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**Unraveling the cold response in Draba**

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## General discussion



Plants have evolved a wide array of responses to deal with cold temperatures. In this thesis we studied the cold response in *Draba* species originating from tropical-alpine, arctic-alpine and temperate regions. We predicted that the cold response pathway of temperate plants would differ from that of their arctic and tropical-alpine relatives (Chapter 1). Adaptation of plants to cold temperatures can involve responses at the genetic and physiological level, and therefore we focused on 1) transcriptional/metabolite changes in response to cold and 2) juvenile survival and electrolyte leakage in response to freezing. Our results demonstrate that differences in the cold response of tropical-alpine, arctic-alpine and temperate species exist. Possible interpretations and suggestions for additional research will be discussed in the paragraphs below.

Central to this thesis are experiments in which different species are exposed to the same environmental conditions in a climate chamber. The conditions in experiments were chosen such that they simulate natural variation in light and temperature of the tropical-alpine region of Ecuador. Similar day lengths and temperatures may occur in spring and fall in a temperate climate and to a lesser extent in an arctic-alpine region. The time trajectory consisted of a 12h photoperiod and a 21/4°C (day/night) temperature regime. All species were subjected to this same regime and *Arabidopsis thaliana* Col-wt was used as a reference species. *Arabidopsis* showed a similar cold response as reported in literature, which confirmed the quality of our set-up and provided the assurance that the obtained *Draba* data were meaningful. The results indicated that, as in *Arabidopsis*, C-repeat binding factors (*CBF*) genes are induced by cold treatment in all *Draba* species. However, our results demonstrated that there may be one big difference: transcript levels in *Draba* were 10 to 20-fold lower than in *Arabidopsis*.

The research also included the investigation of transcript changes as measured under natural conditions in the high Andes of Ecuador. In such an environment more factors could affect the response compared to the laboratory, including weather conditions and micro climate, but also plant size and age. This was a unique and challenging endeavor, as we sampled plants at three high altitude locations during the day and night, and at each location two *Draba* species were sampled. *D. hookeri* was included both in the controlled and the field experiments, so that its responses could be compared. The results demonstrate that *CBF* genes were indeed induced under natural environmental conditions in the field, although the expression patterns between controlled and field experiments differed. Results obtained under controlled conditions in the laboratory do not translate directly into results as found under natural conditions in the field.

### **Tropical-alpine *Draba* species contain cold-responsive genes not found in other species**

In this thesis we report the identification of three classes of *Draba* *CBF* genes labeled *CBFa*, *CBFb*, and *CBFc*. The main goal was to obtain sequence information from cold responsive genes, to be used in later gene expression studies, so the search was not exhaustive. Nonetheless, sequence comparisons showed that these genes comprise three well-supported clusters, albeit with considerable variability within the clusters. In arctic-alpine *D. nivalis* *CBFa* and *CBFc* representatives were found, while tropical-alpine *D. alyssoides* and *D. hookeri* contained *CBFa* and *CBFb* homologs. In the other tropical-alpine species only *CBFa* was detected and temperate *D. muralis* and *D. verna* contained only *CBFc*. Thus, *CBFb* appears to be a gene duplication confined to the tropical *Draba*'s, and the *CBF* gene tree reflected the geographic relationship between the different *Draba* species.

The *Draba* *CBF* genes possess the characteristic structure found in the *Arabidopsis* *CBF* genes: highly conserved nuclear localization signal and AP2-DNA-binding domains, as well as a more variable acidic transcriptional activation domain. The inferred *CBF* proteins all contained the amino acid motif critical for the binding of the *CBF* protein to their known target genes. *COR15b* is one such *CBF* target gene in *Arabidopsis* and in our *Draba* study species we isolated a gene >81% similar to *AtCOR15b* (unfortunately already labeled *COR15a* in another *Draba* (Zhou et al., 2009), Chapter 2). The *CBF* and *COR15* genes in *Draba* and *Arabidopsis* share the structures important for the functionality of the gene, and are therefore predicted to fulfill a similar role in the cold response. Another new *COR15* gene (*Draba* *COR15c*), with an insertion not present in any other known *COR15* gene was cloned from several *Draba* species.

