Dehydrin variability among rhododendron species: a 25-kDa dehydrin is conserved and associated with cold acclimation across diverse species

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Summary

• Here we examine the accumulation pattern of dehydrins in non- vs cold-acclimated leaves of 21 species comprising two divergent groups of *Rhododendron*, Subgenus Hymenanthes and Subgenus Rhododendron. Individuals from five other Ericaceous genera were also evaluated in the same way. Quantitative comparisons of cold-inducibility of a 25-kDa dehydrin and cold acclimation ability in six *Rhododendron* species were also performed.

• Leaf freezing tolerance assay and dehydrin detection and quantification were performed as previously described.

• Eleven dehydrins, ranging from 25- to 73-kDa, were observed among the 21 species, and most were more abundant in winter-collected leaves than in summer-collected leaves. One dehydrin, a 25-kDa protein, was uniquely conserved across most (95%) of the species surveyed, and was absent only in *R. brookeanum*, a tropical species that may not be capable of cold acclimation. The 25-kDa dehydrin was also identified in *Kalmia*, a genus closely related to *Rhododendron* but not in four other less related Ericaceous genera. Comparison of dehydrin profiles in non- and cold-acclimated leaf tissue from six species (three very hardy, and three less hardy, species) indicated a close association ($R^2 = 0.95$) between relative changes in leaf freezing tolerance and 25-kDa dehydrin accumulation.

• The taxonomic and physiological comparisons suggest a central, but as yet unknown, function for the 25-kDa dehydrin in protecting rhododendron leaves from freezing injury.

Key words: cold acclimation, dehydrins, Ericaceae, leaf freezing tolerance, *Rhododendron*, woody plants.

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Introduction

It is well known that the cellular levels of many metabolites increase when plants are exposed to dehydration stresses such as drought, cold, or high salt levels (Levitt, 1980; Ingram & Bartels, 1996). Some of the organic compounds that accumulate in response to these stress factors are proteins called dehydrins (Svensson *et al.*, 2002). Dehydrins have been studied in many plant species, and are characterized by a consensus 15 amino acid sequence known as K-segment, near the carboxy terminus and additional copies upstream of the terminus, in many cases, as a slightly modified 14 amino acid consensus (Close, 1996). Dehydrin proteins and their transcripts have been shown to accumulate during seasonal development of freezing tolerance (cold acclimation) in barks, xylems, buds, shoot apices, and seedlings of a number of woody plant species (Arora & Wisniewski, 1994; Muthalif & Rowland, 1994; Salzman *et al.*, 1996; Cai *et al.*, 1995; Artlip *et al.*, 1997; Welling *et al.*, 1997; Rinne *et al.*, 1998; Levi *et al.*, 1999; Sauter *et al.*, 1999; Kontunen-Soppela *et al.*, 2000; Sarnighausen *et al.*, 2002; among others).

By contrast with herbaceous plant species and model systems such as *Arabidopsis*, where transformation systems (Artus *et al.*, 1996; Jaglo-Ottosen *et al.*, 1998) or mapping populations (Pan *et al.*, 1994; Choi *et al.*, 1999; Ismail *et al.*, 1999)

have been used to establish a more causal relationship between dehydrin gene expression and freezing or chilling tolerance, their presumed function in woody plant winter survival is primarily based on correlative data.

We have been using broadleaf, evergreen members of the genus *Rhododendron* as a system for studying cold acclimation and freezing tolerance in woody plants (Lim *et al.*, 1998a, 1998b, 1999). These evergreen species and cultivars provide an opportunity whereby cold acclimation physiology could be studied in over-wintering leaf tissues without the interference of endodormancy transitions that occur in other tissues (buds) of deciduous woody perennials (Lang, 1987; Arora *et al.*, 1992). Lim *et al.* (1999) first studied the role of dehydrins in *Rhododendron* cold hardiness, using a super-hardy species (*R. catawbiense*), a less hardy species (*R. fortunei*) and their F_2 progenies. They reported that levels of a 25-kDa dehydrin were closely associated with differences in leaf freezing tolerance among F_2 segregants, and suggested that this dehydrin could serve as a genetic marker for cold hardiness in this interspecific population.

The research presented here was conducted to determine: whether the 25-kDa rhododendron dehydrin (which we propose to term RCA25 for 'rhododendron cold-acclimation' protein of 25-kDa) is present in other *Rhododendron* species or related genera; and whether its accumulation is associated with cold hardiness status across a diverse array of species.

