. For each of the three cultivars, LFTs were determined for a single ortet and three propagated ramets. For dehydrin analysis, leaves from the older ortets were compared to a leaf bulk from the three younger ramets.

Leaf freezing-tolerance (LFT) determination

Leaf discs (1 cm in diameter) were punched from individual plants (24 discs per plant) and frozen to various treatment temperatures (3 discs per treatment temperature) to obtain electrolyte leakage data as described in Lim et al. (1998a). Ion leakage data transformations, Gompertz functions fitting, and statistical analyses were also performed as described by Lim et al. (1998a) to obtain values for T_{max} – the temperature at which the maximum rate of freezing injury occurs. In this study, T_{max} is used synonymously with LFT as indicators of CH.

Protein extractions

Extraction protocols described for other woody plants (Arora and Wisniewski 1994; Arora et al. 1997) were modified to optimize protein extraction from *Rhododendron* leaves. Leaf tissue (2 g) was ground finely in LN_2 and stored at –80°C until used. Tissue was extracted with PVPP (35% of tissue weight) in a borate buffer (50 mM sodium tetraborate, 50 m*M* ascorbic acid, 1 m*M* PMSF, pH 9.0) using a 1:4 (w:v, tissue+PVPP: buffer) extraction ratio. Crude extracts were shaken on a gyratory shaker at 4°C for 30 min followed by centrifugation at 26,000 g_n for 15 min at 4°C. To improve protein yields, the pellet was resuspended, shaken, and centrifuged as described above. The pellet was resuspended a third time, then placed on the shaker for 1 h at 4°C followed by a final centrifugation for 2 h as described above. Supernatant (soluble proteins) was collected and filtered twice through 0.45 µm and 0.20 µm filters.

Protein measurement

The method of Esen (1978) for determining total protein content in crude leaf extractions from rhododendrons proved more reliable than the Bradford assay (Arora and Wisniewski 1994) based on our observation of SDS-PAGE profiles. Equal aliquots (5 µl; triplicates) from various extractions and BSA standards (0–4 mg/ml; triplicates) were spotted on Whatman No. 1 chromatography paper and stained with 0.1% Coomassie brilliant blue dye R-250. After brief rinsing with water and drying, the stained spots were eluted with 1% SDS, and absorbance of the dye-protein complex was measured at 600 nm.

SDS-PAGE and immunoblotting

Concentrating the protein in the crude extracts was necessary in order to improve band intensity and resolution on SDS-PAGE gels. Proteins were precipitated from 1.3 ml samples of extract by adding TCA (10% of volume) and centrifuging at 16,000 g_n for 30 min at 4°C. Protein pellets were washed three times with cold acetone by centrifuging at 16,000 g_n for 30 min. During the second acetone wash a sterile sealed pipette tip was used to physically break the pellets. The pellets were broken once again before being air-dried. Dried protein pellets were rehydrated with 100 µl of SDS-PAGE sample buffer and mixed quickly using a sterile sealed pipette tip. Concentrated protein samples were heated in boiling water for 3 min and then gently vortexed for 1 h followed by centrifugation at 16,000 g_n for 30 s to precipitate non-protein material. The breaking of pellets significantly improved band intensities and resolution on SDS-PAGE (visual observation), as did the procedure of multiple resuspension and shaking of the crude extract described above.

Equal amounts of total protein (30 µg) were separated by discontinuous SDS-PAGE and visualized by Coomassie stain as described by Arora et al. (1992). For immunoblots, separated protein (15 µg) from gel loadings were transferred to 0.45-µm nitrocellulose membranes as described by Arora and Wisniewski (1994) and probed with 1:500 dilution of the antibody directed against a synthetic peptide of the 15 amino acid consensus sequence (EKKGIMDKIKEKLPG) that is highly conserved at the C-terminus of dehydrin protein from several plant species (Close et al. 1993; antibody was kindly provided by Dr. Close). Immunoreactive bands were detected by alkaline phosphatase assay using ProtoBlot Western Blot AP Kit (Promega).

Gel imaging and optical density measurements

Gels were recorded digitally using the Optimas image analysis system (Optimas Inc., Edmonds, Wash.). The immunoreactive bands were assigned gray values by the Optimas system, and the optical densities (O.D.) were calculated by taking the inverse log of the integrated gray values. Mean and standard errors of optical densities were based on three separate assignments of gray threshold values.

