Natural Variation in Cold Adaptation and Freezing Tolerance in Arabidopsis thaliana

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NATURAL VARIATION IN COLD ADAPTATION AND FREEZING TOLERANCE IN *ARABIDOPSIS THALIANA*

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ABSTRACT: Plants have spread to almost everywhere in the world. As they disperse, they meet many different environments to which they may be able to adapt. For a plant species to adapt to a new environment, genetic variation is needed. The individuals differ from each other in their genetic composition, which often means differences in phenotypes. Those individuals that manage to reproduce will form the next generation. With different conditions in different environments, it will not be the same phenotypes that reproduce everywhere. In that way, plant species will form into a mosaic of locally adapted populations varying genetically as the species disperses.

After the last ice age plants have started to disperse away from the equators. With increasing latitudes come increasing challenges to migrating plants. As plant species disperse northwards along this gradient of varying conditions individuals are selected for cold adaptive traits like flowering time and freezing tolerance, acquired by cold acclimation. In this way, genetic variation from the original populations for these traits becomes sorted out along a latitudinal cline.

The aim of this thesis was to understand how selection along a latitudinal gradient has shaped natural variation in cold adaptive traits in plants dispersing northwards, and specifically, to investigate what variation can be observed in phenotypes for these traits and how these traits correlate with genetic variation in genes known to be involved in cold acclimation.

In this study significant variation was found in a sample of the model plant Arabidopsis thaliana accessions in cold adaptive traits flowering time and freezing tolerance. A clear latitudinal cline in the cold adaptive traits freezing tolerance for A. thaliana was observed.

Analysis of nucleotide polymorphism for the cold responsive ICE1 (inducer of CBF expression 1) transcription factor revealed a haplotype structure with two allelic clades as well as unusually high levels of synonymous polymorphism.

Nucleotide polymorphism analysis for the transcription factors CBF1, CBF2 and CBF3 (C-repeat binding factors) that play a key role in regulating the expression of a group of target genes known as the “CBF regulon” showed a distinct geographical haplotype structure. One haplotype was dominant in southern accessions while in the other northern accessions overrepresented. There was a significant effect of CBF haplotype on both freezing tolerance and flowering time even after correcting for latitude.

Significant differences in CBF expression levels were found between the different CBF genes as well as between different accessions. Sequence variation at CBF was shown to have a significant effect on expression levels of CBF2. No clear correlations were found between CBF gene expression and freezing tolerance or temperature sensitivity for any of the accessions used in the study. This highlights the complex relationship between sequence variation in candidate genes and gene expression, and the problems associated with unraveling the genetic basis of ecologically important traits.

KEY WORDS: Arabidopsis thaliana, cold acclimation, freezing tolerance, flowering time, latitudinal clines, genetic variation, evolutionary genetics


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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their corresponding Roman numerals.

I  Bos, A.C.G. and Ingvarsson P.K. Natural variation in freezing tolerance and genetic correlations with flowering time in *Arabidopsis thaliana*. (Manuscript)

II Bos, A.C.G. and Ingvarsson P.K. Nucleotide polymorphism in *ICE1*: a regulator of cold induced freezing tolerance in *Arabidopsis thaliana*. (Manuscript)

III Bos, A.C.G. and Ingvarsson P.K. Nucleotide polymorphism in the *CBF* transcription factor gene sequence. (Manuscript)

IV Ingvarsson, P.K. and Bos, A.C.G. Natural genetic variation in CBF gene expression. (Manuscript)
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INTRODUCTION

THE MEANING OF LIFE

All living organisms, plants and animals alike, have a single goal in life: To create the next generation. Those who do so will be represented by a larger proportion of their genes in the generations to come. This is, in short, what evolution is about.

Plants have spread to almost everywhere in the world. As they disperse, they meet many different environments to which they may be able to adapt. For a plant species to adapt to a new environment, genetic variation is needed. The individuals differ from each other in their genetic composition, which often means differences in phenotypes. Those individuals that manage to reproduce will form the next generation. With different conditions in different environments, it will not be the same phenotypes that reproduce everywhere. In that way, plant species will form into a mosaic of locally adapted populations varying genetically as the species disperses.

After the last ice age plants have started to disperse away from the equators. With increasing latitudes come increasing challenges to migrating plants. Seasons become more distinct, growing seasons become shorter, and temperatures lower. Wintertime brings shorter days and lower temperatures, while even summer temperatures are generally lower at high latitudes although daylengths are longer. As plant species disperse northwards along this gradient of varying conditions individuals are selected for who are best adapted to the local prevailing climate and manage to reproduce. In this way, variation from the original populations gets sorted out along a latitudinal cline. Latitudinal clines have been observed in adaptive traits, for instance in responses to light (Stenoien et al. 2002), growth rate (Li et al. 1998), freezing tolerance (Zhen and Ungerer 2007) and in vernalization sensitivity (Stinchcombe et al. 2005).