The *CBF* sequence data were used to estimate the frequency of synonymous and non-synonymous mutations, in order to detect traces of past selection. This indicated that the acidic region of *CBF* genes may not have been under high selective pressure. The conservation of the other protein domains support the conclusion that the variation in cold tolerance is not caused by changes in *CBF* proteins. *CBF* genes are regarded as an essential part of the cold response pathway and appear to be ubiquitous in plants (McKhann et al., 2008). Freezing sensitive tomato that cannot cold acclimate has been found to have a functional *CBF* cold response pathway, but a reduced *CBF* regulon in comparison to *Arabidopsis* (Zhang et al., 2004). In a study of the natural variation in *CBF* gene expression in the Versailles core collection of *A. thaliana*, the freezing sensitive Cvi-0 accession was found to contain lower *CBF* expression levels compared to other accessions (McKhann et al., 2008), which was attributed to a deletion in the *CBF2* promoter. Whether similar promoter differences explain the transcriptional differences between *Draba* and *Arabidopsis* remains to be investigated. A study of the promoter regions of the different *CBF* genes may reveal if instead this could be responsible for differences in the kinetics of *CBF* expression.

### **Kinetics of cold-response gene expression in *Draba* and *Arabidopsis***

Expression analyses of the *CBF* and *COR15a* genes from *Draba* demonstrated that both in *Draba* and *Arabidopsis* *CBF* and *COR15* are induced in response to cold. However, a closer look at the expression of both genes showed that the kinetics of the *CBF* and *COR15* genes varied among species, even within *Draba*. The most abundant *CBF* in the temperate *D. muralis* and *D. verna* (*CBFc*) showed a faster induction than the *CBF* in the arctic-alpine *D. nivalis* and tropic-alpine *D. hookeri* (*CBFa*). This difference in induction of both *CBF* genes did not translate into a different timing of *COR15a* induction among the *Draba* species, so that the lag between the *CBF* and *COR15a* expression peaks was not constant across species.

*ZAT10*, a gene thought to be induced in parallel to *CBF* in response to cold (Vogel et al., 2005), showed a diurnal rhythm of expression in both *Draba* and *Arabidopsis*. The diurnal peak in expression early in the dark period coincided with the start of the cold treatment. Therefore it was difficult to assess the early induction of *ZAT10*. However, a sustained expression in the following hours indicated that this gene is influenced by cold in *Draba*. Several *COR* genes are co-expressed with *COR15a/b* in response to low temperature in *Arabidopsis* (Hajela et al., 1990), which suggests that *COR15a/b* might act in concert with other *COR* genes to enhance tolerance to freezing in plants. It is thus possible that the *CBF* genes in *Draba* regulate a different part of the *CBF* regulon in comparison to *Arabidopsis*. Additional cold-responsive genes that do not depend on the *CBF* pathway have been iden-

tified in *Arabidopsis*, suggesting that cold acclimation is associated with the activation of multiple low temperature regulatory pathways (Seki et al., 2001; Seki et al., 2003; Gilmour et al., 2004; Vogel et al., 2005). In fact, Fowler and Thomashow (2002) found that only 12% of the cold-responsive genes are members of the *CBF* regulon. For our investigation of the *CBF* and *COR* genes present in *Draba* we used a candidate gene approach and investigated the most abundant genes present in cDNA from cold-treated plants only. Although this method did allow us to pick up the candidate cold responsive genes, it excluded the possibility of finding components of alternative cold response pathways. Given the recent advances in molecular analyses, future studies will be able to target more complete expression profiles, for instance using 454 pyrosequencing, so that all possible cold responsive genes present within a cold treated species can be studied.

