Unraveling the cold response in Draba

von Meijenfeldt, N.

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Freezing tolerance in *Draba*; acclimation responses in species from different geographic regions

N. von Meijenfeldt, P.H. van Tienderen & M.A. Haring
In this chapter we investigated the freezing tolerance of Draba species from different geographical regions and compared it to that of A. thaliana. Freezing tolerance was measured via juvenile survival and electrolyte leakage experiments. In addition, we determined the acclimation response of species to a cold pretreatment before testing freezing tolerance. Our results showed that seedlings of species from differing geographical regions vary in their basal tolerance to freezing. D. hookeri from the high Andes of Ecuador was most sensitive to freezing. The electrolyte leakage experiments revealed the same trend. The cold acclimation response was strongest in seedlings of temperate species A. thaliana, D. muralis and D. verna. Arctic-alpine D. nivalis displayed a less pronounced acclimation response, while D. hookeri displayed the weakest response. Electrolyte leakage experiments confirmed this trend in acclimation response. When the seedlings were exposed to a 21/4°C cycle for one week as acclimation treatment, the response was weaker than after a continuous cold period of 4°C. Finally, a positive correlation between freezing tolerance and glucose content was observed among all species. In addition, Draba species displayed a positive correlation between frost tolerance and total sugar as well as proline content.

One important constraint on the growth and distribution of plants are freezing temperatures. Especially the frequency with which frost occurs; that is all-year round, seasonally, or episodically, is of influence. In equatorial uplands and mountains, plants may frequently encounter nocturnal frost throughout the entire year (Larcher, 2003). Plants growing in this environment must maintain freezing tolerance mechanisms different from those of overwintering plants occurring in temperate regions, which encounter freezing on a seasonal basis. Arctic-alpine regions, in turn, are characterized by long winters and a short and cold growing season (Billings, 1974). Plants living in these different environments can be expected to have different survival strategies. Both temperate species D. muralis and D. verna are winter annuals, while the arctic-alpine D. nivalis and the tropical-alpine D. hookeri are perennial species. This difference in life strategy between the species is indicative for specific adaptations to their natural habitat. As these Draba species occur in different geographical regions it can be expected that the various species have developed different mechanisms to cope with frost. We hypothesized that tropical-alpine D. hookeri that encounters subzero temperatures on a daily basis will have a different response compared to the temperate Draba species that encounter subzero temperatures on a seasonal scale, whereas, given its circumpolar distribution, we expected D. nivalis to be most freezing tolerant (see Chapter 1, FIGURE 4).

A particular characteristic of tolerance to freezing, is the phenomenon of acquired tolerance (John et al., 2009). Many temperate species acquire an increased freezing tolerance when they are first exposed to low but non-freezing temperatures; a process known as cold acclimation (Thomashow, 1999). In the temperate climate zone, cold acclimation in plants is initiated by the gradual decrease in temperature and photoperiod that occurs in autumn. Through cold acclimation the necessary mechanisms are triggered to acquire freezing tolerance, which determines the capacity of plants to survive the winter. Low temperatures initiate signaling pathways that control the expression of genes encoding determinants that are necessary for freezing tolerance (Knight et al., 1999; Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006; Chinnusamy et al., 2007). In Arabidopsis, overexpression of each of the three CBF genes induces the CBF regulon and enhances plant freezing tolerance by increasing the levels of cryoprotectants such as proline and sucrose in the cells (Gilmour et al., 2000; Strand et al., 2003; Gilmour et al., 2004; Reyes-Diaz et al., 2006). In addition, a major QTL for freezing tolerance has been mapped to the genomic region containing the CBF genes in Arabidopsis (Alonso-Blanco et al., 2005). Besides Arabidopsis,
cold-acclimation has been shown to improve the freezing tolerance of other Brassicaceae, such as *Thellungiella salsuginea* (Griffith et al., 2007), *Thlaspi arvense* (Sharma et al., 2007), and *Brassica napus* (Jaglo et al., 2001). In addition, Hannah et al. (2006) and Zhen & Ungerer (2008) demonstrated that freezing tolerance in natural *Arabidopsis* accessions correlates positively with habitat winter temperatures.

When temperatures drop below zero, ice crystals form in the intercellular spaces and cell walls of plant tissues. As a result, cytoplasmic water from inside the cell is drawn to the growing mass of extracellular ice. Freezing injury is, therefore, mainly an injury caused by cellular dehydration, which has a damaging effect on membrane structure and cellular functions (Uemura et al., 1995; Xin and Browse, 2000). The primary manifestation of freezing injury in plants is observed in the membrane systems (Steponkus, 1984). This highlights the importance of cryoprotective agents. Their accumulation lowers the water potential and increases the cryostability of the plasma membrane, making it less sensitive to freeze-induced destabilization (Uemura et al., 1995).

In Chapter 3 we studied the transcriptional response to cold in four different *Draba* species. For all investigated species we found that a low temperature (4°C) transiently induced expression of *CBF* and one of its target genes; *COR15*. However, in *Draba* the level of expression of these cold-responsive genes was 10-20x lower than in *Arabidopsis*. In addition, levels of proline and soluble sugars such as sucrose, glucose, fructose and raffinose were measured. In all *Draba* species sugar content increased upon cold treatment. In *D. muralis* raffinose levels increased upon cold treatment, while in *D. hookeri* and *D. nivalis* a constitutively low level was detected. Proline levels did not change in *Draba* species upon cold treatment, *D. muralis* had constitutively high levels. To investigate the possible relation between *CBF* and *COR15* gene expression and changes in metabolites we determined the freezing tolerance and the acclimation capacity of *Arabidopsis* and *Draba* species. To this end we performed juvenile survival and electrolyte leakage experiments.

Because seedlings of many species are less tolerant of extreme environmental conditions compared to adults, the degree of freezing tolerance can determine successful establishment and thereby limit species distributions (Loik and Redar, 2003). Thinner tissues of young plants coupled with a higher sensitivity to freezing can result in damaged membranes and tissues, reduced physiological function and even death (Sakai and Larcher, 1987 as cited by Lambrecht et al., 2007).