REPRODUCTION

To transfer their genes to the next generation plants have two options, vegetative or sexual reproduction. Vegetative reproduction simply puts copies of the parent into the world. This is done in numerous, sometimes ingenuous ways and allows a plant to
rapidly increase its numbers. In this case all individuals are genetically identical to their
parent, and this strategy works well when genotypes are well adapted to a local
environment. Alternatively, new individuals can be produced from genetic combinations
of two individuals, i.e. sexual reproduction. Sex allows for combinations of genes in the
offspring that did not exist in either parent, enhancing the chance of producing
individuals that are better suited to an environment. One disadvantage with sex is the
need to find a partner. Especially when a plant species is dispersing into previously
uncolonized environments, this can become a problem. Alternatively, many plants have
the option of selfing, that is, having sex with themselves. Selfing limits the advantage of
creating new combinations but on the other hand it ensures reproduction. In addition,
when well adapted to a local environment, selfing increases the chance of well adapted
offspring.

Being sessile, plants reproduce sexually by using flowers. Genetic material is
transferred as pollen, allowing for the transfer of genetic material between different
individuals. After pollination, seeds are formed and out of these new individuals will
arise. Besides the advantages of sexual reproduction allowing for new genetic
combinations in the offspring, seeds allow plants to spread their offspring over larger
distances than is possible with vegetative growth. Seeds are also a way of surviving
unfavorable periods of time. Seeds can lay in the ground, up to many years, waiting for
the conditions to become favorable before they germinate.

Timing of flowering

For successful reproduction, especially with increasing latitudes it is essential for
plants to get the timing of their flowering right. Flowering too early in the season might
mean fewer resources available for the production of seeds, resulting in fewer seeds
which means fewer offspring. Flowering too late might mean that seeds do not complete
development before the end of the growing season, resulting in no offspring at all.
Plants have evolved several ways of sensing when the time for reproduction is suitable.
They can detect factors such as drought, day length, light quality and temperature
(Simpson and Dean 2002). One important mechanism used by plants for timing of
reproduction is vernalization. Vernalization means that plants need to undergo a period
with low temperature (i.e. winter) before they can start flowering and produce seeds. Vernalization sometimes gets mixed up with stratification, in which a cold period breaks seed dormancy. This is also an adaptation in plants to get timing right in temperate regions, but in this case seed germination.

**COLD ADAPTATION**

Cold and in particular freezing temperatures are abiotic factors that are known to play an important role during the life cycle of many plant species and it limits both the length of the growing season and in many cases the geographic distribution of species. Freezing temperature damages plants as the water in the plant cells freezes to ice and expands. Furthermore, it has thermal effects on the plasma membrane, proteins and other macromolecules (Steponkus 1984). Nevertheless, plants have successfully adapted to cold environments and many species have colonized both sub-arctic regions and high-altitude habitats. Plants have adapted to cold through a variety of systems. Some adaptations are aimed at avoiding freezing while other adaptations make the plant tolerate freezing better. One way of avoiding freezing is insulation. Plants develop woody barks or hairy leaves, which insulate against the cold. The exposed bark is dead, so frost does not harm it. Hairs trap air, which keeps colder air out more easily. In another way to avoid freezing, deciduous plants lose their leaves in fall, reverting to just their trunks, stems or branches. Yet other plant species die back to ground level, and keep their energy stored within their roots where underground temperatures are higher.

**ACQUIRING FREEZING TOLERANCE**

Instead of avoiding being frozen, plants can increase the freezing tolerance in their vegetative parts. Many plant species growing at higher altitudes or latitudes are able to withstand freezing temperatures better if they are first exposed to low, but non-freezing, temperatures for a period of time, a process known as cold acclimation. This process is well studied because the alterations observed upon cold acclimation could reveal the molecular basis of freezing tolerance in plants (Steponkus 1984, Thomashow 1999). After exposure to a cold period a wide range of changes take place in the plant cells. The composition of the membrane lipids is altered to reduce freezing damage (Webb et al. 1994, Uemura and Steponkus 1994). A range of low-molecular weight organic
solutes accumulate such as saccharides, that temper the effects of freezing damage (e.g. Xin and Browse 1998, Wanner and Juntila 1999), or glycine betain, that can stabilize the plasma membrane and protein structure (reviewed in Sakamoto and Murata 2001). A range of cryoprotective proteins may also accumulate (reviewed in Thomashow 1999, Smallwood and Bowles 2002).