Materials and Methods

Plants

Non- and cold-acclimated leaves from a total of 28 taxa comprising 21 *Rhododendron* species were obtained from The Holden Arboretum's David G. Leach Research Station in Madison, Ohio, USA (Table 1). These species were selected from two *Rhododendron* subgenera – Subgenus Hymenanthes, the nonscaly leaved or elepidote rhododendrons, and Subgenus Rhododendron, the scaly leaved or lepidote species. For cold hardy species, nonacclimated (NA) leaves were field-collected during summer (July and August) while cold acclimated (CA) leaves were collected from the same individuals during late December and January. For less hardy species unable to tolerate outdoor winter conditions at the site (*R. arboreum*, *R. decorum*, and *R. brookeanum*), plants in containers were held in cold storage (2–4°C) following acclimation outside through late fall. Field and container plants received irrigation and fertilizer as needed throughout the growing season. NA (June) and CA (January) leaves from three evergreen members of *Ericaceae* (*Kalmia latifolia*, *Leucothoe fontanesiana* and *Pieris floribunda*) were also obtained from the Madison, Ohio, location. Leaf samples from two other genera in the same family (*Arctostaphyllos uva-ursi* and *Vaccinium macrocarpum*) were summer and winter collected in the wild, near Morgantown, West Virginia.

Relative cold-hardiness estimations

Leaf freezing tolerance (LFT) was determined according to a leaf disk method previously reported (Lim *et al.*, 1998a,b). For CA samples, the leaf disks were cooled at relatively slow rates following ice nucleation at −1.5°C, and sampled at treatment temperatures ranging from −10°C to −52°C. Cooling rates varied with sample temperatures: $-1°C\ h^{-1}$ from −1.5 to −4°C; $-2°C\ h^{-1}$ from −4°C to −10°C; and $-7°C\ h^{-1}$ thereafter. A similar protocol was used to freeze NA samples, except that they were cooled down to only between −10°C to −12°C. Ion leakage calculations, percentage injury estimations, Gompertz functions fitting, and determination of T_{max} (temperature causing maximum rate of injury and defined as leaf freezing tolerance) were performed as described by Lim *et al.* (1998a).

T_{max} was selected (instead of LT_{50}) as the quantitative measure of LFT because we believe that physiologically, T_{max} is more descriptive. LT_{50} is the temperature that causes 50%-injury and is generally considered to be a value that represents the critical temperature (approximates killing temperature) of cold hardiness of the tissue evaluated (Levitt, 1980). It is arguable, however, that LT_{50} represents a temperature that causes 100% injury to half of the total tissue area or causes all cells to be half-injured. T_{max} is the temperature that causes maximum rate of injury where any lowering of temperature beyond T_{max} results in diminishing rates (Lim *et al.*, 1998a). Moreover, Lim and coworkers determined that T_{max} estimations made by Gompertz function (based on an ion-leakage assay) were highly correlated to the visual LT_{50} estimates of LFT in *Rhododendron*. Evidence is accumulating in the literature supporting the use of T_{max} as a quantitative cold hardiness index (Lim *et al.*, 1998a and references therein).

Dehydrin detection and quantification

Total protein was extracted from rhododendron leaves, precipitated, washed, and solubilized in SDS-PAGE loading buffer per Lim *et al.* (1999). The method described by Esen (1978) was used for determining total protein content in the loading buffer samples, using BSA standards. This protein assay (extracts spotted on a filter paper, stained with Coomassie Brilliant Blue R-250, dye-protein complex eluted with SDS, and absorbance measured) is virtually free from interference by common laboratory reagents including SDS-PAGE loading buffer and other nonproteinaceous substances. Separation of proteins by SDS-PAGE electrophoresis and subsequent visualization of Coomassie Brilliant Blue G-250 stained protein bands followed standard protocols (Lim *et al.*, 1999). Proteins were transferred from duplicate gels on to nitrocellulose membranes and immunoblots were obtained using antidehydrin antibody (against the consensus K-segment; Close *et al.*, 1993) provided by Dr Tim Close (UC-Riverside). Immunoblotting proceeded as described (Lim *et al.*, 1999) with the exception that 3% nonfat dry milk in Tris-buffered

Table 1 *Rhododendron* species used in this study, their corresponding cold-acclimated leaf freezing tolerance (LFT), and various dehydrin proteins that accumulate in acclimated leaves

