Unraveling the cold response in Draba

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1 General introduction
Exposure to cold temperatures is a common environmental stress for plants. Plants have developed an array of responses on physiological, cellular and molecular level that allow them to cope with cold stress. Understanding the mechanisms that provide plants with the ability to survive cold will provide us with tools to explain evolution of species as a result of different climatic conditions. From an economical perspective this knowledge can help improve cold tolerance of agriculturally important crops (Thomashow, 2001). Knowledge of the natural variation in non-crop species could help to gain insight into the cold response of plants, which could then be utilized to enhance cold tolerance in crop species. In this thesis we have chosen *Draba* as a model plant species to study the responses to cold. It is a genus with a wide geographical distribution and species can be found in temperate, arctic-alpine and tropical-alpine habitats. *Draba* species, thus, provide a wide range of potential adaptations to different seasonal patterns of exposure to cold and freezing temperatures.

**Draba**

*Draba* L. is a cosmopolitan genus, comprising over 360 species (Warwick and Al-Shehbaz, 2006) distributed in the arctic, subarctic, alpine, and most mountainous regions of the world. *Draba* is in fact the largest genus of the Brassicaceae family (Koch and Al-Shehbaz, 2002) and belongs to the tribe Arabidae (Al-Shehbaz et al., 2006; Figure 1). It is one of the genera that is distributed throughout northern temperate latitudes and at high elevations in temperate, arctic-alpine and tropical-alpine habitats.

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**Figure 1. Position of Draba within the tribal classification of the Brassicaceae** (modified from Al-Shehbaz et al., 2006).
Central and South America (Pfitsch, 1994). A potential force driving speciation in *Draba* could be polyploidization which is very common in this genus (Brochmann and Elven, 1992). Molecular evidence has confirmed that many arctic taxa are allopolyploid, often formed after recurrent polyploidization events (Abbott and Brochmann, 2003). Accordingly, Jordon-Thaden and Koch (2008) hypothesized that the genus’ ability to speciate frequently via polyploidy resulted in high species richness, which was promoted through adaptation to a wide range of extreme ecosystems and environmental fluctuations during glacial cycles. Pfitsch (1994) had suggested earlier that the differences in growing season occurring across the geographical range of *Draba* have contributed to the radiation into life forms that are unique to the genus.

Recent phylogenetic studies estimated the genus *Draba* to have diverged from its closest existent relatives around 5mya (I. Jordon-Thaden, pers. comm.). In addition, a phylogenetic classification of *Draba* covering the entire geographical range of the genus demonstrated the presence of three major groups of true *Draba* species, together referred to as ‘Core’ *Draba* (Jordon-Thaden et al., 2010). With the exception of one species, all South American tropical-alpine species belong to the Core *Draba*. The South-American *Draba* species are thought to have originated from several ancestral species; an Asian connection through the Bering Bridge and repeated north-south migrations and subsequent hybridization is plausible (Koch and Al-Shehbaz, 2002). Such migrations from North-America to the Andes by temperate plant genera, such as *Draba*, are postulated to have occurred after uplift of the northern Andes (Smith and Cleef, 1988; Hughes and Eastwood, 2006). Today, around 65-70 *Draba* species grow in the Andes region, from Colombia and Venezuela to Patagonia (Al-Shehbaz, 1991). The Andean Cordillera is the largest extension of low temperature areas within the tropics (Sarmiento, 1986 as cited by Jørgensen and Ulloa Ulloa, 1994). Ecuador constitutes an important part of this habitat in the Andes, with in total 17 *Draba* species, 11 of which are considered endemic to Ecuador (Jørgensen and Léon-Yanez, 1999).

The *Draba* species investigated in this thesis are species from three geographical regions; arctic-alpine *D. nivalis*, tropical-alpine *D. hookeri*, *D. alyssoides*, *D. aretioides*, *D. splendens*, and *D. steyermarkii*, and temperate *D. muralis* and *D. verna*. *D. nivalis* and all five tropical-alpine species are part of the Core *Draba* (Jordon-Thaden, in prep.), while *D. verna* is one of the early diverged *Draba* species found basal to the Core species (Jordon-Thaden et al., 2010). *D. verna* was formerly known as *Erophila verna* and its exact phylogenetic position within the genus remains uncertain. From sequence data of 169 different *Draba* species, Jordon-Thaden et al. (2010) concluded that *D. muralis* was basal to all the other species and quite distinct. The authors, therefore, proposed to place this species outside the genus *Draba*, in the genus *Drabella*. Further analyses to strengthen this hypothesis are ongoing and in this study we will refer to this species as *Draba muralis*. The phylogenetic relationship between the *Draba* species discussed in this thesis is illustrated in Figure 2.