Results

Group 1 plants

LFT estimates among parents and F_2 segregants in 1997 were similar to those obtained in a 1996 survey (Lim et al. 1998b). T_{max} values for the super cold-hardy parent (*R. catawbiense*), the moderately cold-hardy *parent (R. fortunei*), and the F_1 hybrid 'Ceylon' with intermediate hardiness were –52.0°C, –31.4°C, and –43.4°C, respectively (Table 1). The 1997 screen of 51 F_2 progeny resulted in a –18°C to –48°C range in T_{max} values which were normally distributed around a mean T_{max} of –32.1°C (data not shown). Subsets of progeny used to make tissue bulks of "low", "medium", and "high" CH phenotypes differed significantly in mean T_{max} by at least 10°C (Table 1).

Coomassie-stained SDS-PAGE profiles of total proteins and their anti-dehydrin immunoblots for the two parents, F_1, and F_2 "bulks" are presented in Fig. 1A, B. Anti-dehydrin immunoblots of parents and F_1 plants revealed a group of dehydrins differing in molecular weight and level of accumulation in the cold-acclimated state. The highest O.D. value derived from a 25-kDa dehydrin that was present in the super-hardy *R. catawbiense* parent, absent in an equal loading of protein from the moderately-hardy *R. fortunei* parent, and present at intermediate levels in the F_1 hybrid 'Ceylon', which also displayed an intermediate LFT (Fig. 1B, Table 1). Levels of the 25-kDa dehydrin in cold-acclimated leaves from *R. catawbiense* were about five fold higher than in non-acclimated leaves from the same source. Both parents and the F_1 exhibited low level accumulation of an ap-

Table 1 Leaf freezing-tolerance (T_{max}) and corresponding levels of a 25-kDa dehydrin in a *Rhododendron* population segregating for FT

Group 1 plants	*n*[a]	Mean T_{max} (°C)±SE[b]	Mean O.D. of 25-kDa dehydrin±SE[c]
Parents			
R. catawbiense, P_1	1	–52.0±1.3 [a]	0.95±0.03 [a]
R. fortunei, P_2	1	–31.4±1.5 [i]	0.00±0.00 [r]
R. 'Ceylon', $P_1 \times P_2$, F_1	1	–43.4±3.4 [cdefgh]	0.63±0.04 [bcd]
Bulked and individual F_2 progenies			
F_2-low group (L)	5	–20.5±1.0[k]	0.40±0.05[eghikmnq]
F_2-medium group (M)	5	–32.1±0.2[i]	0.57±0.04[cfg]
F_2-high group (H)	5	–43.7±1.7[defg]	0.76±0.04[b]
L_1	1	–21.3±0.5[k]	0.00±0.00[r]
L_2	1	–22.5±0.7[jk]	0.26±0.07[hikmnpq]
L_3	1	–22.5±0.8[jk]	0.00±0.00[r]
M_1	1	–31.6±0.7[i]	0.37±0.08[deghikmnpq]
M_2	1	–31.8±1.2[i]	0.00±0.00[r]
M_3	1	–32.6±1.9[i]	0.58±0.03[ce]
H_1	1	–39.3±1.2[h]	0.32±0.02[lm]
H_2	1	–40.9±1.3[efgh]	0.75±0.03[b]
H_3	1	–47.1±1.7[bd]	0.45±0.01[fh]

[a] *n* Number of plants. Three replicate discs were measured at each treatment temperature
[b] Estimated by using the Gompertz function fitted to percentage adjusted injury data; mean and SE estimated by replicates (24 leaf discs) using the Jackknife method (Lim et al. 1998a). Mean separation in column was determined by multiple *t*-test. Values having different letters are significant at $P<0.05$
[c] Using Optimas System; mean and SE estimated from three separate gray values assigned. Mean separation in column was determined by multiple *t*-test. Values followed by different letters are significant at $P<0.05$