The ability to cold acclimate and to develop freezing tolerance varies both within and among plant species, depending on the local growth climate (Thomashow 1999). Plants that are usually exposed to freezing temperatures have acquired the ability to develop cold tolerance but this ability is reduced or lacking in plants growing in regions where freezing temperatures never occur. (Thomashow 1999, Smallwood and Bowles 2002, Sung et al. 2003). It is therefore not surprising that clinal variation along a latitudinal gradient in freezing tolerance has been shown (e.g. Zhen and Ungerer 2007).

**OBJECTIVES OF THE THESIS**

The aim of this thesis is to learn more about how selection along a latitudinal gradient has shaped natural variation in cold adaptive traits in plants dispersing northwards, and specifically, to investigate what variation can be observed in phenotypes for these traits and how these traits correlate with genetic variation in genes known to be involved in cold acclimation. More specifically the objectives will be addressed to as follows:

- **In Paper I** I quantify the natural variation in the cold adaptive traits, freezing tolerance and flowering time, in *Arabidopsis thaliana* (L.) Heynh., and look for latitudinal clines in these traits. I also test for associations between these environmentally important traits.

- **In Paper II and Paper III** I quantify the natural genetic variation in genes that are known to be involved in the cold acclimation process of *A. thaliana* (*ICE1* and *CBF*) and I examine the correlations between the genetic variation and the phenotypic variation in cold adaptation traits (I).

- **In Paper IV** I studied the natural variation in expression levels of *CBF1, CBF2*
and CBF3 in *A. thaliana* to see if there was significant variation depending on latitude of origin and if variation in expression levels of these important components of the cold acclimation process could explain the differences in the phenotypic variation in cold adaptive traits that were observed in Paper I.

**Study system**

*Arabidopsis thaliana*

The model organism for my studies is *Arabidopsis thaliana* (L.) Heynh. (English: thale cress). It is a small annual weed in the family Brassicaceae. Being native to Europe, central Asia and northwestern Africa it is found at many different latitudes. It is also naturalized in many places in the world. Its leaves form a rosette at the base of the plant with leaf lengths between 1.5 and 5 cm, while the inflorescence normally reaches up to 25 cm. The flowers are around 3 mm in diameter, arranged in a corymb. The fruit is called a siliqua or pod and contains around 20-30 seeds. One individual plant can produce thousands of seeds. *A. thaliana* can, under ideal conditions, complete its entire life cycle in six weeks. The central stem that produces flowers grows after about three weeks. *A. thaliana* is highly selfing which means it mainly pollinates itself in contrast to most of the other members of the Brassicaceae family, which are self-incompatible. The selfing nature of *A thaliana* may lead to inbreeding depression but selfing has advantages as well. Being a colonizer, *A. thaliana* disperses to new areas and selfing gives reproductive assurance and removes dependence on pollinators (Jarne and Charlesworth 1993). *A. thaliana* seldom outcrosses (Abbott and Gomes 1989) and most studied geographic accessions (or ecotypes) show little or no heterozygosity.

*A. thaliana* was chosen as a model plant because it's small size and low growing demands make it easily grow in pots in greenhouses or even in petri dishes in the lab. Additionally, it is very suitable for genetic research. It has one of the smallest genomes known in plants and the complete genomic sequence is available (*Arabidopsis* Genome Initiative, 2000) which simplifies designing primers for genotype studies. Also much research has been done to assign functions for *A. thaliana*’s approximately 27,000 genes and 35,000 proteins. *A. thaliana*’s distribution range also makes it possible to choose
geographical accessions from many different latitudes which are conveniently ordered from several stock centers. In this study, I used a selection of 38 accessions (also called ecotypes) of *A. thaliana*. The majority of these accessions (33) were collected from the wild or ordered from the VNAT collections at INRA, Versailles. The locations of origin were chosen so that the accessions represent a latitudinal transect throughout Europe, ranging from the Canary Islands (28° N) to the northern-most range limit of *A. thaliana* (62-63° N). The remaining accessions (5) represent naturalized locations of origin. The accessions are described in more detail in Table 1.

**FLOWERING TIME AND VERNALIZATION**

The shift from vegetative growth to flowering marks a major transition in the life cycle of plants as their meristems shift from vegetative growth to reproductive development. Being an annual weed and therefore flowering only once, the timing is critical for reproductive success in *A. thaliana*. It is regulated by different signals such as the developmental state of the plant and by environmental signals, such as daylength and temperature.