Rhododendron species	Genotype used[1]	Leaf freezing tolerance (°C)[2]	Dehydrins (kDa)										
			25	28	30	32	34	37	41	46	50	64	73
Subgenus Hymenanthes													
R. adenogynum Diels	57–1193^{H}	(–29)	X								X		
R. arboreum Sm.	64/118^{R}	(–20)	X								X		
R. brachycarpum D. Don ex G. Don	79/056^{R}	–60	X	X						X	X	X	
R. brachycarpum	(Roslyn form)^{H}	ND	X	X						X	X	X	
R. brachycarpum ssp. tigerstedtii Nitz.	82/109^{R}	ND	X	X						X	X	X	
R. catawbiense Michaux	'Catalgla'^{H}	(–53)	X										
R. decorum Franch.	75/170^{R}	ND	X	X									
R. dichroanthum ssp. *scyphocalyx* Cowan	no #^{H}	(–23)	X	X	X	X							
R. oreodoxa var. *fargesii* (Franch.) DF Chamb.	L78-506^{H}	ND	X								X	X	
R. fortunei Lindley	L62-560^{H}	(–38)	X	X					X	X	X	X	
R. fortunei	64-718-81^{H}	ND	X	X					X	X	X	X	
R. makinoi Tagg	79–181^{H}	ND	X		X				X		X		
R. maximum L.	65–285^{H}	(–52)	X		X						X		X
R. maximum	84–238^{H}	ND	X		X						X		X
R. maximum 'Mt. Mitchell'	L51-500^{H}	ND	X		X						X		X
R. metternichii [*R. degronianum* ssp. *heptamerum* (Carrièrre) Hahn]	L82-515^{H}	(–48)	X	X							X		
R. vernicosum Franch.	L78-508^{H}	(–25)	X	X				X			X		
R. degronianum ssp. *yakushimanum* (Nakai) Hara	67–76^{H}	–40	X								X		
R. degronianum ssp. *yakushimanum*	'Koichiro Wada'^{H}	ND	X								X		
R. degronianum ssp. *yakushimanum*	'Mist Maiden'^{H}	ND	X								X		
Subgenus Rhododendron													
R. brookeanum	82/210^{R}	(–7)							X[3]				
R. dauricum L.	67–143^{H}	–50	X		X						X		
R. hirsutum L.	79-259-85^{H}	ND	X					X					
R. keiskei Miquel	L59–63A^{H}	–25	X					X					
R. minus Michaux	L62-570^{H}	ND	X		X						X	X	
R. mucronulatum Turcz.	L82-551^{H}	–50	X				X				X		
R. myrtifolium Schott & Kotschy	60–336^{H}	ND	X										
R. russatum Blaf.f & Forrest	L78-543^{H}	–40	X			X				X			

[1]Names indicate selected species forms (cultivars). Numbers with letter superscripts indicate accessions from H (The Holden Arboretum collection) or R (originating from Rhododendron Species Botanical Garden with the RSBG accession number). [2]LFT values in parentheses were estimated in our laboratory as T_{max} (the temperature causing maximum rate of injury), whereas those without parentheses are leaf LST (lowest survival temperatures) values from Sakai *et al*. (1986). ND – no data available. [3]does not increase in cold acclimated leaves compared with the nonacclimated ones (the only case in this study where a dehydrin accumulation was not higher in winter-collection compared with summer-collected leaves).

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saline plus Tween 20 was used in place of gelatin as the blocking buffer. Parallel blots were incubated with preimmune serum (provided by Dr Tim Close, UC-Riverside) to verify that the band recognition on antidehydrin immunoblots was not a result of nonspecific binding of secondary antibody.

The immunoblots were recorded using a digital image analysis system (Alpha Innotech Corporation, San Leandro, CA, USA), and integrated optical density (OD) values for the RCA25 band were determined using the system's software. In this procedure, an OD value that quantifies protein band-intensity is an integrated density value (IDV) determined from the number of pixels/band and the sum of intensity of each pixel. In order to establish the relationship between protein abundance (amount loaded) and OD reading, measurements of the RCA25 band intensity were made on a dilution series ranging from 1 μg to 17 μg total protein from CA leaves of *R. catawbiense*. From these calibration curve data (not shown) it was determined that protein loadings of up to 7 μg had a linear relationship to OD values. 7 μg protein loadings were used for all the immunoblots performed in this study.

The quantitative association between RCA25 abundance and level of LFT was examined by comparing NA and CA leaf tissue from six *Rhododendron* species in a replicated experiment. Three very hardy species (*R. catawbiense*, *R. maximum* and *R. metternichi*; with the cold acclimated T_{max} of −53°C, −52°C and −48°C, respectively) and three less hardy species (*R. arboreum*, *R. dichroanthum* and *R. vernicosum* with the T_{max} of −20°C, −23°C and −25°C, respectively) were selected for comparison. Immunoblots for each species were run in three replications (three separate gels from the same extraction) and the mean optical density values of the RCA25 bands were recorded for non- and cold acclimated leaves.

Results and Discussion

Multiple dehydrins were observed across diverse *Rhododendron* taxa

A total of 11 dehydrins were observed in the survey of 21 *Rhododendron* species (Table 1). They ranged in molecular weight from 25-kDa to 73-kDa, and were detected, for the most part, in both non- and cold-acclimated leaf tissue. Species contained from one to as many as six different dehydrins, with a median value of 3.0 per taxon. With the single exception of a 41-kDa dehydrin in *R. brookeanum*, accumulation of the 11 dehydrins was higher (based on visual assessment) in CA/winter-collected than NA/summer-collected leaves (Table 1).