In general, *Draba* plants have small flowers and seeds without any morphological adaptations for long-distance dispersal (Skrede et al., 2008). The genus is morphologically diverse and shares numerous physical features with other members of the Brassicaceae family (Beilstein and Windham, 2003). *Draba* plants carry a variety of trichomes, presenting an important mode of identification of many species. *D. nivalis*, *D. hookeri*, *D. muralis*, and *D. verna* are all compact rosette plants with white flowers. Of the tropical-alpine species, the majority of experiments were conducted with *D. hookeri*, because this species has a rapid
life cycle and is mostly selfing. We will not discuss the other tropical-alpine species here, but refer to Chapter 5 for a description of their growth forms. D. nivalis and D. hookeri are perennial species, while D. muralis and D. verna are winter annuals. As winter annuals, the latter two species are sometimes classified as therophytes, passing the dry season (i.e., summer) as seeds and overwintering as a rosette. All four species are mainly self-pollinating, with occasional outcrossing having been demonstrated for D. nivalis and D. verna (Turesson, 1929 and Winge, 1933 as cited by Andel et al., 1986; Brochmann, 1993; Ekman, 1932 as cited by Grundt et al., 2006).

As already mentioned, Draba is a polyploidy-rich genus (Al-Shehbaz, 1987 as cited by Skrede et al., 2008) consisting of species with various ploidy levels. D. nivalis is the only diploid species in this study (Brochmann et al., 1993). It is a circumpolar species, which probably originated within the last one million years (Grundt et al., 2004; 2006; Figure 3). All five tropical-alpine Draba species investigated are tetraploid (Table 1). From these species D. hookeri, D. aretioides, D. splendens, and D. steyermarkii are endemic to Ecuador, while D. alyssoides occurs in the high Andes from Venezuela to Peru (Tropicos.org, 2010; Figure 3). Because all four endemic species are threatened by habitat loss they are listed on the IUCN Red List as either endangered, vulnerable, or near threatened species (León-Yánez and Pitman, 2003). The temperate Draba muralis, also a tetraploid, occurs in southern and central Europe, northwestern Africa, Turkey, and Caucasus (Ratcliffe, 1960; Jalas et al., 1996 as cited by Jordon-Thaden et al., 2010; Figure 3). D. verna has been identified as a species with highly variable chromosome counts (2n = 14, 28, 30, 32, 36, 38, 39, 52, 58, and 64; Warwick and Al-Shehbaz, 2006; Jordon-Thaden and Koch, 2008), but the population used in this thesis is tetraploid (Table 1). Additional research would be needed to discover if the highly variable chromosome counts in D. verna have functional consequences. D. verna is originally a Eurasian species which has expanded to a weedy, cosmopolitan species of the temperate region (Weeda et al., 2003; Figure 3). It is the only Draba species that occurs worldwide, and Jordon-Thaden et al. (2010) suggest that its weediness could have contributed to its relatively inconclusive genetic inheritance and multiple ploidy level reports. On the other hand, Arabidopsis thaliana, also a weed with a broad distribution, is known to be diploid across its entire range.

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**Figure 2. Phylogenetic relationships between the studied Draba species and Arabidopsis thaliana.** Maximum-likelihood phylogenetic tree analysis of the combined trnL-F and ITS nucleotide sequences based on the best-fit GTR+ substitution model, as adapted from Chapter 2, Figure 3. Only bootstrap values >60% (based on 1000 replicates) are indicated above the branch points. Branch lengths indicate genetic distance.
Since the completion of the sequencing of its genome in 2000, A. thaliana has been the model organism for genetic as well as molecular and cell biological studies of flowering plants. Therefore, research on the cold response pathway of Arabidopsis has become the blueprint for investigating cold induced responses of other (crop) species. Although Draba is not part of the same phylogenetic lineage as Arabidopsis (see FIGURE 1), the relatedness between both genera allows for genomic comparisons. For example, a pairwise cpDNA sequence comparison showed that ITS sequences of Draba and Arabidopsis are 85% identical.