There is a strong selection on plant varieties spreading to new environments for adaptations in flowering time that give a reproductive advantage in seed production. *A. thaliana* has evolved two major flowering strategies (Michaels et al. 2003). Summer annuals (also called early flowering) will flower very rapidly and can complete a whole lifecycle within 4 to 6 weeks while winter annuals (late flowering) need a long period of low temperatures after which they rapidly initiate flowering, a process known as vernalization (Michaels et al. 2003, Michaels et al. 2004, Johansson et al. 2002). It is generally believed that the summer annual is an adaptation to warm and dry environments allowing germination and flowering to occur rapidly in spring before conditions become too dry. The winter annual is supposedly more adapted to colder environments where it germinates and grows in summer and autumn and then survives winter under the snow as a rosette. When spring comes, the plant shifts from vegetative to reproductive growth and produces seeds (Pigliucci 2002).
Detailed studies have revealed that two genes play a major role in the vernalization process (Johansson et al. 2000, Le Corre et al. 2002, Michaels et al. 2004, Shindo et al. 2005). Fig. 1 shows a simplified signaling pathway for the vernalization process. In winter annuals with an intact copy of the \textit{FRIGIDA} gene, high levels of \textit{FLC} mRNA accumulate, which inhibit flowering. Vernalization reduces \textit{FLC} RNA levels and clears the way for the plant to flower. Even after the vernalization period has ended, \textit{FLC} RNA levels stay low. Most summer annuals have been shown to have \textit{FRIGIDA} genes containing one (or both) of two deletions which lead to loss of function in the \textit{FRIGIDA} gene (Johansson et al. 2000). In these cases, no \textit{FLC} RNA accumulation takes place and these plants can flower without vernalization (Johansson et al. 2002, Michaels et al. 2003).
**FREEZING TOLERANCE AND COLD ACCLIMATION**

Cold temperatures in winter and early spring are important abiotic factors that limit the dispersal northwards of *A. thaliana* (Hoffman 2002). Despite this, *A. thaliana* has managed to spread as far north as 62-63° N. One factor that allows *A. thaliana* to grow in cold climates is freezing tolerance which is induced in a process known as cold acclimation (Thomashow 1999). These processes are initiated by low but non-freezing temperatures and are marked by large-scale changes in gene expression in *A. thaliana* (Hannah et al. 2006, Chinnusamy et al. 2007). The cold acclimation signaling pathway (Fig. 2) and its components have been the subject of many studies. In these, it has been shown that *CBF1, CBF2* and *CBF3* (*C-repeat binding factors*) play an important role in upregulating a wide range of cold regulated genes also known as *COR* genes. Together these genes are referred to as the *CBF* regulon. The *CBF* transcription factors bind a sequence in the promotor of the the *COR* genes, known as the C-repeat (CRT), dehydration-responsive element (DRE) or low temperature element (LTRE). *CBF* in turn are regulated by several different genes. One of these upstream regulators is *ICE1* (*Inducer of CBF Expression 1*) (Chinnusamy et al. 2003, Lee et al. 2005, Chinnusamy et al. 2007).

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**Figure 2.** Simplified signaling pathway for cold acclimation.
METHODS

Paper I

• The flowering time for all of the accessions was determined with and without vernalization. The experiment was aborted after 200 days when all but two of the accessions had flowered. These accessions were scored as having a flowering time of 200 days.

• All the accessions were subsequently screened for the presence of a deletion in the FRIGIDA gene to determine whether or not they possessed an active copy of the gene.

• Furthermore, the freezing tolerance acquired by cold acclimation was measured for the accessions by exposing them to different subzero temperatures (0ºC, -6ºC or -9ºC). The amount of electrolyte leakage due to freezing damage was used as a measure for frost tolerance.

• Clinal effects and correlations between freezing tolerance, temperature sensitivity and flowering time were then tested for.

Paper II

• The ICE1 gene was amplified (by way of Polymerase Chain Reaction technique (PCR)) and sequenced for the same accessions as in Paper I and the sequences were compared.

• Statistical analyses were performed to estimate nucleotide polymorphism and to test for neutrality (no selection pressure). A genealogy was also constructed for the ICE1 region.

• Tests for associations between genetic variation at ICE1 and freezing tolerance (I) were performed.

Paper III

• Parts of the CBF region were amplified and sequenced.

• Statistical analyses were performed to estimate nucleotide polymorphism, linkage disequilibrium and tests of neutrality were obtained.

• Associations between sequence variation at the CBF region and the diverse
phenotypic traits were tested for.

**Paper IV**

- *CBF1, CBF2* and *CBF3* expression was measured upon cold induction. Semi-quantitative RT-PCR (Reverse transcriptase PCR).
- The data on the *CBF* expression levels were analyzed with the aim of detecting effects of the *CBF* or *ICE1* haplotypes identified in the previous papers (II and III).

**MAJOR RESULTS AND DISCUSSION**

One major objective of evolutionary genetics is to understand the genetic control behind ecologically important traits, traits that allow an organism to survive and reproduce in natural environments (Tonsor et al. 2005, Stinchcombe and Hoekstra 2008). Although many of these traits are quantitative and likely have a complex genetic background it might be possible to acquire a more complete understanding of what factors shape genetic variation in ecologically important traits (Stinchcombe and Hoekstra 2008). Spatially variable selection, favoring different genotypes at different sites has been shown to maintain variation among populations despite considerable gene flow, leading to phenotypic and genotypic clines. (Le Corre and Kremer 2003). A good example of a selective force changing across the landscape is for instance photoperiod or temperature correlating with latitude.