When levels of *CBF*, *COR15*, and *ZAT10* expression were compared between *Arabidopsis* and *D. hookeri*, the gene expression levels of the latter species appeared to be 10-20x lower than those of *Arabidopsis*. Unfortunately, it was not possible to design a primer that could amplify all *Draba* and *Arabidopsis* *CBF*, *COR15*, or *ZAT10* genes simultaneously, so expression levels in other *Draba* species could not be compared. However, the RNA gel blot analysis did confirm that *CBF* expression levels were low across all investigated *Draba* species, relative to *Arabidopsis* (Chapter 2). This important observation suggests that *Draba* does not rely on *CBF* for its cold responses.

### Cold response at the metabolite level

Investigating the cold response at transcriptional level is an interesting first step, but only relevant if it leads to a response at the metabolite level. Proline and soluble sugar levels of *Draba* and *Arabidopsis* were, therefore, measured and analyzed. The resulting pattern of metabolite accumulation in *Arabidopsis* in response to cold was similar to that reported in literature (Wanner and Juntila, 1999; Gilmour et al., 2000; Klotke et al., 2004; Zuther et al., 2004). To our surprise, metabolite levels in *Draba* did not resemble that of *Arabidopsis*. Raffinose, a compatible solute known to accumulate during cold acclimation in *Arabidopsis* (Zuther et al., 2004) does not appear to accumulate in two of the three *Draba* species upon cold treatment. Proline levels, which increase in response to cold in *Arabidopsis* did not change in response to cold treatment in *Draba*. Interestingly, *D. muralis* displayed constitutive high proline levels, irrelevant of the temperature treatment. Therefore, there appears to be no clear-cut correlation between soluble solute accumulation and gene expression. *D. verna*, a widespread, early flowering winter annual, with a *CBF* and *COR15* expression pattern that most resembled that of *Arabidopsis*, had low metabolite levels that increased marginally in response to cold. In *D. verna*, raffinose and proline levels did not accumulate after cold treatment.

The other temperate *Draba* species in this study, *D. muralis*, contained constitutively high proline levels throughout the temperature trajectory. Analysis of this species physiological response to sub-zero temperatures showed that this species had a high freezing tolerance (Chapter 4). A freezing tolerant mutant that accumulated high levels of proline has also been reported in *Arabidopsis* (Xin and Browse, 1998). This mutant had a mutation in the *eskimo 1* (*esk1*) locus and since other levels of amino acids remained low the authors proposed constitutive accumulation of proline to be a component of the mutant's freezing tolerance. Metabolite levels were higher in *D. muralis* than in the other *Draba* species studied. However, according to Hannah et al. (2006) high metabolite content is not sufficient for improved freezing tolerance. In general, freezing tolerance appears to be a multigenic trait

(Rohde et al., 2004). Furthermore, the other freezing tolerant *D. verna* did not display constitutively high proline levels. In fact, *D. verna* had similarly low proline and total sugar levels as the freezing sensitive *D. hookeri*. Whether the constitutive proline expression in *D. muralis* contributes to the species' high freezing tolerance requires further investigation. Proline synthesis is costly and a plant cannot utilize carbon invested in proline for growth. As a result, plants constitutively accumulating proline could display reduced growth. Indeed, Xin and Browse (1998) found the *esk1* mutants to be smaller in size, but further comparable in chronology of development to wild type plants when grown under 22°C temperature conditions. When grown under a temperature regime of 4°C both wild type and *esk1* mutants showed similar morphological features in response to the cold treatment. If proline accumulation would be costly to *D. muralis*, we would expect to see slower growth compared to the other species under standard (21/15°C) than under cold (21/4°C) temperature conditions. This was not the case. *D. muralis* showed a reduction in growth under the 21/4°C temperature regime, similar to the other *Draba* species. Any differences in cold acclimation mechanisms between the various species apparently did not translate into differences in growth.