### Table 1. Overview of the different species studied and their ploidy levels.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Site</th>
<th>Collection by</th>
<th>Life cycle</th>
<th>Year</th>
<th>Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. nivalis</td>
<td>USA</td>
<td>Alaska</td>
<td>R. Eissen</td>
<td>Annual</td>
<td>n.a.</td>
<td>2n</td>
</tr>
<tr>
<td>D. muralis</td>
<td>Germany</td>
<td>Mount Healy</td>
<td>R. Elsen &amp; H.H. Grundt, University of Oslo</td>
<td>Perennial</td>
<td>1998</td>
<td>2n</td>
</tr>
<tr>
<td>D. verna</td>
<td>Germany</td>
<td>The Netherlands</td>
<td>A.C. Hilt-Adam, University of Amsterdam</td>
<td>Annual</td>
<td>2005</td>
<td>4n</td>
</tr>
<tr>
<td>D. hookeri</td>
<td>Ecuador</td>
<td>Cotopaxi National Park</td>
<td>N. von Meijendijk, University of Amsterdam</td>
<td>Perennial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. alyssoides</td>
<td>Ecuador</td>
<td>Rara Pichincha</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. alyssoides</td>
<td>Ecuador</td>
<td>Cotopaxi National Park</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. splendens</td>
<td>Ecuador</td>
<td>Rara Pichincha</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. steyermarkii</td>
<td>Ecuador</td>
<td>Cotopaxi National Park</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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Plants in a cold climate

Plants face many environmental stresses amongst which exposure to low temperatures is very common in many habitats. A plant’s ability to withstand and respond to such low temperatures is crucial to its survival. Various physiological mechanisms to enhance cold tolerance have evolved. This tolerance can be enhanced by a process known as cold acclimation. It is instigated by low, but non-freezing temperatures that enhance the plants tolerance to sub-zero temperatures (Smallwood and Bowles, 2002). Research has shown that when a plant is exposed to low temperatures, a signaling cascade is triggered which results in an increased ability of the plants to withstand frost (Gilmour et al., 1998; Medina et al., 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Fowler and Thomashow, 2002; Xiong et al., 2002; Zarka et al., 2003). This whole process is generally referred to as the cold response pathway of plants (Jaglo et al., 2001; Thomashow, 2001; Fowler and Thomashow, 2002).

Which adaptations are required for survival will depend on whether frost occurs all year round, periodically in the course of seasons, or on a daily basis. The year-round growing season of the tropical-alpine region differs considerably from the seasonal temperate and arctic-alpine regions and therefore one can predict that the patterns of cold tolerance differ among those regions (Figure 4). In temperate climatic zones, plants prepare for freezing temperatures in winter by ceasing their growth in autumn as the temperature gradually drops and day length decreases (Larcher, 2003). Through this process the plants acclimate and prepare for the oncoming frost, reaching maximum cold acclimation in winter (Ruelland et al., 2009). Especially in the autumn and spring a difference is expected between the tolerance during the day and at night (Figure 4A). Arctic-alpine regions are generally characterized by long, cold winters where even during summer months the possibility exists that temperatures drop below zero. Plants occurring in these regions have to be better prepared for frost than those growing in temperate regions, where periods of frost only occur periodically during winter and early spring. Thus, they are expected to reach high tolerance levels in the arctic winter (although snow cover may alleviate the need for extreme tolerance), but also have to be able to deal with cold spells during the growing season (Figure 4B). In contrast, in equatorial high altitudinal (i.e., tropical-alpine) mountains the climate is characterized by extreme diurnal variation in temperatures, from freezing at night to 25-30°C in the afternoon. Such temperature fluctuations result in a daily freeze-and-thaw cycle that has been

![Figure 4](image-url)
referred to as ‘summer every day and winter every night’ (Hedberg, 1964 as cited by Luteyn, 1999). Night frost can occur at all times of the year, usually lasting for a few hours only. Tropical-alpine plants will need to react rapidly to these regular temperature fluctuations, irrespective of the time of year (FIGURE 4C). One could hypothesize that it can be advantageous to have certain key components of the cold response pathway already present in the plant’s cells. In temperate regions where temperature changes in general occur more gradually and are overall less frequent and more predictable, it can, however, be too costly for plants to have components constitutively present in the cell without a direct use or necessity for them. The cost of constitutively producing osmoprotectants, for instance, might be a reduction of growth, since both processes involve the same carbon source. For example, overexpression of Arabidopsis CBF (C-repeat binding factor, a cold response regulating transcription factor) caused ‘stunted’ growth, a decrease in seed yield and a delay in flowering (Liu et al., 1998; Gilmour et al., 2000). Natural selection will result in adaptation to local circumstances, by outweighing the costs versus the benefits of constitutive cold tolerance. Plants have, therefore, finely tuned temperature responses shaped by evolution, to suit the specific environment in which they occur, and in order to achieve a high fitness (Wang and Hua, 2009). We, therefore, predict that the cold response pathway of temperate plants differs from that of their arctic and tropical-alpine relatives. By removing plants from their natural environment and placing them in a ‘common’ and controlled environment, it can be established whether differences are determined at a genetic and/or physiological level. If tropical-alpine, arctic-alpine and temperate species have different cold responses under controlled conditions this would point to adaptation at the genetic level. However, in the case that no differences in the cold response of these species is found when placed under controlled conditions, this would be indicative of a common physiological adaptation.