**Natural variation and latitudinal clines in freezing tolerance and flowering time**

While common in other organisms, in the model plant species *Arabidopsis thaliana* the existence of clines has been subject of debate. Stinchcombe et al. (2004) and Caicedo et al. (2004) showed a latitudinal cline in flowering time in *A. thaliana* but all accessions used in these studies had a functional *FRIGIDA* allele, which plays a major role in regulating flowering time in *A. thaliana*. On the other hand, Stenøien et al. (2002), in a study on Norwegian ecotypes, and Shindo et al. (2005) in a study of 192 more widespread accessions found no significant correlations between flowering time and latitude of origin. According to Shindo et al. (2005) the differences between their results and the studies by Stinchcombe et al. (2004) and Caicedo et al. (2004) might be
explained by differences in experimental procedures or the use of different accessions. Shindo et al. (2005) stressed that latitude is a rather crude environmental variable as local climatic conditions probably vary even for populations at similar latitudes.

Table 1. Geographic origin of the used accessions.

<table>
<thead>
<tr>
<th>Label</th>
<th>Origin</th>
<th>Accession No.</th>
<th>Latitude</th>
<th>January mean temp</th>
<th>January min temp</th>
<th>Flowering time w/o vernalization</th>
<th>Flowering time w vernalization</th>
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<tbody>
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<td>E1</td>
<td>Färdjstaeden, Sweden</td>
<td>*</td>
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<td>-1</td>
<td>3</td>
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<td>na</td>
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<td>E2</td>
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*Field collected by P. Ingvarsson, **Field collected by A. Bos

1*Nottingham Arabidopsis Stock Centre accession number (N00000) or INRA, Varieties accession number (NVAR000)
2Mean or minimum January temperature from [www.weatherbase.com](http://www.weatherbase.com)
3not available

In Paper I, however, I found a clear clinal variation in freezing tolerance with the northern accessions being the most freezing tolerant. The variation in freezing tolerance in my sample of *A. thaliana* accessions could be explained significantly better by latitude than by temperature at the site of origin. Possibly, the minimum or mean temperatures at the site of origin might not be the most ecologically relevant temperature influencing freezing tolerance or else the available minimum temperatures for my sample of accessions was not measured as precise as latitude. Recently, Zhen and Ungerer (2007) in a dataset with 70 accessions, also showed that freezing tolerance varies with the geographic origin of accessions, with northern accessions being more tolerant.
In Paper I considerable variation in flowering time was also observed in the sample of accessions (Table 1). A significant clinal variation in flowering time was found but this was mostly explained by eight Swedish accessions, all of which are late flowering. When excluding the Swedish accessions, there was no significant cline in flowering time. This concurs with the findings of Nordborg et al. (2005) who also found that several Scandinavian accessions have very long flowering times.

Furthermore, I found a significant positive genetic correlation between flowering time and freezing tolerance, suggesting that accessions with longer flowering times are more freezing tolerant. A negative correlation was found between temperature sensitivity (i.e. sensitivity to a change in freezing temperature) and flowering time (I). This suggests that early flowering accessions are more sensitive to changes in freezing temperatures, which follows intuitively since accessions from southern regions tend to have shorter flowering times while accessions from northern region are mostly late flowering and are more freezing tolerant. For those accessions, small differences in freezing temperature should have a minor effect on their ability to withstand cold.

The cause of the genetic correlations found between freezing tolerance/temperature sensitivity and flowering time could be either pleiotropy or linkage disequilibrium (LD). In highly selfing A. thaliana LD may extend over hundreds of kb (Nordborg et al. 2002). There is also substantial population structuring in A. thaliana that may reinforce associations between loci influencing flowering time and frost tolerance (Nordborg et al. 2005, Zhao et al 2007). This suggests that linkage and/or population structure could be significant forces in shaping observed genetic correlations between frost tolerance and flowering time. Similarly, given what is known about the genetic basis of flowering time variation and the genetic control of low temperature responses in A. thaliana, pleiotropy is at first unlikely (Thomashow 1999, Sung et al. 2003). However, concordant patterns of spatially variable selection are known to result in the build-up of associations between (potentially unlinked) loci across populations (Le Core and Kremer 2003). This suggests that the strong genetic correlations I observed between flowering time and frost tolerance could be a byproduct of natural selection acting in parallel on flowering time and freezing tolerance, two important life history traits.
I quantified the nucleotide variation at the *ICE1* locus (II) and the *CBF* locus (III) for my accessions of *Arabidopsis thaliana*. A strong haplotype structure with very little intra-haplotype variation was found as has been observed in *A. thaliana* in earlier studies (Hanfstingl et al. 1994), and this was initially thought to be the result of balancing selection (Hanfstingl et al. 1994). However, demographic history and the selfing nature of *A. thaliana* confound tests of neutrality (Nordborg et al. 2005). Furthermore, high levels of selfing reduce both effective population size and the effective rate of recombination (Charlesworth 2003). Because of the low effective recombination observed in *A. thaliana*, it is not uncommon to observe a strong haplotype structure in samples taken from a single population (Nordborg 2000). Such haplotype structures may resemble patterns observed under balancing selection and confound neutrality tests such as Tajima's D (Tajima 1989). In addition, there is evidence of a large-scale genetic population structure in *A. thaliana* likely reflecting historical processes such as isolation into glacial refugia and postglacial dispersal northwards (Nordborg et al. 2005, Schmid et al. 2005).