### Differences in freezing tolerance and cold acclimation

Once we had investigated the cold response at a molecular level we were interested in finding out what the possible implications of our results were on the freezing tolerance of *Draba*. When tropical-alpine, arctic-alpine and temperate seedlings were grown under equal light and temperature conditions before being exposed to freezing temperatures, certain species proved to be more freezing tolerant than others. Interestingly, our control species *A. thaliana* Col-wt proved to be more freezing tolerant than even the most frost resistant *D. muralis* and *D. verna*. *D. nivalis* showed a less pronounced freezing tolerance, while *D. hookeri* proved to be the species most sensitive to freezing. Our results suggest that selection pressures for freezing tolerance within the genus *Draba* are indeed diverse. In all species except the tropical-alpine *D. hookeri*, freezing tolerance was enhanced by cold acclimation. Under non-acclimated conditions no significant differences in freezing tolerance was detected between the species. This indicates that, in contrast to the results found in 71 *Arabidopsis* accessions (Zhen and Ungerer, 2008), no intrinsic biochemical and physiological factors contribute to variation in this phenotype for the *Draba* species studied. The observed differences in the freezing tolerance of acclimated tropical-alpine, arctic-alpine and temperate *Draba* species suggested adaptations to the temperature responses of their natural habitats at a genetic level. In *Arabidopsis* high acclimated freezing tolerance has been shown to be correlated with low minimum habitat temperatures (Hannah et al., 2006; Zhen and Ungerer, 2008).

The acclimation response of plants in the experiments was triggered by lowering the temperature during the night. However, it is possible that this was not sufficient to elicit an acclimation response. For many species, different or additional environmental cues, for example the combination of cold temperatures and short days, are needed to stimulate a robust acclimation response (Loik and Redar, 2003). Karlson et al. (2003), for example, found photoperiod to be a reliable environmental cue that regulates the first stage of cold acclimation in temperate woody plants. Also the duration of the acclimation period may be of influence. 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 may explain the limited acclimation response found in arctic alpine *D. nivalis*. It is possible that

*D. nivalis* seedlings may require a longer acclimation period, a decrease in the overall day/night temperature regime, or a decrease in photoperiod for optimal cold acclimation to occur, to prepare for the cold winter.

### ***Draba hookeri* is sensitive to freezing**

Tropical-alpine *D. hookeri* proved to be the most freezing sensitive of all investigated *Draba* species. In addition, this species displayed a poor ability to enhance its freezing tolerance via cold acclimation. Tropical-alpine plants have no use of a gradual method to increase its freezing tolerance throughout the year, in contrast to temperate or arctic-alpine plant species. As a result *D. hookeri*, through relaxation of selective pressures, may have lost its ability to cold acclimate. Because temperature fluctuations from 25-30°C during the day to freezing at night may occur any day of the year in a tropical-alpine climate, there is no need or time for plants to cold acclimate. Despite its low freezing tolerance, *D. hookeri* thrives in its native habitats, suggesting that conditions could be less extreme than in a different climatic region, or for the other species. Temperatures in the high Andes of Ecuador never stay below zero for extensive periods of time and mainly occur during the night (Azocar et al., 1988). Soils and rocks may buffer extremes through their heat capacity, and furthermore, the compact rosette of *D. hookeri* may reduce heat loss.

The induction of the *CBF* and *COR15* genes in *D. hookeri* did not result in high metabolite levels or in enhanced freezing tolerance, implying that this pathway may differ functionally in tropical-alpine species. Creating transgenic *Draba* plants with *CBF* genes from *Arabidopsis*, or vice versa, would help to investigate this. It has been found that overexpression of *Arabidopsis CBF* genes results in the constitutive expression of the *CBF* regulon and an increase in freezing tolerance without a cold stimulus (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000). Effects of *CBF* overexpression and knockouts on accumulation of proline and soluble sugars, as well as freezing tolerance would be worthwhile to study.

According to Hannah et al. (2006) the overall magnitude of the *CBF* regulon response is important in determining freezing tolerance in *Arabidopsis*. Other *Draba* species were not as freezing sensitive as *D. hookeri*, so the link between low *CBF* transcript levels and freezing tolerance is not apparent in *Draba*.