**MATERIALS AND METHODS**

**Plant growth conditions**

The *Draba* and *Arabidopsis* species used in this study are the same as described in Chapter 3. All seeds were surface sterilized as described in Chapter 2 and subsequently sown on 0.5 MS, 1% (w/v) Daishin agar plates (Duchefa Biochemie, Haarlem, The Netherlands). For the juvenile survival experiments the plates were divided into five equal parts in which ten seeds per species were sown in random combinations next to each other. Seeds were vernalized as described in Chapter 3 with slight modifications in the length of vernalization. For the juvenile survival experiment all species were vernalized for two weeks prior to transfer to the growth chamber. For the electrolyte leakage experiment different vernalization treatments were applied to different species. This was to ensure that all species had large enough leaves to conduct the electrolyte leakage experiment, while simultaneously ensuring good germination rates. *D. hookeri, D. nivalis* and *D. verna* were vernalized for two weeks after which
they were transferred to a growth chamber. In the mean time *D. muralis* and *A. thaliana* were vernalized for an additional week. One week after transfer to the growth chamber all seedlings for the electrolyte leakage experiment were transferred to sterilized sowing soil.

The standard growth conditions in the growth chamber were the same as described in Chapter 3: a 12L/12D photoperiod with a 21/15°C temperature regime. Illumination was set at 150µEinstein/m².

**Plant freezing survival**

Survival was measured as percentage survival of plants after freezing at different temperatures. Half of the 3week old plants of *D. hookeri, D. muralis, D. nivalis, D. verna, and A. thaliana* were cold-acclimated at 4°C for 48hours under the standard 12h photoperiod while the other half remained in the standard growth conditions. After acclimation and four hours into the night all plants were transferred to a Microclima climate cabinet (Snijders Microclima 1000; Snijders Scientific, Tilburg, The Netherlands) programmed to decline from 21 to −1°C at a rate of 1°C/h. The non-acclimated plants were transferred to the Microclima at 15°C, the cold acclimated plants when the temperature had dropped to 4°C, to avoid sudden changes. All plates were placed in a complete randomized design in the Microclima. Once at −1°C, the temperature was maintained there for six hours. After one hour equilibration at −1°C, ice chips were applied to all agar plates in order to ensure ice nucleation. Temperature in the Microclima was then programmed to gradually decline from −1 to −15°C at a rate of 1°C/h. The temperature trajectory of the Microclima was monitored using a datalogger (Onset HOBO Pro H08-032-08, Onset Computer Corporation, MA, USA). Plates were removed at 2°C intervals over a temperature range of −5 to −13°C for non-acclimated plants and −7 to −15°C for cold acclimated plants so that survival curves could be fitted. Per acclimation treatment (non- versus cold acclimation) three agar plates containing ten plants/species were removed per time point. The experiments were replicated independently.

Once removed, the plants were thawed for 12hrs in the dark at 4°C and then placed back into a growth chamber set at the standard temperature and light conditions. Photographs of the plants were taken prior to cold-acclimation, one day prior, one day after, one week after, and two weeks after the freezing treatment. Survival was visually scored by monitoring the fate of the frozen leaves over the next two weeks. Plants that still contained one or more green leaves after two weeks were counted as survivors. The percentage freezing survival per species was calculated as: (number of survivors / total number of plants) * 100. Data presented are mean values of three independent agar plates from the two experimental replicates.

Based on the results, response curves were fitted for each survival experiment according to the equation: \( N_T = N_0 / (1 + e^{\mu + \beta T}) \), where \( N_0 \) is the initial number of plants, \( N_T \) the number of survivors at temperature \( T \), and \( \mu \) and \( \beta \) are parameters estimated with Excel’s Solver tool by minimizing the squared differences between the observed and fitted number of dead plants. Lethal temperature for 50% death (LT\(_{50}\)) was estimated from the fitted response curves by setting \( N_T = 0.5 \ N_0 \) and solving for \( T \), resulting in \( LT_{50} = \mu / \beta \).

\( \Delta LT_{50} \) was calculated as the difference between the LT\(_{50}\)’s of the cold-acclimated and non-acclimated plants.
Determination of freezing tolerance of leaves

Injured cells are unable to maintain the chemical composition of their contents and therefore release electrolytes through damaged membranes. When injured leaves, or other plant parts, are placed in water the electrolytes present within the cell will leak into the water. The conductivity of the resultant solution can then be measured, which provides a means for quantifying the amount of cell damage caused by freezing. This method is commonly referred to as the electrolyte leakage (EL) method (Murray et al., 1989) and with this method freezing-induced leaf injury was determined. To investigate the possible role of cold-acclimation on enhancing freezing tolerance two different acclimation treatments were applied to the plants during two independent experiments. First, 5.5week old *D. hookeri*, *D. nivalis*, and *D. verna* and 4.5week old *D. muralis* and *A. thaliana* plants were acclimated for 48h at 4°C as described in the paragraph above. Alternatively, plants of these ages were acclimated for one week at a 21/4°C (day/night) temperature regime and 12h photoperiod, while non-acclimated plants were kept at the standard light and temperature conditions. Thus, in the 48h 4°C acclimation experiment plants were 4.5 or 5.5weeks old when EL was measured, while in the one week 21/4°C acclimation experiments plants were 5.5 or 6.5weeks old.