A few taxonomic similarities and differences were noted based on the dehydrin profiles. The two major groups represented – Subgenus Hymenanthes (13 species) and subgenus Rhododendron (8 species) – shared 7 of the 11 dehydrins (64%). Three proteins were unique to the Hymenanthes group (28-, 46-, and 73-kDa dehydrins), while the 34-kDa form in *R. mucronulatum*, was unique to the group comprising Subgenus Rhododendron. Dehydrin variability within species was nonexistent in the three instances where multiple accessions were evaluated – *R. brachycarpum*, *R. maximum*, and *R. yakushimanum* (Table 1).

Our data are consistent with previous observations (Close *et al.*, 1993) that dehydrins are typically encoded by a multigene family that can vary among plant taxa. The present finding of interspecific (intrageneric) differences in *Rhododendron* parallel observations of intergeneric and intraspecific dehydrin variability in other plants (Close *et al.*, 1993; Muthalif & Rowland, 1994; Wisniewski *et al.*, 1996; Sarhan *et al.*, 1997; Nylander *et al.*, 2001; among others).

Among species with the highest level of leaf freezing tolerance, no consistent dehydrin profile was observed (Table 1). In Subgenus Hymenanthes**,** for example, six dehydrins were distributed among the three hardiest species: one in *R. catawbiense* (LFT = −52°C), four in *R maximum* (LFT = −52°C), and five in *R. brachycarpum* (LFT = −60°C). Less cold hardy species such as *R. dichroanthum* and *R. vernicosum* were not characterized by fewer dehydrins than the median value of 3. Cold hardiness therefore appears to be independent of total dehydrin diversity within species, and may be influenced more by the expression of these proteins (abundance) and/or qualitative differences in their function at the cellular level.

A 25 kDa dehydrin is conserved among *Rhododendron* species with leaf freezing tolerance, and also appears in *Kalmia*

Only a few dehydrins were detected at high frequency in our survey of species. Most notably, the 25-kDa dehydrin (RCA25) was found in all but one (95%) of the taxa (Table 1) and accumulated at higher levels in CA/winter collected tissues compared to NA/summer-collected ones (Table 1; Figs 1 and 2). A 50-kDa dehydrin was next in frequency, occurring in 13 of 21 (62%) species in both subgenera. The 28-kDa protein was observed in 6 of 21 (28%) of the species, but only in subgenus Hymenanthes.

The only species that lacked RCA25 was *R. brookeanum*, a tropical Indonesian plant that appears incapable of cold-acclimation. Leaf-freezing tests indicated that CA *R. brookeanum* leaves had a T_{max} of −7°C (Table 1), which is roughly equivalent to NA freezing tolerances (−3°C to −6°C) determined previously in many rhododendron species, cultivars, and progenies (Holt & Pellet, 1981; Anisko & Lindstrom, 1995; Lim *et al.*, 1998a, 1998b; and also the present study, Fig. 2). Other reports indicate that tropical species belonging to the same taxonomic group, subgenus Rhododendron section Vireya, were injured by slight freezing even after cold hardening at 0–5°C (Sakai *et al.*, 1986). In addition, the single dehydrin observed in *R. brookeanum* immunoblots, a 41-kDa protein, did not appear to be up-regulated in leaf tissues following fall acclimation and subsequent storage at 2–4°C (Table 1; blots not shown).