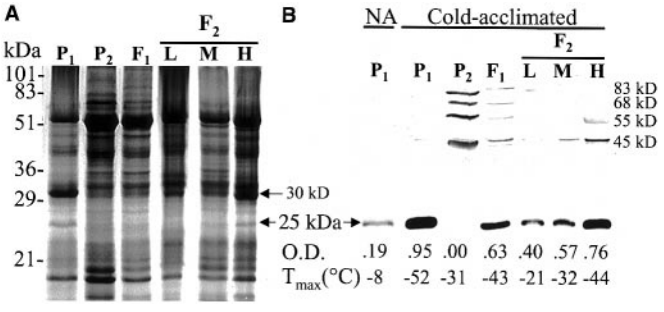

Fig. 1A, B Group 1 plants. **A** SDS-PAGE profiles of total soluble proteins from cold-acclimated leaves (30 µg per lane). P_1 = *R. catawbiense*, P_2 *R. fortunei*, F_1 = *R. catawbiense* × *R. fortunei* 'Ceylon', F_2 = 'Ceylon' selfed; *L*, *M*, and *H* correspond to the low, medium, and high freeze-tolerant F_2 bulks. **B** Anti-dehydrin immunoblots of parents, F_1 and bulks of F_2 progenies. Protein (15 µg) was loaded in each lane. *NA* Non-acclimated, *O.D* optical densities, T_{max}=quantitative measure of leaf freezing-tolerance

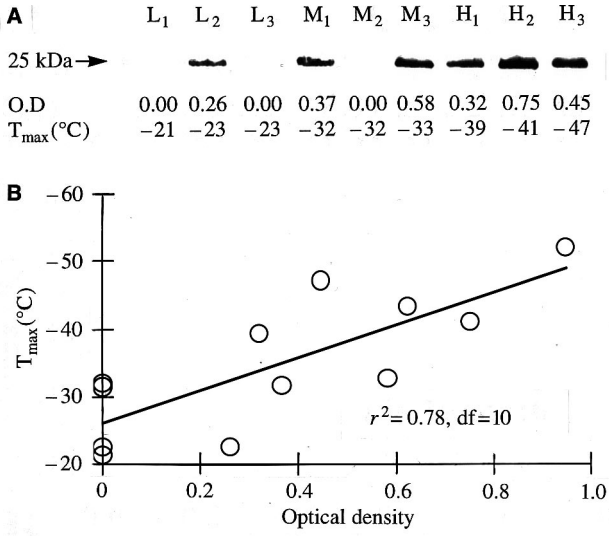

Fig. 2A, B Group 1 plants. **A** Anti-dehydrin immunoblots of 9 individual F_2 progenies. Protein (15 µg) was loaded in each lane **B** Regression analysis of LFT on dehydrin O.D. in the population comprised of parents, F_1, and 9 F_2 progenies. *O.D.*= Optical densities, T_{max} quantitative measure of leaf freezing-tolerance

proximately 45-kDa dehydrin. Three higher molecular weight dehydrins (approx. 55, 68, and 83 kDa) appeared in total proteins from *R. fortunei* but not *R. catawbiense*. These three dehydrins also accumulated in the F_1 hybrid at levels too low for O.D. determination but intermediate in appearance to the parental levels. None of these dehydrins were detected when parallel samples were probed with pre-immune serum (data not shown).

None of the bands detected by anti-dehydrin immunoblotting corresponded to major bands on Coomassie-stained SDS-PAGE protein gels from cold-acclimated parents and the F_1 plant (Fig. 1A). The relatively abundant 25-kDa dehydrin on Western blots appeared as a faint band on the corresponding protein gel. The reason for the apparent lower dehydrin intensities on SDS-PAGE (observed for leaf tissues) is not clear. However, other papers have reported similar low intensities of leaf dehydrins as compared to bark or xylem dehydrins from woody plants (Arora et al. 1996; Artlip and Wisniewski 1997). In contrast, a 30-kDa protein with no dehydrin homology was very abundant in acclimated leaves from

Table 2 Leaf freezing-tolerance (T_{max}) and corresponding levels of a 25-kDa dehydrin in *Rhododendron* plants varying in physiological and chronological age