Three uniform leaf disks per plant per species were cut (Ø 0.5mm) from non-acclimated or cold-acclimated plants with use of a leaf disc borer and placed into test tubes containing 100μl de-ionized water. Test tubes were subsequently placed in a completely randomized design in a –1°C waterbath (Lauda Ecoline RE 312, Lauda Germany) for 1h after which ice crystals were added to nucleate freezing. After an additional 2h of equilibration at –1°C the samples were gradually cooled in increments of –1°C/h. Two samples per species and per acclimation treatment were removed every two hours starting at –3°C until the last samples were removed at –13°C. The temperature trajectory of the waterbath was monitored with the same datalogger as described above. Once removed the samples were stored on ice until all samples had been collected and left to thaw overnight in the cold room at 4°C. After thawing, all samples, including the unfrozen controls kept at 4°C (EL unfrozen), were incubated in 1ml de-ionized water with gentle shaking (125motions/min) at room temperature for 2h. Electrolyte leakage from the leaves was measured using a Radiometer CDM80 (Radiometer Copenhagen, Denmark) conductivity meter with a Radiometer CDC114-type conductivity cell. The samples were then placed for 1h in a –80°C freezer, thawed for 30min in a 57°C stove, and shaken gently for an additional 2h before the conductivity of the resulting solution was measured to obtain a value for 100% electrolyte leakage (EL 100). As a calibration reference for the conductivity meter a 10x dilution series ranging from 0.1mM to 1M NaCl was always included at the start of all conductivity measurements.

The percentage of electrolyte leakage from frozen leaves was calculated according to the equation as described by Webb et al. (1994):

\[ \%EL = \frac{(EL_{\text{frozen}} - EL_{\text{unfrozen}})}{(EL_{100} - EL_{\text{unfrozen}})} \times 100. \]

The 48h at 4°C acclimation experiment was replicated three times, the one week at 21/4°C experiment two times.

Response curves were fitted for each electrolyte leakage experiment based on a modified Richards’ function as described by Anisko and Lindstrom (1995): \[ \%EL_T = \alpha e^{\beta e^{\kappa T}}, \]

where \%EL_T is the percentage leakage at temperature T, \( \alpha \) is the upper asymptote for \( T \to -\infty \) (100% leakage expected), and \( \beta \) and \( \kappa \) are parameters determining the form of the curve. \( \alpha \), \( \beta \) and \( \kappa \) were estimated with Excel’s Solver-tool by minimizing the sum of squared difference between observed and fitted \%EL values. The temperature at which 50% cell damage
occurred (LT$_{50}$) was estimated from the fitted models by setting %EL$_T$ = 50 and solving for $T$, resulting in: $LT_{50} = \frac{1}{\kappa} * \ln(\frac{1}{\beta} \ln(50/\alpha))$. LT$_{50}$ was, thus, derived from the estimated temperature at which 50% of the leakage occurred according to the fitted curve. ΔLT$_{50}$ was calculated as the difference between the LT$_{50}$'s of the cold-acclimated and non-acclimated plants.

**Growth measurements**

Growth was evaluated by sampling twenty-eight 4 to 4.5 week old plants per species randomly picked in the growth chamber of which fresh weight, dry weight, projected leaf area and total leaf area were recorded. As all investigated species are rosette plants their leaves grow in such a way that they partially overlap. In order to get an idea of this degree in overlap the projected and total leaf area of all plants was measured separately. The projected leaf area was measured on a digital picture of intact plants, the total leaf area on a digital scan of all the individual leaves detached from a rosette. Projected and total leaf area were calculated using image analysis software (Image J; Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2009). A standard size object was included in each picture for calibration. Fresh weight was determined by harvesting and weighing the whole aboveground biomass. After weighing, the plant rosettes were placed for 48h in a 70°C stove, after which the dry weight was measured. Based on these measurements the leaf area per unit leaf mass or Specific Leaf Area (SLA; the quotient of total leaf area to leaf dry weight; mm$^2$/mg) and the Leaf Dry Matter Content (LDMC; the quotient of dry weight to fresh weight) were calculated. To obtain a measure for the initial size of the plants, the projected leaf area of all plants was measured at the start of the week. Then, 14 of the 28 plants per species were harvested to determine the total leaf area, fresh weight and dry weight. Half of the remaining 14 plants were transferred to a 21/4°C temperature while the other half were kept under the standard 21/15°C temperature conditions. A 12h photoperiod was maintained in both temperature treatments. After one week, fresh weight, dry weight and total leaf area was determined for these 14 individuals per species.

**Statistical analysis**

Univariate analyses of variance (ANOVA) with type-III sum of squares were performed for the LT$_{50}$ values derived from survival and electrolyte leakage data, using SPSS v 16.0 (SPSS Inc., Chicago IL). A t-test was performed to analyze the average LT$_{50}$ values between the two different electrolyte leakage experiments, separately for all species.

Post hoc tests were analyzed with LSD, FREGW, and QREGW comparisons. Assumptions of normality and homogeneity of variance were examined with visual plots. Prior to analysis of the growth data, variables Leaf area and Projected leaf area were normalized via log-transformation. The original Leaf area of the plants grown for one week at 21/4°C was predicted from the projection images via a Weighted least squares Linear Regression analysis forced through the origin (Pin 0.05 and Pout 0.10). These values will be referred to as Predicted Leaf area. The corresponding data was analyzed via a univariate ANOVA with species, time, and treatment as fixed factors. For all analyses, $\alpha = 0.05$ level of significance was used.

Based on the soluble sugar and proline data from Chapter 3, a polynomial species contrast analysis was performed between the metabolites and the maximum freezing tolerance as measured by electrolyte leakage in the plants acclimated at 4°C for 48h. The null-hypoth-
thesis was that across species a linear relationship existed between the acclimated LT<sub>50</sub> and the average accumulated metabolite within a 95% confidence interval.

**RESULTS**

*Draba* species are freezing tolerant but react differently to cold acclimation

In a seedling-freezing assay, 3-week old seedlings grown on agar plates were exposed to a freezing trajectory of −1°C/h with a minimum temperature of −15°C. Plants were removed at 2°C intervals over a temperature range of −5 to −15°C. Plant survival was scored after two weeks of growth under standard conditions and representative results are shown in Figure 1. By quantifying the mortality, the freezing tolerance of the different species could be visualized and the temperature at which 50% of the juveniles survived (LT<sub>50</sub>) was calculated. With a decrease in temperature an increase in seedling mortality was observed (Figure 2). When grown under standard conditions (21/15°C day/night, 12h photoperiod), levels of freezing tolerance did not significantly differ between 3-week old *Draba* and *Arabidopsis* seedlings (Table 1, F<sub>4,5</sub> = 1.935 n.s.). The temperature at which 50% survival occurred was determined to lie between −5 and −6°C (Figure 2 and Table 1).