**Cold stress and acclimation**

Cold and especially frost impairs the metabolic activity, growth, and viability of plants and thus sets limits to the distribution of species (Larcher, 2003). In addition, the occurrence of species in cold climates is determined by their survival as seedlings. Seedlings of many species are less tolerant to extreme environmental conditions compared to adults (Lambrecht et al., 2007). The degree of cold tolerance during the seedling stage can determine successful establishment and thereby limit species distributions to certain microsites and contribute to the geographic range of a species, as well as speciation.

In order to survive in less hospitable environments a variety of physiological mechanisms and morphological adaptations to enhance cold tolerance have evolved. Morphological adaptations can contribute to a better insulation and/or thermal buffering. For instance, hairiness and a more compact growth form may result in a large difference between ambient and leaf temperatures at night and reduce radiative cooling. Typical frost-adapted species are of short stature, have a small leaf area and a high root/shoot ratio (Janska et al., 2010). Cold acclimation involves changes in cell physiology and biochemistry, including extensive alterations in lipid, protein and metabolome composition, many of which are brought about by changes in gene expression (Thomashow, 1999; Chinnusamy et al., 2007). Membrane fluidity is an important determining factor for cell adaptation to low temperature stress (Levitt, 1980 as cited by Lee et al., 2002). The membrane systems constitute that part of the cell that is first exposed to low temperatures, and is also a primary site of freeze-induced injury (Ouellet, 2007). This injury results largely from the severe dehydration associated
with freezing (Steponkus, 1984). Freezing temperatures create a situation for plants that is similar to drought. As temperatures drop below zero, ice formation is first initiated in the intercellular spaces due to the extracellular fluid having a higher freezing point (lower solute concentration) than the intracellular fluid (Thomashow, 1999). As a result, the intercellular osmolyte concentrations increase as water is withdrawn from the cell due to formation of extracellular ice. In non-acclimatized cells, membrane contraction occurs as a response to the water loss, which later makes the cells susceptible to ‘expansion induced lysis’ at the onset of a thawing period. The accumulation of ice in the intercellular spaces can also potentially result in the physical disruption of cells and tissues caused in part by the formation of adhesions between the intercellular ice and the cell walls and membranes (Levitt, 1980 as cited by Thomashow, 1998).

Multiple mechanisms are involved to protect cells from potentially irreversible freezing-induced damage. Some of these mechanisms include changes in lipid composition and the accumulation of compatible solutes with cryoprotective properties such as proline and soluble sugars including sucrose, glucose, raffinose and fructose (Thomashow, 1999). These cryoprotective substances may also act as molecular chaperones to maintain membrane integrity and function. By the expression of a specific set of genes and induction of the biosynthesis of such osmoprotectants, plants can prevent the occurrence of freezing-induced injury.

**CBF cold pathway and the CBF regulon**

Much insight has been obtained in recent years about how plants sense low temperatures and channel this information to bring about changes in gene expression, protein synthesis and biosynthesis of osmoprotectants that increase freezing tolerance. Cellular membranes are fluid structures, and cold temperatures can reduce their fluidity, resulting in increased rigidity (Chinnusamy et al., 2007). Phospholipids are the main constituents of cell membranes and, it is here where early cold response signals are thought to occur (Smallwood and Bowles, 2002). Chinnusamy et al. (2007) propose membrane rigidification and/or other cellular changes, which could induce a calcium signature and activate protein kinases, to be involved in the low temperature perception of plants.