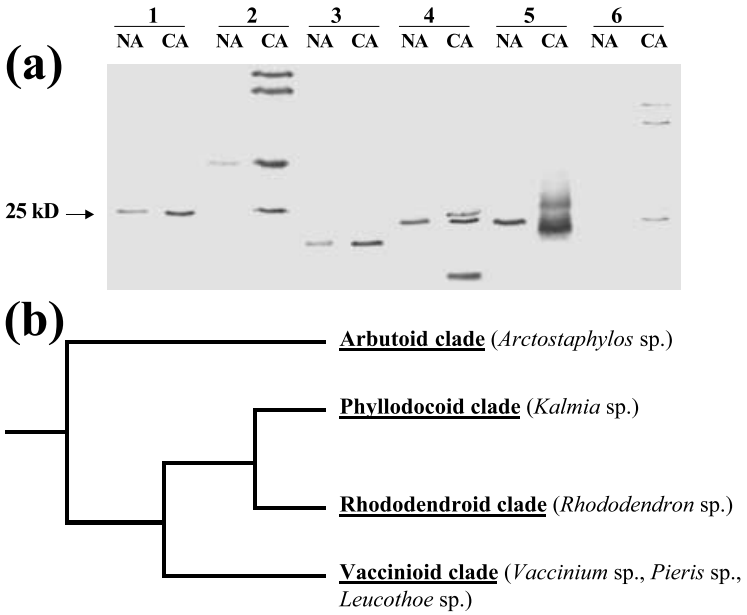

Fig. 1 (a) Anti-dehydrin immunoblot profile of leaf proteins from six Ericaceae species: (1) *R. catawbiense* (2) *Kalmia latifolia* (3) *Vaccinium macrocarpon* (4) *Leucothoe fontanesiana* (5) *Pieris floribunda* (6) *Arctostaphyllos uva-ursi*. The 25-kDa Rhododendron dehydrin (RCA25) is indicated by an arrow. (b) Cladogram representing phylogenetic relationships within Ericaceae family (adapted from Kron, 1997). All lanes were loaded on an equal protein basis (7 μg/lane). CA, cold acclimated; NA, nonacclimated.

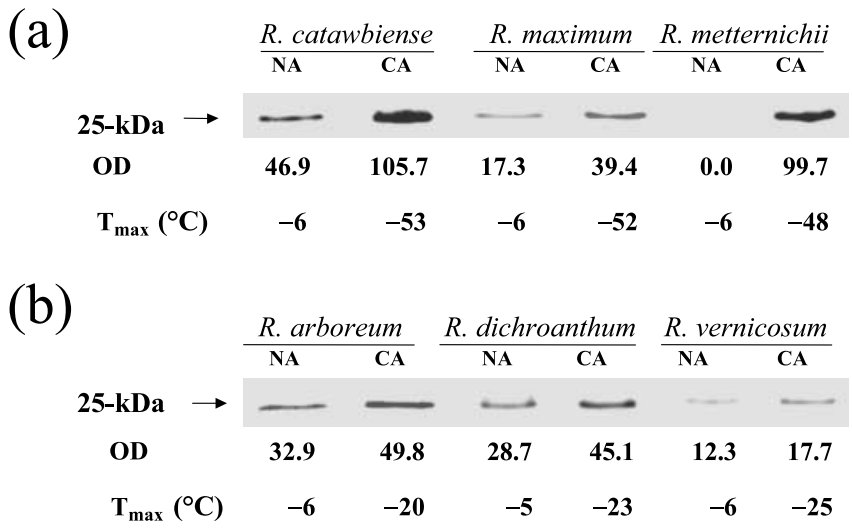

Fig. 2 Anti-dehydrin immunoblots for the 25-kDa dehydrin (RCA25) (indicated by arrows) of leaf proteins from three super hardy (a) and three less hardy (b) *Rhododendron* species. All lanes were loaded on an equal protein basis (7 μg/lane). The optical density for the RCA25 band and the T_{max} (temperature causing maximum rate of injury, a measure of leaf freezing tolerance) value for non- and cold acclimated leaves of each species are indicated. OD, optical density, a quantitative measure of RCA25 band intensity; NA, nonacclimated; CA, cold acclimated.

A survey of dehydrins from NA and CA leaves of five other ericaceous genera revealed that mountain laurel, *Kalmia latifolia*, also contained a cold-induced RCA25 (Fig. 1a). The other four genera – *Arctostaphyllos*, *Vaccinium*, *Leucothoe*, and *Pieris* – lacked the RCA25 but contained other dehydrins that appeared to be more abundant in the CA condition. The band in lane 4/CA (*Leucothoe*) resolving very close to 25-kDa appears to be due to non-specific immune reaction based on the corresponding immunoblots incubated with preimmune serum (data not shown) and is therefore not RCA25. Of the five genera, *Kalmia* is the most closely related to *Rhododendron* (Fig. 1b).

From these taxonomic comparisons, it is possible to postulate a key role for the RCA25 dehydrin in protecting *Rhododendron* leaves from freezing injury. This protein appears to have been conserved in two subgenera that have probably diverged at least 20 million yr ago (D. Chamberlain, pers. comm.) and have Asian and North American temperate zone distributions, but is absent in a tropical species that appears to be incapable of cold-acclimation. This conserved status is based in our study on the consistent appearance of proteins of equivalent molecular mass, not on any peptide or nucleic acid sequence. In addition, it should be noted that the only super cold-hardy genotype containing a single dehydrin, *R. catawbiense* 'Catalgla', has the RCA25. Thus there may have been selection pressure over evolutionary time to maintain this particular dehydrin in *Rhododendron*. The reasons for its conserved status are unknown, but may involve some unique

functional property of the RCA25, either alone or in interactions with other dehydrins, during freeze-induced dehydration stress.