**Figure 1.** Juvenile survival of non-acclimated and acclimated *Arabidopsis* and *Draba* seedlings prior to and after treatment with subzero temperatures. Images taken at the start of treatment (A) and two weeks after freezing treatment of −5 (B), −7 (C), or −9°C (D) for non-acclimated individuals. Images of acclimated individuals were taken at the start of acclimation treatment (E) and two weeks after freezing treatment of −7 (F), −11 (G), or −15°C (H). Ten juveniles per species were grown on agar plates at 21/15°C under 12h light and 12h dark. During the freezing treatment temperature was lowered by 1°C/h and once removed plates were left at 4°C overnight to defrost before being returned to standard growth conditions. Plants were cold acclimated by exposing half of the plates with juveniles to 4°C for 48h prior to freezing under the standard light regime.
Cold acclimation by growing the seedlings 48h at 4°C resulted in an increase in freezing tolerance of both *Draba* and *Arabidopsis* seedlings (see Figure 1 and 2), and species-specific differences in freezing tolerance became more apparent. Cold acclimation caused a shift in the survival curve, resulting in lower LT50 values, i.e., cold-acclimation had a significant overall effect on the LT50 in all species ($F_{1,18}=63.027^{***}$) resulting in enhanced freezing tolerance. However, the degree to which the freezing tolerance increased differed significantly between species (Figure 1 and 2, Table 1, $F_{4,5}=32.518^{**}$). The LT50 after

**FIGURE 2.** Cold acclimation enhances the freezing tolerance of juvenile *Draba* species. Average juvenile survival obtained from three independent agar plates containing ten individuals per species of two biological replicates. Survival of four *Draba* species (A-D) and *Arabidopsis* (E) was scored two weeks after freezing treatment. Plants were cold acclimated by exposure for 48h to 4°C under standard light/dark cycles. Dashed lines represent non-acclimated individuals, solid lines acclimated individuals.
acclimation, ranged from –7.5°C for *D. hookeri* to –12.3°C for *A. thaliana*. Thus, *Draba* species differed from each other and from *Arabidopsis* in their ability to cold acclimate, as apparent from the difference between the LT50 of cold acclimated and non-acclimated plants (the ΔLT50; TABLE 1, F4,5 = 12.613** for all species, F3,4 = 18.540** for *Draba* only). Temperate *A. thaliana* and *D. verna* appeared to benefit the most from cold acclimation with ΔLT50s of –6.2 and –6.3°C, respectively. Temperate *D. muralis* and arctic-alpine *D. nivalis* had intermediate ΔLT50s of –5.1 and –4.1°C respectively, while tropical-alpine *D. hookeri* showed the smallest ΔLT50; i.e., –2.5°C.

**Cold acclimation decreases freezing induced electrolyte leakage in *Draba***

For comparison with the whole plant survival, freezing induced damage at the tissue level was measured via an electrolyte leakage experiment. This method is used as a measure for plasma membrane disruption, thought to be the primary site of injury during freezing (Sharma et al., 2007). The electrolyte leakage values (as % of the maximal electrolyte leakage in leaf discs) were plotted against the temperature (FIGURE 3). The lower the temperature, the higher the amount of electrolyte leakage measured. In control plants the temperature at which 50% of maximum leakage occurred, varied between –3 and –4°C (FIGURE 3 and TABLE 2). Significant differences in electrolyte leakage were detected among the five species (TABLE 2, F4,10 = 5.808*). Cold pretreatment had a significant effect on LT50 (F1,27 = 15.512**). Similar to the juvenile survival experiment, 48h acclimation at 4°C shifted the electrolyte leakage curves to the right and lowered the temperature at which freezing damage occurred (FIGURE 3). In three of the five species; i.e., *A. thaliana*, *D. muralis*, and *D. nivalis*, cold acclimation significantly decreased the temperature at which 50% of maximal electrolyte leakage occurred (TABLE 2). The effect was strongest in *A. thaliana*, ΔLT50 = –3.2°C. As in the survival experiment, *D. hookeri* showed the weakest cold acclimation response of all species. Univariate ANOVA analysis of all ΔLT50 values confirmed that cold acclimation responses at the tissue level differed between species (TABLE 2, F4,9 = 9.813**).

As the same acclimation treatment was used in the juvenile survival and electrolyte leakage experiments, ΔLT50 values can be compared between these two assays to quantify acclimation responses in freezing tolerance. The effect of assay method was evident in this analysis, i.e, the effect of acclimation was more pronounced for survival than for leakage (F1,14 = 174.052***). At the species level a significant difference in ΔLT50 was found (F4,14 = 18.495***). This implied that, averaged over the two experimental methods, species bene-

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**TABLE 1. Overview of species specific responses to cold acclimation at the whole plant level in *Draba*.** Average lethal temperatures from two independent experiments in which 50% of non-acclimated (non-acc) or acclimated (acc) juveniles survived (LT50) in response to freezing treatment. ΔLT50 is the calculated difference between the LT50’s of acclimated and non-acclimated samples. Univariate ANOVA; P < 0.05 *, P < 0.01 **, P < 0.001 ***, n.s. = non significant.

<table>
<thead>
<tr>
<th>Species</th>
<th>LT50 Non-Acclimated</th>
<th>ΔLT50</th>
<th>Significant difference in non-acc and acc LT50s</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. thaliana</em></td>
<td>12.3</td>
<td>6.2</td>
<td>***</td>
</tr>
<tr>
<td><em>D. verna</em></td>
<td>-11.5</td>
<td>5.2</td>
<td>***</td>
</tr>
<tr>
<td><em>D. muralis</em></td>
<td>-11.3</td>
<td>6.2</td>
<td>***</td>
</tr>
<tr>
<td><em>D. nivalis</em></td>
<td>-10.2</td>
<td>-6.2</td>
<td>***</td>
</tr>
<tr>
<td><em>D. hookeri</em></td>
<td>-7.5</td>
<td>-5.0</td>
<td>**</td>
</tr>
</tbody>
</table>

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fitted differently from acclimation. *D. verna* displayed the strongest effect of experimental method on its calculated ΔLT50 resulting in a relatively small but significant interaction between species and experimental method (F4,14 = 4.427*).