In the model species, *Arabidopsis thaliana*, the C-repeat Binding Factor (CBF) cold response pathway plays a central role in cold tolerance. It is characterized by rapid induction of a cluster of genes encoding the CBF1-3 transcription factors, which themselves induce the CBF regulon. Within 15 minutes after transferring plants to cold temperatures the CBF genes are induced (Gilmour et al., 1998; Liu et al., 1998). Low temperature induction of CBF1-3 is transient and quickly followed by expression of *Cold responsive (COR)15a/b* genes, 2-4h after the onset of the cold treatment (Gilmour et al., 1998). As part of its cryoprotective properties, the *COR15a* encoded protein stabilizes membranes against freezing injury and forms oligomers in the chloroplast stroma (Artus et al., 1996; Steponkus et al., 1998; Nakayama et al., 2007). *COR15* together with other *COR* genes comprise the CBF regulon; i.e., CBF-targeted cold-induced genes (Fowler et al., 2005), and the expression of this CBF regulon confers cold tolerance in plants. Vogel et al. (2005) identified a zinc finger transcription factor, ZAT10, that is induced within 1h upon cold treatment and appears to be regulated in parallel to the CBF-pathway in response to cold. Lee et al (2002) propose that the presence of two zinc finger specific binding sites in the *COR15a* promoter indicate a possible interaction between ZAT10 and *COR15a*. 
Upstream of the CBF genes, two genes, *Inducer of CBF expression* (ICE) and *High expression of osmotically responsive genes* (HOS) have been identified as potential cold signal transduction components. Chinnusamy et al. (2003) found ICE1 to be a constitutively expressed transcription factor that becomes sumoylated (i.e., post-translationally modified by Small Ubiquitin-related Modifier or SUMO proteins) by cold stress, which is critical for its activation of CBF transcription. At warm temperatures ICE1 is in an ‘inactive’ state, while upon exposure to cold it is modified and thereby becoming activated. HOS1, that is also constitutively expressed, negatively regulates the expression of cold responsive genes by ubiquitination and proteosomal degradation of ICE1 and possibly other regulators (Lee et al., 2001; Dong et al., 2006). Figure 5 gives a simplified, schematic overview of the known cold-responsive transcriptional network in *Arabidopsis*. This network is much more complex, but in order to discuss the results of our research we limit ourselves to those components portrayed in Figure 5. In fact, it should be noted that plant cold tolerance and acclimation are multigenic, quantitative traits and gene expression profiling with whole genome arrays in *A. thaliana* indicates that it involves changes in the expression levels of several hundred genes (Hannah et al., 2005; Vogel et al., 2005; Hannah et al., 2006; Kaplan et al., 2007). In addition, several CBF-independent regulons that affect cold acclimation have also been identified (Zhu et al., 2004; Xin et al., 2007). This illustrates that the CBF-regulon is an important, but not exclusive pathway involved in the cold response in plants.

**Outline of this thesis**

The original title of my thesis project, funded by NWO-WOTRO was ‘Biodiversity and adaptation in the genus *Draba* in the Páramo of the Ecuadorian Andes’. Through a combination of ecological, evolutionary and developmental genetic approaches we aimed to unravel how tropical-alpine *Draba* species, occurring along different altitudinal gradients, cope with their extreme habitat conditions. In mountainous regions the diurnal variation in temperature increases with increasing elevation. Ecuadorian *Draba* species were reported to occur over a wide altitudinal range (Jørgensen and Ulloa Ulloa, 1994; Jørgensen and Léon-Yanez, 1999; Luteyn, 1999), thus making an interesting model to investigate differences in cold adaptation mechanisms. A first field visit to the high Andes of Ecuador in 2005, however, revealed that the encountered *Draba* species did not occupy such a wide altitudinal range. Temperature differences of more than 2–4°C between the lower (3850m) and upper (4318m) populations in, for example, Cotopaxi National Park were not encountered in the field. This, in addition to several administrative problems that complicated our ability to conduct research in Ecuador, resulted in a shift in focus of the project. Instead of restricting the research to tropical-alpine species only, the research was broadened to also include arctic-alpine and temperate *Draba* species.

Due to the wide geographical range covered by the genus *Draba*, it presents an ideal tool to compare the cold response of species originating from different geographical regions. This thesis will, therefore, focus on variation in the cold response pathway in *Draba* species from the tropical-alpine, the arctic-alpine and the temperate region. The project is unique in its approach of studying cold adaptation across various geographical regions by the combination of different disciplines and assessing the plant’s cold responses under tropical-alpine field conditions. In addition, to date the cold response of plants has mainly been studied under standard growth conditions of a constant 21°C temperature regime and a long day photoperiod, i.e., lab conditions optimal for *Arabidopsis thaliana*. In this thesis we chose to imitate the natural temperature and light regimes encountered by *D. hookeri* in the...
Ecuadorian Andes and conducted our experiments under a 12h photoperiod and a 21/4°C (day/night) temperature regime. In temperate and arctic-alpine regions such temperature and light conditions are encountered in spring and fall. Throughout the thesis, with the exception of the field experiments conducted in Ecuador, A. thaliana ecotype Columbia wild type (Col-wt) was included as a frame of reference.