*ICE1* haplotypes and natural variation in cold tolerance showed no correlation (II). This might be expected as only a few of the mutations that were detected in *ICE1* are functionally important. There are only 5 non-synonymous mutations of which only one is not a singleton. Furthermore, none of the non-synonymous mutations occur in the functional domains of *ICE1* identified by Chinnusamy et al. (2003). Alternatively, natural variation in cold tolerance among the ecotypes I have studied could be explained by variation in expression levels of *ICE1*, and therefore, gene expression of *ICE1* in *A. thaliana* after cold acclimation should be examined. Recently, Kanaoka et al. (2008) showed that *ICE1* plays an integral role in plant development as an essential initiator of stomatal differentiation in *A. thaliana* (Kanaoka et al. 2008). This suggests that *ICE1* could be a key gene integrating environmental input and developmental programs in plants (Kanaoka et al. 2008). In line with this observation, the *ICE1* gene is under strong purifying selection (K_A/K_S =0.1) and has few segregating non-synonymous
polymorphisms suggesting that it is evolving under strong functional constraint.

At the *CBF* region, one haplotype (H1) was more common among southern accessions while the other haplotype (H2) was more common among northern accessions (III). Southern accessions (haplotype H1) showed two to three-fold higher levels of nucleotide polymorphism and there is a tendency for mutations in these accessions to segregate at lower frequencies. This concurs with findings from Zhen and Ungerer (2008) and Mckhann et al. (2008) who also found increased levels of nucleotide polymorphism in *A. thaliana* accessions from southern Europe. A possible explanation is that *CBF* is under lower selective pressure in southern ecotypes, which are less exposed to low temperatures, resulting in reduced efficiency of purifying selection. However, the differences found in polymorphism and mutation segregation between haplotypes in Paper III are not significant.

I did find a strong *CBF* haplotype effect on freezing tolerance. However, because of the geographic structuring of the two *CBF* haplotypes it is hard to determine whether this haplotype effect is directly caused by sequence variation at the three *CBF* genes or caused by segregating, but unmeasured, variation elsewhere in the *A. thaliana* genome that is confounded by the haplotype designations that I used in these analyses (Stranger and Mitchell-Olds 2005).

I did found a strong effect of *CBF* haplotype on flowering time. This might at first seem rather surprising, but is in fact in line with the significant positive genetic correlation between flowering time and freezing tolerance in Paper I. As discussed above, the presence of a strong population structure in *A. thaliana*, suggests that the genetic correlation I observe is generated by linkage disequilibrium. However, flowering and cold tolerance appear to be at least partly controlled by the same genetic pathways (Kim et al. 2004, Franklin and Whitelam 2007), suggesting that pleiotropy may also play a role in explaining the haplotype effect of *CBF* on flowering time. For instance, the autonomous pathway gene *FVE* is a negative regulator of *CBF* expression and a positive regulator of *FLC*, a repressor of flowering in *A. thaliana* (Kim et al. 2004). Kim et al. (2004) demonstrated that plants sense intermittent cold stress through *FVE* and respond by delaying flowering mediated through an increased expression of
Similarly, plants sense light quality (i.e. the ratio of red (R) to far-red (FR) light) through phytochromes and the photoperiodic pathway (Corbesier and Coupland 2005). Interestingly, *CBF* gene expression also appears to be under partial phytochrome control and a low R:FR ratio increases *CBF* gene expression in *A. thaliana* (Franklin and Whitelam 2007). This increase in *CBF* expression is large enough that freezing tolerance develops at temperatures which would normally not induce cold acclimation (Franklin and Whitelam 2007). These results suggest that the possible links between freezing tolerance and flowering time need to be studied in greater detail and the roles of population structure and pleiotropy in linking *CBF* variation to naturally occurring variation in these traits need to be clarified.