Group 2 and 3 plants	*n*[a]	Mean T_{max} (°C)±SE[b]	Mean O.D. of 25-kDa dehydrin±SE[c]
Physiological-aged[a]			
R. maximum, juvenile seedlings-1996	7	–38.5±2.0^{gh}	–
R. maximum, mature plants-1996	3	–50.2±0.2^{abc}	–
R. maximum, juvenile seedlings-1997	5	–36.3±2.4^{hi}	0.31±0.02^{ln}
R. maximum, mature plants 1997	3	–43.5±0.9^{def}	0.76±0.03^{b}
Chronological-aged			
R. 'Hawaii', ramets	3	–18.8±1.9^{k}	0.18±0.02^{op}
R. 'Hawaii', ortet	1	–41.1±1.2^{efgh}	0.40±0.00^{jk}
R. 'Swansdown', ramets	3	–24.7±1.1^{j}	0.45±0.00^{fi}
R. 'Swansdown', ortet	1	–45.7±2.0^{de}	0.64±0.02^{bc}
R. 'PinkParasol', ramets	3	–40.3±1.4^{fgh}	0.73±0.03^{bc}
R. 'Pink Parasol', ortet	1	–45.4±1.4^{d}	0.73±0.03^{b}

[a] *n*=Number of plants. Three replicate discs were measured at each treatment temperature
[b] Estimated by using the Gompertz function fitted to percentage adjusted injury data; mean and SE estimated by replicates (24 leaf discs) using the Jackknife method (Lim et al. 1998a). Mean separation in column was estimated by multiple *t*-test. Values followed by different letters are significant at $P<0.05$
[c] Using Optimas System, mean and SE estimated from three separate gray values assigned. Mean separation was estimated in column by multiple *t*-test. Values followed by different letters are significant at $P<0.05$
[d] 1997 plants were evaluated in late February

R. catawbiense compared to *R. fortunei*; this protein was also abundant in the "high" LFT bulk from F_2 progenies (Fig. 1A) and in the 3 individuals constituting this bulk (data not shown).

Among F_2 seedlings, the 25-kDa dehydrin was the only protein clearly associated with differences in CH. Comparisons of the F_2 tissue bulks grouped by phenotypic class – "low", "medium", and "high" LFT – indicated a 50–100% increase in the 25-kDa dehydrin level as the CH status increased (Table 1, Fig. 1B). When individual progeny from these F_2 bulks were evaluated, most but not all of the offspring displayed increased dehydrin accumulation at higher levels of LFT (Table 1, Fig. 2A). Regression of T_{max} on dehydrin O.D. values for this population (Fig. 2B) resulted in a significant positive relationship (r^2=0.78, df=10, $P<0.05$), an indication that 25-kDa dehydrin levels were reasonably predictive of acclimated LFT status in this population.

The high-molecular-weight dehydrins unique to the *R. fortunei* parent and present in the F_1 hybrid 'Ceylon' (Fig. 1B) were essentially absent among F_2. This result may be due to age factors described in more detail below. Since the physiologically mature F_1 plant displayed weak signals for these dehydrins in the cold-acclimated state, it is possible that juvenile F_2 seedlings (2–3 years old) contained undetectable levels at comparable protein loadings.

Group 2 plants

There was a significant difference in LFT and dehydrin levels between juvenile seedlings and mature plants of *R. maximum* collected from a wild population in the cold-acclimated condition. In two sampling years, mature plants (approx. 30 year old) were more cold hardy than the juvenile seedlings (approx. 3-years old) by an average of –9.5°C T_{max} (Table 2). Immunoblots of leaf bulks from the 1997 collection indicated an approximate 2.4-fold increase in levels of the 25-kDa dehydrin in mature plant leaves relative to juvenile plants (Table 2, Fig. 3B).

Two other dehydrins (29 kDa and 55 kDa) were faintly detected by immunoblots of cold-acclimated protein extracts of *R. maximum*. These dehydrins accumulated at very low levels during the cold-acclimated state in both juvenile seedlings and mature plants and did not appear to be associated with FT levels (complete immunoblots not shown). The homology of a *R. maximum* 55-kDa dehydrin to other similar-sized dehydrins in other *Rhododendron* groups has not yet been confirmed by running them on the same gel. Additionally, a 30-kDa non-dehydrin protein strongly accumulated at high levels in the mature and more cold-hardy tissues as compared to juvenile and less-hardy tissues (Fig. 3A).