Cold acclimation ability and 25-kDa dehydrin accumulation are closely associated

Anti-dehydrin immunoblots of RCA25 at varying levels of CA and subsequent LFT are shown in Fig. 2. In the comparisons of three very cold hardy species (Fig. 2a) and three less hardy species (Fig. 2b), the RCA25 in each genotype is more abundant in the CA than NA state, based on OD values. To determine whether a general relationship between RCA25 and LFT exists across species, regression of OD values on T_{max} was performed, using either absolute values ($n = 12$), or standardized values given as fold increases in cold-acclimated T_{max} and RCA25 O.D. relative to the nonacclimated state (ΔT_{max} and ΔOD, respectively, $n = 5$). Only five of the six species could be used for the standardized data set, because *R. metternichii* had no detectable RCA25 band in nonacclimated leaves at the protein loadings used in these experiments. The regressions using absolute values ($OD = 0.14 - 1.2T_{max}$, $R^2 = 0.55$, df = 11) and standardized data ($\Delta OD = 0.88 + 0.16\Delta T_{max}$, $R^2 = 0.95$, df = 4), were both significant ($P < 0.01$), although a stronger relationship was obtained by standardizing the cold-acclimated values relative to nonacclimated ones (Fig. 3a,b).

Data on the correlation between dehydrin abundance and the level of freezing tolerance in tissues across diverse species are scarce. However, differences in the freezing tolerance among cultivars (for example, *Vaccinium* sp. and *Tritcum aestivum*) have been found to be positively correlated with the accumulation levels of specific dehydrins (Arora *et al.*, 1997; Danyluk *et al.*, 1998). This is the first report, to our knowledge, to demonstrate a correlation between the degree of cold acclimation ability (ΔT_{max}) and the cold-inducibility (ΔOD) of a specific dehydrin.

Lim *et al.* (1999) suggested that the presence/absence and/or the abundance levels of 25-kDa dehydrin could serve as a biochemical marker to distinguish between super hardy and less hardy *Rhododendron* genotypes. This work was based on a segregating population from a controlled, interspecific cross, where F_2 progeny shared a similar genetic background. The regression data from six diverse *Rhododendron* species further suggests that accumulation of this particular dehydrin can be used to 'predict' the extent of cold acclimation and subsequent winter leaf freezing tolerance. Taken together with the finding that the RCA25 is the only 'universal' dehydrin among cold-acclimating rhododendrons in our survey, these data are concordant with our hypothesis that this particular dehydrin plays a central, but unknown, role in rhododendron cold hardiness.

From a physiological perspective, overall plant cold hardiness (or winter survival) is a complex trait that results from the interaction between several biochemical, physiological and morphological factors. Research to date involving transformation

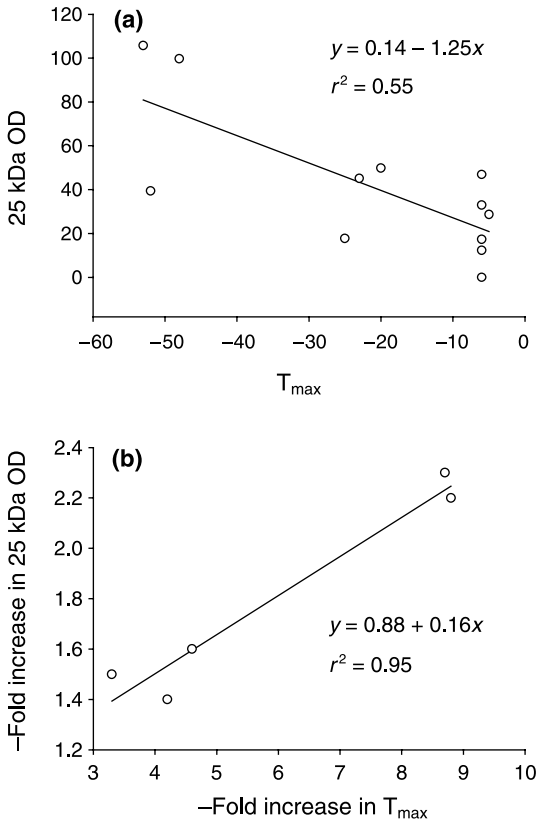

Fig. 3 Association of freezing tolerance and RCA25 abundance. (a) Regression of 25-kDa OD on T_{max} using absolute values from Fig. 2a,b. (b) Regression of OD on T_{max} using –fold increase from nonacclimated to cold-acclimated conditions (ΔOD and ΔT_{max}, respectively) in leaves. OD, optical density, a quantitative measure of RCA25 band intensity; T_{max}, temperature causing maximum rate of injury, a measure of leaf freezing tolerance.

strategies has not identified single structural genes that 'confer' the cold hardy phenotype (Xin & Browse, 2000 and references therein). Similarly, although constitutive expression of a regulatory gene (CBF) significantly improved cold and drought tolerance at whole plant level (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999), this effect is associated with multiple cellular changes such as increased expression of many cold-regulated genes, including dehydrins, as well as an increase in the cellular pools of proline and carbohydrates (Gilmour *et al.*, 2000).