Because the tropical-alpine species, *D. hookeri* rarely encounters long periods of cold we also investigated whether an alternative cold acclimation period, an exposure for 1 week to a 21/4°C day/night temperature regime with a 12h photoperiod, would enhance its freezing tolerance in the electrolyte leakage experiment. The results obtained from two biological replicates showed that this treatment did not increase the cold tolerance of *D. hookeri* (TABLE 2 and APPENDIX 1). The LT50 of the cold acclimated *D. hookeri* plants did not differ

**FIGURE 3.** Cold acclimation reduces the amount of cell damage due to freezing in *Draba* species. Average electrolyte leakage obtained from two independent samples from three biological replicates of four *Draba* species (A-D) and *Arabidopsis* (E). Cold acclimation treatment and representation of data as described in FIGURE 2.
significantly from the LT$_{50}$ of the non-acclimated plants (TABLE 2). In this experimental setting no significant differences in $\Delta$LT$_{50}$ were detected between species (TABLE 2, F$4,5 = 3.887$ n.s.).

When comparing the acclimation treatment of 48h at 4°C to a one week period at 21/4°C a significant difference in $\Delta$LT$_{50}$s was found between species (F$4,14 = 8.443^{**}$). Species, thus, responded differently to the applied acclimation treatment. In addition, the interaction between species and acclimation treatment was significant (F$4,14 = 4.124^*$). Acclimation at a constant 4°C temperature for 48h resulted in a significantly stronger effect on the freezing tolerance of *Arabidopsis thaliana* (F$1,3 = 16.396^*$). Despite a suggestive difference in $\Delta$LT$_{50}$ between the 48h at 4°C and one week at 21:4°C acclimation treatments, these did not prove to be statistically significant for the *Draba* species.

Both in juvenile survival (F$4,9 = 8.603^{**}$) and in electrolyte leakage of leaves (48h at 4°C F$4,17 = 8.894^{***}$, or one week at 21:4°C F$4,9 = 5.956^*$) species reacted differently to the cold pretreatment. Independent of the acclimation treatment, for all species the temperature at which electrolyte leakage occurs is higher than the temperature that causes seedling mortality. Because the different *Draba* species were different in size and the smallest species *D. hookeri* proved to be the most freezing sensitive, we investigated how a one week exposure to a 21/4°C (day/night) temperature regime influenced growth parameters in all species.

### Table 2. Overview of species specific responses to cold acclimation at the cellular level in *Draba*.

Average lethal temperatures at which 50% cell damage (LT$_{50}$) occurred in leaf disks of non-acclimated (non-acc) or acclimated (acc) plants in response to freezing treatment. LT$_{50}$ values presented are averages of three experimental replicates for the 48h at 4°C acclimation and two experimental replicates for the 1 week at 21/4°C acclimation treatment. $\Delta$LT$_{50}$ calculation and representation of significance levels as described in TABLE 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>LT$_{50}$ Non-Acclimated</th>
<th>LT$_{50}$ Acclimated</th>
<th>$\Delta$LT$_{50}$</th>
<th>Significant difference in non-acc and acc LT$_{50}$'s</th>
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<tr>
<td><em>A. thaliana</em></td>
<td>-3.7</td>
<td>-6.9</td>
<td>-3.2</td>
<td>***</td>
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<td><em>D. verna</em></td>
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<td>-3.9</td>
<td>-0.6</td>
<td>n.s.</td>
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<td><em>D. muralis</em></td>
<td>-3.9</td>
<td>-5.6</td>
<td>-1.8</td>
<td>***</td>
</tr>
<tr>
<td><em>D. nivalis</em></td>
<td>-3.6</td>
<td>-4.6</td>
<td>-1.0</td>
<td>*</td>
</tr>
<tr>
<td><em>D. hookeri</em></td>
<td>-3.1</td>
<td>-3.4</td>
<td>-0.3</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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Both in juvenile survival (F$_{4,9} = 8.603^{**}$) and in electrolyte leakage of leaves (48h at 4°C F$_{4,17} = 8.894^{***}$, or one week at 21:4°C F$_{4,9} = 5.956^*$) species reacted differently to the cold pretreatment. Independent of the acclimation treatment, for all species the temperature at which electrolyte leakage occurs is higher than the temperature that causes seedling mortality. Because the different *Draba* species were different in size and the smallest species *D. hookeri* proved to be the most freezing sensitive, we investigated how a one week exposure to a 21/4°C (day/night) temperature regime influenced growth parameters in all species.

### Draba and Arabidopsis plants have similar growth characteristics when exposed to cold nights

To investigate whether the observed differences in cold acclimation between the different species could be related to differences in plant size, we monitored plant growth of plants exposed to a 21/4°C day/night regime with a 12h photoperiod during one week. We measured total leaf area (TLA), projected leaf area (PLA), dry weight (DW) and fresh weight (FW) of the five investigated plant species. From these measurements the Specific Leaf Area (SLA; TLA/DW) and the Leaf Dry Matter Content (LDMC; DW/FW) were calculat-
ed. All investigated species had a rosette growth form, which meant that their leaves all showed some degree of overlap. By comparing the total leaf area as measured by adding up all the measurements of each individual leaf, with the projected leaf area; i.e. the area occupied by the entire rosette, an estimate of the leaf overlap could be made.

All species displayed a diminished increase in total leaf area when grown for one week at 21/4°C compared to 21/15°C (Table 3). Leaf area and projected leaf area were positively correlated in all species; as total leaf area increased so did projected leaf area (two-tailed Pearson’s correlation: \(P<0.01\) for all species; Appendix 2A, D, G, J and M). Our results demonstrated that irrespective of the temperature treatment an increase in total leaf area was obtained mostly through elongation of the leaves rather than by leaves becoming wider. This would have resulted in increased overlap of leaves and a smaller projected leaf area. Plants grown under a 21/4°C day-night regime had a smaller increase in both the total leaf area and projected leaf area compared to plants grown under 21/15°C conditions (Appendix 2A, D, G, J and M); 4°C nights appeared to reduce, but not prevent growth.