Group 3 plants

Different aged ortets and ramets of the same cultivar also exhibited some correspondence between T_{max} values and levels of the 25-kDa dehydrin (Table 2, Fig. 4A, B). In all three comparisons, young ramets from 'Hawaii', 'Swansdown' and 'Pink Parasol' had significantly lower LFTs than the ortets. Differences in the 25-kDa dehydrin levels paralleled LFT differences. The older ortets of 'Swansdown' and 'Hawaii' showed a 1.4-to 2.3-fold increase in this dehydrin relative to younger ramets propagated from them (Table 2, Fig. 4B). However, LFT differences in the 'Pink Parasol' comparison (which were the smallest among all three cultivars) were associated with equal amounts of dehydrin. Taken as a whole, this group of cultivars showed a significant, positive relation-

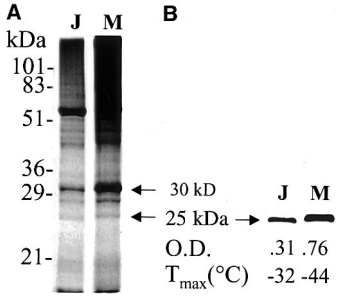

Fig. 3A, B Group 2 plants: wild populations of *R. maximum.* **A** SDS-PAGE profiles of total soluble leaf proteins extracted from cold-acclimated state. Protein (30 µg) was loaded in each lane **B** Anti-dehydrin immunoblots of juvenile seedlings and mature plants. Protein (15 µg) was loaded in each lane. *J* = Juvenile seedlings (approx. 2–3 years old), *M*=mature plants (approx. 30-years old), *O.D.* Optical densities, T_{max} quantitative measure of leaf freezing-tolerance

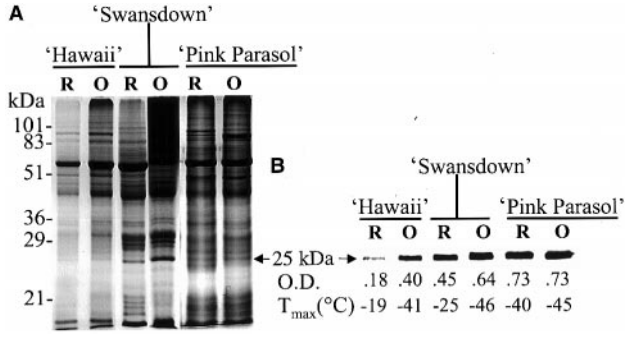

Fig. 4A, B Group 3 plants: *R.* 'Hawaii', R. 'Swansdown' and *R.* 'Pink Parasol' cultivars. **A** SDS-PAGE profiles of total soluble leaf proteins extracted from cold-acclimated state. Protein (30 µg) was loaded in each lane. **B** Anti-dehydrin immunoblots of ramets (rooted-cuttings) and ortets (stock plants). Protein (15 µg) was loaded in each lane. *R* Ramet (approx. 4 years-old), *O* ortet (approx. 30–40 year old), *O.D.* optical densities, T_{max} quantitative measure of leaf freezing-tolerance

ship between T_{max} and accumulation of the 25-kDa dehydrin (r^2=0.81, *df*=4, *P*<0.05).

Five other dehydrins (ranging from 45 kDa to 97 kDa) were detected by immunoblots of cold-acclimated protein extracts from these cultivars (complete immunoblots not shown). Two commonly expressed dehydrins (approx. 45 kDa and approx. 83 kDa) may be homologous to similar-sized proteins noted above in *R. fortunei.* Other dehydrins were cultivar specific (approx. 55 kDa in 'Hawaii' and 'Pink Parasol'; approx. 78 kDa in 'Swansdown' and 'Pink Parasol'; and approx. 97 kDa in 'Hawaii' and 'Swansdown'). With the exception of the 25-kDa form, these dehydrins exhibited equally low accumulation in younger ramets and older ortets and appeared to lack any association with LFT levels.

Discussion

Genetic interpretation of dehydrin profiles

In a previous study, two *Rhododendron* species differing in CH were used as parents to generate F_2 seedlings segregating for LFT (Lim et al. 1998b). In the present report we show that the parental genotypes express cold-acclimation-induced dehydrins and that the number and molecular weight of these proteins varies between the species. This result is consistent with reports on other plant taxa in which dehydrins comprise a variable gene family that is coordinately regulated by low temperatures (Houde et al. 1992a; Robertson et al. 1994; Rowland et al. 1996; Choi et al. 1999).