In vitro studies have shown that many dehydrins (spinach COR85, maize DHN1, wheat WSC120, peach PCA60 and *Citrus unshiu* CuCor 19) can protect cold-labile LDH enzyme from freeze-thaw deactivation (Kazuoka & Oeda, 1994; Houde *et al.*, 1995; Close, 1996; Wisniewski *et al.*, 1999; Hara *et al.*, 2001) and that a birch dehydrin can preserve α-amylase activity under low water activity (Rinne *et al.*, 1999). Studies of *in vitro* cryoprotection using purified RCA25, coupled with data on gene sequence, expression profiles, and subcellular localization may provide insights into the functional

relevance of this dehydrin in *Rhododendron* freezing tolerance. In all likelihood, the RCA 25 alone is not sufficient to confer tolerance to freezing stress, but it may be a necessary component of biochemical interactions with other cryoprotectant metabolites or other dehydrins. As noted above, presence and accumulation of greater number of dehydrin proteins in *Rhododendron* does not necessarily translate into more cold hardy tissue (Table 1). This suggests that different cold-induced rhododendron dehydrins may not contribute equally towards improving cold tolerance – some might be more potent stress-protector than others.

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References

Anisko T, Lindstrom OM. 1995. Applying the Richards function in freezing tolerance determination with electrolyte and phenolic leakage techniques. *Physiologia Plantarum* **95**: 281–287.

Arora R, Rowland LJ, Panta GR. 1997. Chill responsive dehydrins in blueberry: are they associated with cold hardiness or dormancy transitions? *Physiologia Plantarum* **101**: 8–16.

Arora R, Wisniewski M. 1994. Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch.): a 60-kilodalton bark protein in cold acclimated tissues of peach is heat-stable and related to the dehydrin family of proteins. *Plant Physiology* **105**: 95–101.

Arora R, Wisniewski M, Scorza R. 1992. Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch.). I. Seasonal changes in cold hardiness and polypeptides of bark and xylem tissues. *Plant Physiology* **99**: 1562–1568.

Artlip TS, Callahan AM, Bassett CL, Wisniewski ME. 1997. Seasonal expression of dehydrin gene in sibling deciduous and evergreen peach (*Prunus persica* [L.] Batsch.). *Plant Molecular Biology* **33**: 61–70.

Artus NN, Uemura M, Steponkus PL, Gilmour S, Lin C, Thomashow MF. 1996. Constitutive expression of the cold-regulated *Arabidopsis thaliana COR 15a* gene affects both chloroplast and protoplast freezing tolerance. *Proceedings of the National Academy of Sciences, USA* **93**: 13404–13409.

Cai Q, Moore GA, Guy CL. 1995. An unusual group 2 LEA gene family in citrus responsive to low temperature. *Plant Molecular Biology* **29**: 11–23.

Choi D-W, Zhu B, Close TJ. 1999. The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allelic types, chromosome assignments, and expression characteristics of 11 *Dhn* genes of cv. Dicktoo. *Theoretical and Applied Genetics* **98**: 1234–1247.

Close TJ. 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum* **100**: 291–296.

Close TJ, Fenton RD, Moonan F. 1993. A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Molecular Biology* **23**: 279–286.

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F. 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell* **10**: 623–638.

Esen A. 1978. A simple method for quantitative, semiquantitative and qualitative assay of protein. *Analytical Biochemistry* **89**: 264–273.

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

Hara M, Terashima S, Kuboi T. 2001. Characterization and cryoprotective activity of cold-responsive dehydrin from *Citrus unshiu*. *Journal of Plant Physiology* **158**: 1333–1339.

Holt MA, Pellet NE. 1981. Cold hardiness of leaf and stem organs of rhododendron cultivars. *Journal of American Society for Horticultural Science* **106**: 608–612.

Houde M, Daniel C, Lachapelle M, Allard F, Laliberte S, Sarhan F. 1995. Immunolocalization of freezing-tolerance-associated proteins in the cytoplasm and nucleoplasm of wheat crown tissue. *Plant Journal* **8**: 583–593.

Ingram J, Bartels D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 377–403.

Ismail AM, Hall AE, Close TJ. 1999. Allelic variation of a dehydrin gene cosegregates with chilling tolerance during seedling emergence. *Proceedings of the National Academy of Sciences, USA* **96**: 1356–1357.

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. 1998. *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280**: 104–106.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnology* **19**: 287–291.

Kazuoka T, Oeda K. 1994. Purification and characterization of Cor 85-oligomeric complex from cold-acclimated spinach. *Plant and Cell Physiology* **35**: 601–611.

Kontunen-Soppela S, Taulavuori K, Taulavouri E, Lähdesmäki P, Laine K. 2000. Soluble proteins and dehydrins in nitrogen-fertilized Scots pine seedlings during deacclimation and the onset of growth. *Physiologia Plantarum* **109**: 404–409.