When exposed to cold nights all species showed increased leaf dry matter content compared to plants grown under warm nights (Table 3). This increase in LDMC signifies that dry weight increased more than fresh weight, for instance through a reduced cell expansion and water uptake in cold nights (Table 3). In addition, while plants grown under a 21/4°C temperature regime had increased leaf dry matter content, their specific leaf area decreased (Appendix 2B, E, H, K and N). SLA, calculated as the leaf area per unit leaf weight, gives an indication of leaf structural strength and thickness, and is usually negatively correlated with LDMC (Roche et al., 2004). In all species, exposure to 4°C nights, thus, resulted in more compact but stronger leaves containing less water. Despite being the smallest species,

### Table 3. Effects of cold nights on growth of *Arabidopsis* and *Draba*.

Average Total Leaf Area (TLA), Leaf Dry Matter Content (LDMC; DW/FW) and Dry Weight (DW) were measured at the start of the temperature treatment, after one week at standard growth conditions (21/15°C) and after one week with cold nights (21/4°C).

<table>
<thead>
<tr>
<th>Species</th>
<th>TLA (mm²)</th>
<th>LDMC (mg/mg)</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start 1w 21/15°C 1w 21/4°C</td>
<td>Start 1w 21/15°C 1w 21/4°C</td>
<td>Start 1w 21/15°C 1w 21/4°C</td>
</tr>
<tr>
<td>At</td>
<td>181491    632954 350611</td>
<td>0.080 0.088 0.114</td>
<td>31.4 136.0 98.0</td>
</tr>
<tr>
<td>Dv</td>
<td>132910    309488 243816</td>
<td>0.078 0.077 0.099</td>
<td>31.3 81.3 48.9</td>
</tr>
<tr>
<td>Dm</td>
<td>171399    444298 299433</td>
<td>0.115 0.115 0.155</td>
<td>44.4 127.2 110.8</td>
</tr>
<tr>
<td>Dn</td>
<td>95780     213801 135393</td>
<td>0.102 0.100 0.137</td>
<td>18.7 47.0 45.4</td>
</tr>
<tr>
<td>Dh</td>
<td>47189     103198 102300</td>
<td>0.083 0.081 0.096</td>
<td>10.5 23.8 33.2</td>
</tr>
</tbody>
</table>

### Table 4. Output from the univariate analysis of variance (ANOVA) for the log-transformed Predicted Leaf Area (PLA).

Analysis was performed with a type-III sum of squares in SPSS with species, time, and treatment as factors. An \(\alpha=0.05\) level of significance was applied. Representation of significance levels as described in Table 1.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>4</td>
<td>2.126</td>
<td>230.598</td>
<td>***</td>
</tr>
<tr>
<td>Time</td>
<td>1</td>
<td>3.333</td>
<td>361.385</td>
<td>***</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.245</td>
<td>26.606</td>
<td>***</td>
</tr>
<tr>
<td>Species * Time</td>
<td>4</td>
<td>0.045</td>
<td>4.846</td>
<td>**</td>
</tr>
<tr>
<td>Species * Treatment</td>
<td>4</td>
<td>0.048</td>
<td>5.195</td>
<td>**</td>
</tr>
<tr>
<td>Time * Treatment</td>
<td>1</td>
<td>0.200</td>
<td>21.618</td>
<td>***</td>
</tr>
<tr>
<td>Species * Time * Treatment</td>
<td>4</td>
<td>0.200</td>
<td>0.098</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Error 190
*D. hookeri* showed a similar increase in leaf dry matter during the cold nights as the bigger *D. verna*. Overall, *A. thaliana* and *D. muralis* were the largest species.

Since a destructive method was used to obtain measurements for TLA this parameter could not be measured at the start of the experiment for the plants that were to be exposed to the two different temperature regimes. Total Leaf Area was, therefore, predicted for these individuals. When the predicted leaf area of the species at the start and the end of the one-week temperature treatment were compared an increase in leaf area was noted (Appendix 2C, F, I, L and O). Size differences among species, as measured by PLA, were highly significant (Table 4, $F_{4,190} = 230.598^{***}$). Statistical analysis also confirmed that all plants displayed significant growth after the one week period (Table 4, $F_{1,190} = 361.385^{***}$), with *Arabidopsis* being the fastest grower. The plants also all had a reduced growth due to cold (Table 4, $F_{1,190} = 21.658^{***}$), whereas the interaction of species, time, and treatment was not significant (Table 4, $F_{4,190} = 0.098$ n.s.), indicating that all species experienced a similar reduction of growth due to the cold nights.

In summary, during a one-week period individuals of all investigated species increased their predicted leaf area whether they were exposed to a 21/4°C or a 21/15°C day/night temperature treatment. Under standard growth conditions, the slopes of the growth curves are similar among the different *Draba* species. All species have grown at a similar rate. This pattern is maintained when plants are grown under a 21/4°C temperature regime. Over an equivalent period and under identical growth conditions; i.e. standard or cold, *A. thaliana* showed a faster growth rate in this single experiment than *Draba*.