Several dehydrins were present in only one of the two species – a 25 kDa variant found in *R. catawbiense* and 55-, 68-, and 83-kDa forms observed in *R. fortunei*. Because the immunoblots are detecting temperature-induced proteins, the parental differences in dehydrin profiles (presence vs. absence of a specific molecular-weight protein) could be attributed to regulatory genes rather than dehydrin-encoding structural genes. For example, *R. catawbiense* and *R. fortunei* could share identical dehydrin genes under the control of regulatory genes which respond differently to low temperatures. At present, we favor an interpretation of the data based on structural gene differences between the parents. Studies with cold-responsive regulatory elements (transcriptional factors) suggest that they promote coordinate expression of a suite of cold-regulated genes, some of which also encode dehydrin or dehydrin-like proteins (Jaglo-Ottosen et al. 1998). There is no evidence in the literature, to date, supporting the scenario described above – an identical dehydrin gene common to two cold-acclimating species but differentially induced by cold in only one of them. It is likely, then, that the dehydrin profiles observed in acclimated leaf tissue from each parent, in this study, represent the expression of the full set of cold-regulated dehydrins. It is important to note, however, that different dehydrin genes within the same genotype may be differentially regulated by altering the type of stimulus, such as low temperature, water-stress, ABA, etc (Choi et al. 1999).

The limited F_1 and F_2 data in this study also suggest that presence versus absence (+/–) of the 25-kDa dehydrin, and possibly some of the other higher MW dehydrins, is due to structural gene differences. The F_1 interspecific hybrid displayed all parental bands, indicating either a dominant or codominant inheritance of dehydrin presence. Accumulations of several dehydrins in the F_1 (based on O.D. or visual estimates) were intermediate to parental levels, suggesting a gene dosage effect, which could result from codominant expression of 'presence' and 'absence' alleles at the corresponding loci. The F_1 hybrid may be heterozygous for alleles that are homozygous for the presence trait (+/+) in one parent and homozygous for dehydrin absence (–/–) in the other, resulting in high, intermediate, and null levels of protein accumu-

lation among genotypes. Given this model, the expectation for the F_2 is three dosage phenotypes segregating in a 1:2:1 ratio, or a 3:1 ratio for presence versus absence of a dehydrin. The 25-kDa dehydrin was absent in 3 of 9 F_2 progeny, giving a 2:1 ratio for presence versus absence – this fits reasonably well to the 3:1 expectation (χ^2=0.15; 0.5<*P*<0.75). However, the total sample was too small to determine whether dehydrin accumulation formed a continuous distribution or fell within distinct low, intermediate, and high levels in the 1:2:1 ratio as predicted. Nevertheless, at least 1 progeny had high levels of accumulation (O.D. ≅0.75), which in addition to the null genotypes, suggests that both parental genotypes were recovered in the F_2 generation. No information could be gleaned from the higher MW dehydrins contributed by *R. fortunei* because they did not accumulate to detectable levels in individual F_2 seedlings.

In maize, allelic variation at one of two dehydrin loci has a similar presence/ absence phenotype in some genotype comparisons. At both the *dhn1* and *dhn2* loci, different-sized alleles have been observed, but some individuals lacked a dehydrin band at the *dhn2* locus (Campbell et al. 1998). In F_1 and F_2 progeny, the presence trait appears in a pattern consistent with dominant expression of structural genes, although some variation due to possible dosage and maternal influence was noted. The authors did not rule out the possibility that the absence trait at *dhn2* could result from regulatory gene control.

Association of freeze-tolerance and dehydrin levels

A number of studies have established a positive correlation between dehydrin accumulation and CH phenotype among selected genotypes (Danyluk et al. 1994; Muthalif and Rowland 1994; Robertson et al. 1994; Cai et al. 1995; Arora et al. 1997; Artlip et al. 1997). In cowpea, the presence or absence of a 35-kDa dehydrin in parents and F_1 hybrids accounted for 19% of the variation in chilling tolerance at germination (Ismail et al. 1997). We postulated in an earlier report (Lim et al. 1998b) that as few as three genes with additive effects could produce the normally distributed CH phenotypes observed in an F_2 population derived from the *R. catawbiense* × *R. fortunei* cross. This group of genes may include the 25-kDa dehydrin from *R. catawbiense* because variation in the accumulation of protein was closely associated with segregation for LFT. Linear regression analysis indicated that 25-kDa O.D. values accounted for 78% of the variation in LFT among parents, F_1, and 9 F_2 progeny. The 25-kDa dehydrin thus appears to be a key component of LFT in rhododendrons, although its function has not been ascertained.