**Natural variation in expression levels of *CBF1*, *CBF2* and *CBF3* in *A. thaliana**

Upon cold induction all three *CBF* genes were rapidly induced, reaching their highest expression approximately one hour after cold exposure, after which expression levels gradually decline. There were, however, substantial differences between ecotypes. Some ecotypes show little effect of cold exposure, responding with a modest induction of *CBF* gene expression whereas others show higher induction for longer periods of time (IV).

I found that *CBF* haplotype variation had a significant effect on gene expression of *CBF2*, with the biggest differences between the haplotypes at one and two hours after exposure to cold. This corresponds to the two time points with maximum *CBF2* gene expression, thus increasing the power to detect differences between haplotypes. Interestingly though, I observed no corresponding increase/decrease in *CBF1* and *CBF3* gene expression between haplotypes despite experimental evidence suggesting that *CBF2* expression negatively regulates *CBF1/CBF3* expression (Novillo et al. 2004). There are weak indications of a *CBF* haplotype effect on *CBF3* gene expression although these differences are not significant (III, IV).

Furthermore, when calculating the genetic correlations between gene expression for the three *CBF* genes I find that these correlations are generally positive, contrary to the expectation of a negative correlation between *CBF2* and *CBF1/3* expression. However, only the *CBF1-CBF3* comparison is significantly different from zero. It is worth noting
that the genetic correlation between *CBF1* and *CBF3* expression is substantially stronger than that between *CBF1* and *CBF2* or between *CBF2* and *CBF3*. This observation is consistent with a strong co-regulation of *CBF1* and *CBF3* and with evidence suggesting that *CBF2* and *CBF1/3* genes have different functions and activate different subsets of the *CBF* regulon (Novillo et al. 2004, 2007) (IV).

I found a negative correlation between *CBF2* expression and freezing tolerance (*r*=-0.151, ns), as expected if high *CBF2* gene expression is associated with reduced freezing tolerance (Novillo et al. 2007). Similarly, genetic correlations between *CBF1* and *CBF3* gene expression and frost tolerance were positive, again as expected from previous experiments (Novillo et al. 2007). While none of these correlations were significant, their general direction is once again in agreement with what is known about the regulatory network of the three different *CBF* genes (Novillo et al. 2004, 2007) (I, IV).

**Conclusions**

*Variation in Adaptive Traits and Latitudinal Clines*

As plants migrated northwards from their refugia after the last ice age they were confronted with an increasing selective pressure by cold temperatures. Initial variation in the plant populations in important cold adaptive traits, such as freezing tolerance and flowering time, was sorted out as locally best adapted individuals managed to reproduce. In this study, I have demonstrated that *A thaliana* contains considerable variation in these evolutionary important traits in accessions from different site of origin, and I showed, in addition that there is a clear latitudinal cline for freezing tolerance (I) as tolerance increases with latitude of origin. I also found a significant cline in flowering time, but here the results were biased by a group of Swedish accessions with extremely long flowering times. Further research is needed to be able to draw conclusions on the existence of a latitudinal cline in flowering time.

*Polymorphism and Correlation with Adaptive Traits*

Acquiring freezing tolerance through cold acclimation coincides with a range of
biochemical changes in the plant (as reviewed Thomashow 1999, Smallwood and Bowles 2002). I show that there is significant polymorphism in some key regulators of this process, *ICE1* and *CBF* and show allelic clades for both of these regulators. (II, III).

The geographic distributions of the *CBF* haplotypes are of special interest with one group consisting of northern accessions and the other of southern accessions. At the same time however, this strong population structure necessarily confounds the strong correlations that I found for *CBF* haplotype and naturally occurring variation in freezing tolerance among accessions of *A. thaliana* (III). The study Zhao et al. (2007) demonstrate how difficult it is to deal with this problem. Another problem is allelic heterogeneity at causal loci. When variation in a trait is caused by a number of different alleles from a single gene (as opposed to a few frequently occurring alleles), association methods, such as I used in this paper, are likely to indicate that there is no major locus for this trait in the region(s) studied. As pointed out by Nordborg and Weigel (2008), this is really another facet of the problem with population structure, because the importance of particular alleles always depends on the reference population. Genetic dissection of complex trait variation in *A. thaliana*, and other species with strong population structure, will thus continue to rely on the use of a combination of techniques, including traditional QTL mapping, association mapping and reverse genetics.