**FIGURE 5. Simplified representation of the hypothetical low temperature-responsive transcriptional network leading to freezing tolerance in A. thaliana.** Low temperature is thought to be first perceived by the cellular membrane. Via membrane rigidification and/or other cellular changes the cold signal might induce phospholipid signaling, leading to a calcium signature and activating protein kinases. This early cold response might lead to a) induction of the CBF-response pathway, b) induction of a CBF-independent pathway via ZAT10, or c) induction of an independent cold response pathway yet to be identified. In the CBF-pathway constitutively induced ICE1 is activated through sumoylation and phosphorylation. This allows ICE1 to bind to the promoter of CBF and induce CBF1-3. They, in turn, bind to the COR promoter leading to the expression of COR15a/b and ultimately to acquired freezing tolerance. HOS1 mediates the ubiquitination of ICE1, thereby regulating CBF expression. As an CBF-independent pathway, ZAT10 is thought to regulate freezing tolerance by binding to the promoter of the COR genes. Rectangles represent genes, ovals proteins, and question marks uncertain elements of the network. Abbreviations: HOS1, high expression of osmotically responsive genes 1; ICE1, inducer of CBF expression 1; CBF, C-repeat binding factor; ZAT10 (=STZ), salt tolerance zinc finger; COR, cold responsive genes; P, phosphorylation; S, SUMO (small ubiquitin-related modifier); U, ubiquitin. The diagram is adapted from that of Chinnusamy et al., 2007.
Chapter 2 describes the variation in the genetic components of the cold response pathway detected in *Draba* by means of a candidate gene approach. As we are dealing with a non-model genus, but a relative to *Arabidopsis*, the molecular tools available from this species presented a starting point. Via RT-qPCR, molecular cloning and sequencing we identified the possible CBF- and COR15-like genes from different *Draba* species. The findings provided us with the molecular tools to investigate the induction of the CBF-regulon in response to cold.

In Chapter 3 a comparison between the expression patterns of members of the *ICE1-HOS1-CBF-COR15a* transcriptional cascade of arctic-alpine, tropical-alpine and temperate *Draba* species is made. We conducted our experiments on juvenile plants grown under a 12h photoperiod and a 21-4°C (day-night) temperature regime. The aim is to investigate whether gene expression profiles in temperate *Draba* species differs from their tropical- and arctic-alpine relatives in response to cold. In addition, we investigate if there are differences at metabolite level. Soluble sugar content and proline levels are measured.

**Figure 6. Geographical map of Ecuador including the three research locations.** From north to south the country is divided into two parallel mountain ranges, the Western and Eastern Cordillera, separated by the high altitude inter-Andean valley with the capital Quito. Both mountain ranges are dominated by volcanoes, some still active, and reaching altitudes above 4500m. The image was adapted from www.language.iastate.edu/sp304/2004/ecuador/terrain_map_mc.gif, 2004.
Next, in Chapter 4, we investigate the species’ cold response at a physiological level. Do differences exist in the ability of Draba species to withstand freezing temperatures and to cold acclimate? This question is answered by means of both a juvenile survival and an electrolyte leakage experiment. The interesting aspect of the juvenile survival experiment is that no matter whether a species is a perennial or a winter annual, if a juvenile does not survive its first winter it will never reach maturity and be able to flower and set seed. Juvenile survival will thus be an important component of the fitness of all species.

Since all experiments described in Chapter 3 and 4 are conducted under controlled conditions in the lab, the question arises as to what happens under natural conditions in the field. Chapter 5 describes the findings of a field study conducted in the high Andes of Ecuador. Three research sites were selected, each harboring two different tropical-alpine Draba species. The three locations are the Rucu and Guagua Pichincha volcanoes, Cotopaxi National Park and Cajas National Park (Figure 6) and the species studied: D. alyssoides, D. aretioides, D. hookeri, D. splendens, and D. steyermarkii. Leaf samples were collected locally and used for gene expression analyzes.

Finally, Chapter 6 discusses the results presented in this thesis and summarizes our findings on the cold response in Draba. Moreover, some suggestions are made for potential routes forward to increase our knowledge.