CH is considered to be a physiologically complex trait under multigenic control (Hayes et al. 1993; Stone et al. 1993; Pellet 1998), and there are undoubtedly other gene products that play major roles in CA of rhododendrons. In this study, a 30-kDa non-dehydrin protein was visualized at lower levels under non-acclimated conditions than under cold-acclimated conditions, was observed at higher levels in *R. catawbiense* than in *R. fortunei*, and appeared to be one of the most abundant proteins in the "hardiest" F_2 bulk. This undetermined protein also displayed a close quantitative association with age-dependent LFT changes in *R. maximum* (a closely related species to *R. catawbiense* with comparable FT). We plan to look more closely at the association of this 30-kDa protein with CH and possibly characterize it in the future.

Age effects on cold hardiness and dehydrin accumulation

Woody perennials typically have lengthy juvenile phases which terminate upon flowering (maturation). Between these developmental phases, plants undergo morphological changes (Hartmann et al. 1997) as well as genetic, physiological, or biochemical changes (Hackett et al. 1990); a process known as "phase-change" or "maturation" (Brink 1962). However, to the best of our knowledge, very little is known about the effect of phase change on adaptive traits such as CH.

In rhododendrons, there is a significant effect of physiological age on both CH and dehydrin accumulation. In the wild population of *R. maximum*, juvenile plants (2–3 years old) were less hardy than mature plants by about 9°C. Parallel differences were observed in the relative abundance of the 25-kDa dehydrin in leaves from juvenile and mature plants. Phase-related, differential expression of proteins (qualitative and quantitative) has been reported for several woody species (Hand et al. 1996 and references within). The present study is the first to report the phase-dependent accumulation of a dehydrin and its correlation with CH. The underlying mechanisms for this age-dependent effect are unknown but may involve regulatory elements (e.g. signal transduction linked with dehydrin expression) or phase changes in nutrient assimilation.

The data from the *R. maximum* population suggest that parent-offspring comparisons in genetic studies of CH can be confounded by age-dependent factors when the offspring are in a juvenile phase. In an earlier study (Lim et al. 1998b), we reported that the average F_2 progeny (2–3 years old) was 12°C less freeze-tolerant than the F_1 parent (approx. 30 years old). This difference now appears to result from developmental rather than genetic factors. For future research and breeding efforts, further studies will be needed to determine how much LFT increases with juvenile-mature phase change, and how well seedling CH predicts mature plant performance.

Chronological age also appeared to have a positive effect on both CH and dehydrin accumulation. In the comparisons of younger ramets versus older stock plants, all of the three cultivars studied showed significantly higher LFT, and two of the three cultivars displayed higher dehydrin levels in the older ortets. In the case of 'Pink Parasol', age differences in LFT were the

smallest among the three cultivar comparisons (only 5°C vs. >20°C for the other two cultivars), but they corresponded to similar abundances of the 25-kDa dehydrin in leaf tissues from ramets vs. ortets. It is conceivable that the differences in dehydrin level (associated with relatively smaller differences in LFT) are marginal and could not be detected by the quantitative method used in this study.

Dehydrins as markers for freezing-tolerance

While the chronological age data are somewhat equivocal with respect to interpretation, it is interesting to note that a pooled analysis of the cultivar comparisons indicated a significant correlation between LFT and 25-kDa dehydrin accumulations. As in the F_2 population, 25-kDa dehydrin level alone is a reasonably good predictor of CH status among a diverse group of *Rhododendron* genotypes. Our data corroborate a suggestion by Houde et al. (1992b) that dehydrins could be used as both quantitative and qualitative markers for the FT phenotype in *Gramineae* and extend their applicability to woody plant systems. The 25-kDa dehydrin present in the super-hardy *R. catawbiense* may have cryoprotectant/chaperone/ion-sequestration properties which exceed the higher MW dehydrins found only in the moderately hardy *R. fortunei* (which lacks the 25-kDa dehydrin). Differential transmission and accumulation of the 25-kDa protein among *Rhododendron* genotypes in the F_2 significantly affected CH status, suggesting that this dehydrin may be a genetic marker for CH. The ability of the 25-kDa dehydrin to serve as a physiological indicator of CH was demonstrated by the parallel changes in protein levels and LFT caused by phenological (acclimation) or developmental (age) factors.

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