**Freezing tolerance of *Draba* is correlated with soluble sugar and proline content**

To determine whether differences in freezing tolerance at the level of electrolyte leakage and juvenile survival could be attributed to the metabolite levels as measured in Chapter 3, we investigated whether a relationship existed with acclimated LT$_{50}$ values. When all species are considered we only found evidence for a significant relationship for both LT$_{50}$’s with glucose content (Table 5). This was less clear for total sugar content: only when we considered the LT$_{50}$ values of *Draba* species in the electrolyte leakage experiment a correlation was found with total sugar content. Although the results presented in Chapter 3 showed that proline levels did not increase in response to cold treatment in any of the *Draba* species, there was a trend among *Draba* species, in that – similar to *Arabidopsis* – the species with

<table>
<thead>
<tr>
<th>Species</th>
<th>LT50 Acc</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Raffinose</th>
<th>Sucrose Sugar</th>
<th>Proline</th>
<th>LT50 Non-Acc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>At</em></td>
<td>12.3</td>
<td>-6.9</td>
<td>0.967</td>
<td>3.549</td>
<td>2.326</td>
<td>0.118</td>
<td>6.960</td>
<td>2.468</td>
</tr>
<tr>
<td><em>Dm</em></td>
<td>11.3</td>
<td>-5.6</td>
<td>1.321</td>
<td>2.531</td>
<td>1.379</td>
<td>0.141</td>
<td>8.067</td>
<td>3.544</td>
</tr>
<tr>
<td><em>Dv</em></td>
<td>11.5</td>
<td>-3.9</td>
<td>1.202</td>
<td>2.232</td>
<td>1.612</td>
<td>0.063</td>
<td>4.816</td>
<td>0.984</td>
</tr>
<tr>
<td><em>Dh</em></td>
<td>7.5</td>
<td>-3.4</td>
<td>0.942</td>
<td>1.146</td>
<td>1.947</td>
<td>0.006</td>
<td>4.122</td>
<td>1.027</td>
</tr>
</tbody>
</table>
a high proline level (D. muralis) also had low LT$_{50}$ values in the electrolyte leakage experiments, whereas the two low-proline species (D. verna, D. hookeri) had a high LT$_{50}$ and were more frost sensitive. In non-acclimated species a positive correlation was detected between raffinose content and both LT$_{50}$ values. Raffinose proved to be a good indicator for low freezing tolerance.

**DISCUSSION**

Many plant species have a broad geographic distribution, where selection pressure for freezing tolerance is expected to be diverse (Zhen and Ungerer, 2008). In Arabidopsis, for example, Hannah et al. (2006) demonstrated that the freezing tolerance of natural accessions was correlated with the habitat winter temperatures. In this chapter, the freezing tolerance of four Draba species from three different geographical regions was examined and compared to that of A. thaliana.

The basal level of freezing tolerance in the juvenile survival experiment did not differ between the species investigated. Upon cold acclimation A. thaliana proved to be the most frost tolerant species. That acclimation could increase survival of A. thaliana ecotype Col-0 to temperatures of about −12°C had been reported previously by Xin and Browse (1998). Of the Draba species, temperate D. verna and D. muralis survived the coldest temperatures, followed by arctic-alpine D. nivalis. Tropical-alpine D. hookeri was most sensitive to freezing. Using electrolyte leakage as an assay, variation in tolerance to sub-zero temperatures occurred between both cold and non-cold acclimated plants from all species. Independent of the experimental procedure A. thaliana ecotype Col-wt remained the most tolerant species. The freezing tolerance based on electrolyte leakage results reported here for Arabidopsis is in agreement with previous studies, despite differences in methodology (Rohde et al., 2004; Hannah et al., 2006; McKhann et al., 2008). In the Draba species, the responses of D. verna disrupted a clean cut correspondence between the LT$_{50}$ values in the juvenile survival experiment and the electrolyte leakage experiment. D. muralis (from a temperate climate zone) displayed the lowest LT$_{50}$ temperature of all Draba species, followed by D. nivalis (from an arctic-alpine climate zone) and D. verna (from a temperate climate zone). D. hookeri (from a tropical-alpine climate zone) was the species most sensitive to freezing.

**Draba hookeri has a weak cold acclimation response**

Our results demonstrate that all investigated Draba species can enhance their freezing tolerance via pre-exposure to cold but non-freezing temperatures, albeit to a different extent. The juvenile survival experiment indicated that all species did benefit from acclimation, with D. hookeri benefitting the least. A similar trend was seen in the electrolyte leakage experiment. A significant species x acclimation treatment interaction term among the two electrolyte leakage experiments indicated that all investigated species differed in their cold acclimation abilities. For A. thaliana the method of acclimation applied had a significantly different effect on its ability to withstand freezing. Uemura et al. (1995) showed that maximum freezing in Arabidopsis was achieved after exposing 14day-old plants to 2°C for one week. Apparently, the week growth in a 21/4°C regime did not enhance the freezing tolerance as effective as the 48h treatment at 4°C. According to Xin and Browse (2000), depending on the plant species, it may take a few days to several weeks to reach maximum levels of freezing tolerance. This demonstrates the influence that the duration of cold acclimation has on a species’ ability to enhance its freezing tolerance. Considering the importance of cold acclimation to a plant’s
ability to survive an extensive winter period, this is not surprising. In *Arabidopsis* acclimated freezing tolerance has been show to be correlated with minimum habitat temperatures (Hannah et al., 2006; Zhen and Ungerer, 2008). For *D. muralis*, *D. nivalis*, and *D. verna* the 48h 4°C or one week 21/4°C cold acclimation treatments did not result in big differences in ΔLT50 values (Table 2). At most a 1°C difference was obtained between both treatments. We do not expect a 1°C difference in cold tolerance to play a role in plant speciation.

The difference in freezing tolerance between the acclimated *D. muralis* and *D. hookeri* ranged from 2.2°C in the electrolyte leakage to 3.8°C in the juvenile survival experiment. *D. hookeri* showed no significant effect of acclimation on electrolyte leakage ΔLT50. The low acclimation capacity found for *D. verna* plants acclimated for 48h at 4°C was probably due to the high variability found in the results for this species, and more experimental replicates are probably needed to characterize this species accurately. Electrolyte leakage results for *D. verna* under a different acclimation treatment (i.e. one week at 21/4°C; Appendix 1) suggest that the observed variation could be due to the methodology rather than a species-specific response.