**Variation in CBF expression levels**

The study shows that there are significant differences in expression levels between the three different *CBF* genes, with *CBF3* being most strongly induced. Differences among the different ecotypes were significant as well. After controlling for these effects, I also find an effect of sequence variation in the *CBF* region on expression of *CBF2*, where a set of 13 ecotypes carrying a specific *CBF* haplotype that show 1.5-fold higher levels of *CBF2* expression. I did not, however, find significant correlations between *CBF* gene expression and freezing tolerance or temperature sensitivity of the different ecotypes tested. The results highlight the complex relationship between sequence variation in candidate genes and gene expression and the problems associated with
dissecting the genetic basis of ecologically important phenotypes (IV).

**UNRAVELING THE RELATIONSHIPS BETWEEN SEQUENCE VARIATION, GENE EXPRESSION AND INDUCTION OF FREEZING TOLERANCE**

Some of the complexities involved in unraveling adaptation to temperatures is shown by the study of Alonso-Blanco et al. (2005) who identified a 1.6 kb deletion in the 5' UTR of *CBF2* in the Cvi (Cap Verde Island) ecotype. This deletion spanned several putative regulatory elements of the *CBF2* gene and resulted in very low levels of *CBF2* expression. Interestingly, the reduction in *CBF2* expression was accompanied by a reduction in frost tolerance, contradictory to the results of Novillo et al. (2004).

Novillo et al. (2004) identified a T-DNA insertion 179 bp upstream of the *CBF2* gene that also resulted in extremely low levels of *CBF2* expression. However, Novillo et al. (2004) found that this resulted in increased frost tolerance, likely caused by constitutive expression of *CBF1* and *CBF3*.

Two recent papers have examined *CBF* gene expression in a diverse set of ecotypes (Mckhann et al. 2008, Zhen and Ungerer 2008). Zhen and Ungerer (2008) found that ecotypes from southern latitudes (below 42°N) exhibited higher levels of polymorphism at non-synonymous and regulatory sites at the three *CBF* genes. This in turn translated into lower rates of induction and lower maximum levels of cold-induced genes downstream of the *CBF* genes (Zhen and Ungerer 2008). Mckhann et al. (2008), in a study of four cold tolerant and four cold sensitive ecotypes, also found elevated rates of *CBF* polymorphism in cold sensitive ecotypes. However, they did not find any clear effects of this on downstream expression of several *COR* genes (Mckhann et al. 2008). These studies, combined with the results obtained in this thesis, highlight the problems of dissecting the genetic architecture of ecological adaptations. In this respect, it is interesting to note that all experiments that have studied the link between *CBF* gene expression, cold and frost tolerance have used very different protocols with respect to both acclimatization and exposure to freezing temperatures (Novillo et al. 2004, 2007, Alonso-Blanco et al. 2005, Mckhann et al. 2008, Zhen and Ungerer 2008, I, II, III)

Adaptation can be mediated through mutations that change the coding region of genes, so called structural mutations. Alternatively, mutations that affect the level of
expression of a gene, known as regulatory mutations can also be adaptive. The relative importance of structural versus regulatory mutations is currently a topic of hot debate (see for example, Hoekstra and Coyne 2007, Mitchell-Olds et al. 2007). The strong haplotype effect I observe for CBF2 gene expression is consistent with cis-acting variation that affects how strongly induced the CBF2 gene is between the two different haplotypes. The haplotype effect I observe is likely not caused by trans-acting polymorphisms in upstream transcription factors, as I do not observe an effect of ICE1 haplotype. However, more work is clearly needed before the possible relationships between sequence variation in CBF genes, CBF gene expression and induction of cold tolerance response and frost tolerance becomes clear.

**Further research**

After this study some interesting questions remain and new have been raised. Further studies could address:

The existence of a latitudinal cline in flowering time as my results suggests. The study sample will need to be extended with accessions displaying longer flowering times from lower latitudes as well as with accessions from higher latitudes with shorter flowering times. Possibly, when the variations in flowering time are examined for winter and summer annuals separately, two latitudinal clines for flowering time might be found. One for accessions with an intact FRIGIDA locus and one for accessions with an intact FRIGIDA locus.

Completing the sequencing of the CBF gene region. My studies suggested that the regions that were hard to sequence contained higher levels of nucleotide polymorphism. Completing the sequence and correlating the variation within the accessions with cold adaptive traits might contribute to understanding the relationships between sequence variation, gene expression and cold acclimation in plants.

Study polymorphism in genes in other pathways that regulate adaptive traits like the ABA (abscisic acid) pathway known to be involved in a range of responses to stress including freezing tolerance or the Autonomous Pathway regulating flowering (Fig. 1). Flowering time control protein FCA is one such possibly interesting candidate. Being
part of the Autonomous Pathway \textit{FCA} in its active form promotes flowering by downregulating \textit{FLC} but it also negatively regulates its own expression and in that case \textit{FLC} is not downregulated and can still block flowering.

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Multilocus analysis of variation and speciation in the closely related species *Arabidopsis halleri* and *A. lyrata* *Genetics* **66**, 373-388.


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