Kron KA. 1997. Phylogenetic relationship of *Rhododendron* (Ericaceae). *American Journal of Botany* **84**: 973–980.

Lang GA. 1987. Dormancy: a new universal terminology. *Hortscience* **22**: 817–820.

Levi A, Panta GR, Parmentier CM, Muthalif MM, Arora R, Shanker S, Rowland LJ. 1999. Complementary DNA cloning, sequencing and expression of an unusual dehydrin from blueberry floral buds. *Physiologia Plantarum* **107**: 98–109.

Levitt J. 1980. *Responses of plants to environmental stresses, Vol. I. Chilling, freezing and high temperature stresses, 2nd edn.* New York, USA: Academic Press.

Lim CC, Arora R, Townsend ED. 1998a. Comparing Gompertz and Richards functions to estimate freezing injury in *Rhododendron* using electrolyte leakage. *Journal of American Society for Horticultural Science* **123**: 246–252.

Lim CC, Krebs SL, Arora R. 1998b. Genetic study of freezing tolerance in *Rhododendron* populations: Implications for cold hardiness breeding. *Journal of American Rhododendron Society* **52**: 143–148.

Lim CC, Krebs SL, Arora R. 1999. A 25-kDa dehydrin associated with genotype- and age-dependent leaf freezing-tolerance in *Rhododendron*: a genetic marker for cold hardiness? *Theoretical and Applied Genetics* **99**: 912–928.

Muthalif MM, Rowland LJ. 1994. Identification of dehydrin-like proteins resposible to chilling in floral buds of blueberry (*Vaccinium*, section *Cyanococcus*). *Plant Physiology* **104**: 1439–1447.

Nylander M, Svensson J, Palva ET, Welin BW. 2001. Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. *Plant Molecular Biology* **45**: 263–279.

Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I, Bedo Z. 1994. Genetic analysis of the components of winterhardiness in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **89**: 900–910.

Rinne PL, Kaikuranta PLM, van der Plas LHW, van der Schoot C. 1999. Dehydrins in cold-acclimated apices of birch (*Betula pubescens* Ehrh.): production, localization and potential role in rescuing enzyme function during dehydration. *Planta* **209**: 377–388.

Rinne P, Welling A, Kaikuranta P. 1998. Onset of freezing tolerance in birch (*Betula pubescens* Ehrh.) involves LEA proteins and osmo-regulation and is impaired in an ABA-deficient genotype. *Plant, Cell & Environment* **21**: 601–611.

Sakai A, Fuchigami LH, Weiser CJ. 1986. Cold hardiness in the genus *Rhododendron*. *Journal of American Society for Horticultural Science* **111**: 273–280.

Salzman RA, Bressan RA, Hasegawa PM, Ashworth EN, Bordelon BP. 1996. Programmed accumulation of LEA-like proteins during desiccation and cold acclimation of over-wintering grape buds. *Plant, Cell & Environment* **19**: 713–720.

Sarhan F, Ouellet F, Vazquez-Tello A. 1997. The wheat wcs120 gene family: a useful model to understand the molecular genetics of freezing tolerance in cereals. *Plant Physiology* **101**: 439–445.

Sarnighausen E, Karlson DT, Ashworth EN. 2002. Seasonal regulation of a 24-kD protein from red-osier dogwood (*Cornus sericea* L.) xylem. *Tree Physiology* **22**: 423–430.

Sauter JJ, Westphal S, Wisniewski M. 1999. Immunological identification of dehydrin-related proteins in the wood of five species of *Populus* and in *Salix caprea* L. *Journal of Plant Physiology* **154**: 781–788.

Svensson J, Ismail AM, Palva T, Close TJ. 2002. Dehydrins. In: Storey KB, Storey JM, eds. *Sensing, signalling, and cell adaptation*. Elsevier Science BV, 155–171.

Welling A, Kaikuranta P, Rinne P. 1997. Photoperiodic induction of dormancy and freezing tolerance in *Betula pubescens*. Involvement of ABA and dehydrins. *Physiologia Plantarum* **100**: 119–125.

Wisniewski M, Close TJ, Artlip T, Arora R. 1996. Seasonal patterns of dehydrins and 70-kDa heat-shock proteins in bark tissues of eight species of woody plants. *Physiologia Plantarum* **96**: 496–505.

Wisniewski M, Webb R, Balsamo R, Close TJ, Yu XM, Griffith M. 1999. Purification, immunolocalization, cryoprotective, and antifreeze activity of PCA60: a dehydrin from peach (*Prunus persica*). *Physiologia Plantarum* **105**: 600–660.

Xin Z, Browse J. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant, Cell & Environment* **23**: 893–902.