**The role of microclimate on freezing tolerance**

Overall, of the five species studied *Arabidopsis* benefits most from acclimation, whereas tropical-alpine *D. hookeri* exhibited a weak acclimation response in the juvenile survival experiment only. One possible explanation could be that acclimation plays little or no role in the tropical-alpine Andean environment where extreme fluctuations in temperature occur on a daily basis. During any given day of the year, rain, ice, snow, and fog may alternate abruptly with clear sunny skies and elevated temperatures (Luteyn, 1999). In addition, temperatures below –10°C may never occur in *D. hookeri*’s natural habitat, or at least not for extensive periods of time. According to Larcher (2003), occasional night frosts as low as –10 to –12°C may occur at all times of the year in equatorial mountains. During our own fieldwork the lowest subzero temperature we recorded in Cotopaxi national park, where *D. hookeri* occurs, was –4.7°C (see Table 1, Chapter 5). This measurement took place during a night when temperatures fluctuated between zero and –4.7°C for more than 10h. This is clearly within the range we have determined in the laboratory experiments (Table 1).

Not all tropical-alpine species are as freezing sensitive as *D. hookeri*. The LT50 of mature *Draba chionophila* plants, a small rosette species growing in the desert páramo of Piedras Blancas, Venezuela, has been estimated between –10 and –15°C (Azocar et al., 1988). In contrast, the only *D. chionophila* seedlings Pfitsch (1994) found during his study in this area were buried in the soil. How long the seedlings stayed buried underground or if this strategy is applied by other *Draba* species remains unknown. Squeo et al. (1996), in turn, demonstrated that freezing injury occurred at differing subzero temperatures between –5 and –20°C in 16 high desert Andean plants originating from the Cordillera de Doña Ana, Chile. The lowest recorded temperature in Piedras Blancas was –10°C, while the coldest month in the Cordillera de Doña Ana was July with an average temperature of –1.8°C. Unfortunately neither articles state anything about the duration of exposure to these temperatures by local plants. However, these examples do illustrate the influence of the microclimate in which tropical-alpine species occur on the species’ freezing tolerance. Local climate is known to be of influence in alpine environments where daytime maxima are lower on windswept ridges and nighttime minima are lower in cirques and alpine valleys due to cold air drainage (Billings, 1974). In arctic-alpine regions plants growing on windward slopes or ridges usu-
ally remain snow-free in comparison to plants occurring beneath a long-lasting snow bank. The former will be more exposed to freezing temperatures than the latter, for which the snow cover functions as an insulation buffer. By thermal insulation snow dampens temperature oscillations; relatively warm temperatures below winter snow cover reduce the need for plants to invest in cryoprotective measures (Körner, 2003). *D. nivalis* was less freezing tolerant than expected based on the geographical region in which it occurs. These results are consistent for both the juvenile survival and the two electrolyte leakage experiments. As a circumpolar species we would have expected *D. nivalis* to be more freezing tolerant as temperatures can drop to below the –15°C tested here.

The *D. nivalis* population used in this study originates from Mount Healy, Alaska and is covered by snow throughout the entire winter (Hanne Hegre Grundt, University of Oslo; personal communication). The presence of snow during the arctic-alpine winters may, thus, explain the lower freezing tolerance of arctic-alpine *D. nivalis* in comparison to both temperate *Draba* species.

**Does plant size matter for frost tolerance?**

Under natural conditions, *D. hookeri* and *D. nivalis* are both small plants with compact rosettes of leaves that occur on gravel substrates, preferably in the vicinity of rocks. Compact growth forms profit from delayed night time cooling due to the heat capacity of either the moisture stored within the rosettes or in the underlying soil or rocks (Körner, 2003). Although the bigger *D. muralis* plants did have the highest and the smaller *D. hookeri* plants the lowest freezing tolerance, the ratio of specific leaf area to leaf dry matter of *D. hookeri* was similar to that of the bigger and more freezing tolerant *D. verna*. The differences in freezing tolerance are, therefore, presumably not size-related. In addition, all *Draba* species showed similar growth rates when grown under a 21/4°C (day/night) temperature regime. In response to cold all species showed reduced growth and formed smaller leaves; a common feature displayed by plants under cold stress (Sharma et al., 2007). In fact, smaller leaves can be more advantageous in cold as the surface area from which heat loss can occur is smaller in comparison to large leaves.

Results of this chapter suggest that there might be a link between average metabolic contents; i.e. soluble sugars and proline, and freezing tolerance. Glucose proved to be a good indicator for freezing tolerance in acclimated species, while raffinose best explained low freezing tolerance in non-acclimated species. Across all *Draba* species, the most freezing tolerant *D. muralis* had the highest average metabolite levels, while the most freezing sensitive *D. hookeri* had the lowest levels. Hannah et al. (2006) found that high metabolite levels in different natural *Arabidopsis* accessions were insufficient for improved freezing tolerance and more research will be needed to investigate the differences in freezing tolerance between *D. hookeri* and *D. muralis* further.

In the next chapter we will investigate the CBF response of *D. hookeri* and four other tropical-alpine *Draba* species under natural temperature conditions as encountered in the high-Andean region of Ecuador.

**Acknowledgements**

We gratefully acknowledge S. Arisz for designing and optimizing the methodology of the juvenile survival experiment. We thank J. van Arkel for helping with the photography and scanning of the plant material.
Appendix 1. Acclimation treatment affects freezing tolerance of Draba and Arabidopsis. Average electrolyte leakage obtained from two independent samples from three (A, C, E, G, and I) and two (B, D, F, H, and J) biological replicates. Plants were cold acclimated by being exposed for 48h to 4°C (A, C, E, G, and I) or for 1week to 21/4°C (B, D, F, H, and J). During both cold acclimation regimes the light regime remained 12L:12D. Representation of data as described in FIGURE 2.
Appendix 2. Effects of cold nights on growth of Draba and Arabidopsis. Correlation between leaf area and projected leaf area (A, D, G, J, and M) and between Specific Leaf Area (SLA) and Leaf Dry Matter Content (LDMC; B, E, H, K, and N) at the start of the temperature treatment (t=0), after one week at standard growth conditions (21/15°C, t=1; normal) and after one week with cold nights (21/4°C, t=1; cold). Correlation of the estimated marginal means of the predicted leaf area at the start (t=0) or end (t=1) of the temperature treatment (C, F, I, L, and O). Values for leaf area, projected leaf area and predicted leaf area were all Log-transformed.