### **Sampling in the field**

This study is the first to investigate the *CBF* cold response pathway in five tropical-alpine *Draba* species under natural climatic conditions in Ecuador. Despite different weather conditions in the various sites, all investigated species showed an induction of *CBF* expression. The average *CBF* expression level measured in adults under natural conditions was (>10-fold) higher than that of juveniles analyzed under laboratory conditions. Since the tropical-alpine *Draba* species are all perennial species and we had no means of estimating the age of these plants, it is uncertain whether this difference can be attributed to plant age. Alternatively, different (parts of the) cold response pathways could be active in different developmental stages of a plant. The interaction between plant development and low temperature implies that some genes are regulated by both environmental factors and developmental cues (Wang and Cutler, 1995). In addition, different individuals had to be sampled at different time points. These are the limitations one faces when stepping out of the controlled laboratory environment and into the plant's natural environment at 4000m altitude in the high Andes. Knight et al. (2004), in their study on ABA induction of the *CBF* pathway, propose that conflicting results between different studies may be caused by differences in

plant age, size or history. In future field experiments, seedlings could be included in the study to compare gene expression in juvenile and adult plants. Ideally, one would place all species in a 'common garden' type of setting to ensure all plants encounter the same conditions and then study gene expression. This would allow us to investigate whether differences in cold tolerance is related to differences at the molecular or physiological level under field conditions. In our experiments, plants with differing growth forms were included and we cannot link gene expression to growth form, due to other variables such as plant age, plant height, microclimate, and exposure.

Our results also revealed *CBF* induction at dawn, suggesting a possible role for *CBF* when temperatures are still low but plants already start to photosynthesize. Savitch et al. (2005), demonstrated a possible involvement of *CBF* pathways in photosynthetic cold acclimation by the overexpression of *BNCBF/DREB1* in *Brassica*. The mutants showed partially regulated chloroplast development to increase photochemical efficiency and photosynthetic capacity. In the controlled experiments no sampling was conducted around dawn so nothing can be said about possible *CBF* induction at this time. This stresses the importance of frequent sampling, especially around shifts in temperature and light, as transient peaks can easily be missed.

### In summary

Although not a model-genus, *Draba* does present an interesting model system with a lot of variation in morphology, physiology and genetics. It allows for comparisons in the cold response of species from different geographical regions, of perennial and annual plant species, and species with differing growth forms. Although we are still far from understanding the cold response of *Draba*, the following main conclusions emerged from this thesis:

- » *Draba* species share genes of the cold response pathway with *Arabidopsis*, but also contain novel genes and show different responses. This merits additional research by, for example, studying *CBF* overexpression/knock-outs in transgenic plants.
- » Gene expression patterns (*CBF*, *COR15*, and *ZAT10*) under controlled circumstances in the laboratory differed from those measured in the field. When investigating plants in the field, genetic variation between individuals, developmental stage, plant age and micro habitat may all influence results. Despite the obvious challenges, more field studies are needed if we want to gain insights in the evolution of cold responses in plants.
- » Tropical-alpine *D. hookeri* has low *CBF* transcript and low metabolite levels in response to cold, does not cold acclimate and is the most freezing sensitive of all investigated *Draba* species.
- » *Arabidopsis thaliana* Col-wt accession from a temperate region was more freezing tolerant than any of the *Draba* species investigated, some originating from extreme habitats. It also showed the strongest acclimation response.
- » Temperate, *D. verna* had *CBF* and *COR15* expression patterns that most resembled that of *Arabidopsis*, but low metabolite levels that increased marginally in response to cold. Cold acclimation greatly enhanced this species' freezing tolerance.
- » The other temperate and most freezing tolerant *Draba* species, *D. muralis*, has constitutively high proline levels, calling for additional research.
- » Arctic-alpine *D. nivalis* had a similar *CBF* expression pattern to *D. hookeri*, but was more freezing tolerant. However, not as tolerant as we expected for a species with an arctic-alpine origin. *D. nivalis* was the species that benefitted most from a one-week 21/4°C acclimation period, suggesting that the duration of the acclimation period may affect species differently.



- » Experiments under controlled conditions show that differences in the freezing tolerance between tropical-alpine, arctic-alpine and temperate *Draba* species are determined at the genetic level.
- » Because the *Draba* species investigated in this thesis differ in freezing sensitivity from *A. thaliana* Col-wt, the genus could provide novel information about cold responses including genes and pathways that may not be found in other (model) taxa.