NATURAL VARIATION IN FREEZING TOLERANCE IN *ARABIDOPSIS THALIANA*

by

YING ZHEN

B.S., Beijing University, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Elucidating the molecular basis of adaptive phenotypic variation represents a central aim in evolutionary biology. Using the model plant species *Arabidopsis thaliana*, I studied the intra-specific variation in freezing tolerance among natural accessions across its native range. Considerable variation in freezing tolerance among 71 selected accessions was observed both with and without a prior cold acclimation treatment, suggesting that both differences in cold-acclimation capacity and in intrinsic physiology contribute to this variation. A highly significant positive relationship was observed between freezing tolerance and latitude of origin of these accessions. This clinal pattern of variation is found to be attributable, at least in part, to relaxed purifying selection on *CBF/DREB1* genes in the species' southern range. These *CBF/DREB1* genes encode transcriptional activators that play a critical role in the ability of *A. thaliana* plants to undergo cold acclimation and thereby achieve maximum freezing tolerance. Relative to accessions from northern regions, accessions of *A. thaliana* from the southern part of their geographic range exhibit significantly higher levels of nonsynonymous polymorphisms in coding regions of *CBF/DREB1* genes. Relaxed selection on the *CBF/DREB1*s in southern accessions also has resulted in mutations in regulatory regions that lead to abrogated expression. These mutations in coding and regulatory regions compromise the function of CBF/DREB1 transcriptional activators during the cold acclimation process, as determined by reductions in rates of induction and maximum levels of expression in the downstream genes they regulate. These mutations could be selective neutral or beneficial in southern accessions depending on whether there is an allocation cost associated with cold acclimation. The fitness benefit and possible allocation cost of cold acclimation was examined in freezing and freezing-free environments using natural accessions exhibiting contrasting abilities of cold acclimation as well as transgenic *CBF* gene over-expression or knockdown/knockout lines. The extent to which cold acclimation benefits the plant in presence of freezing temperature is revealed, but a cost of cold acclimation wasn't detected in the absence of freezing temperature under our experimental design, which suggests that these mutations in *CBF* genes in southern accessions might be neutral to natural selection.

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Elucidating the molecular basis of adaptive phenotypic variation represents a central aim in evolutionary biology. Using the model plant species *Arabidopsis thaliana*, I studied the intra-specific variation in freezing tolerance among natural accessions across its native range. Considerable variation in freezing tolerance among 71 selected accessions was observed both with and without a prior cold acclimation treatment, suggesting that both differences in cold-acclimation capacity and in intrinsic physiology contribute to this variation. A highly significant positive relationship was observed between freezing tolerance and latitude of origin of these accessions. This clinal pattern of variation is found to be attributable, at least in part, to relaxed purifying selection on *CBF/DREB1* genes in the species' southern range. These *CBF/DREB1* genes encode transcriptional activators that play a critical role in the ability of *A. thaliana* plants to undergo cold acclimation and thereby achieve maximum freezing tolerance. Relative to accessions from northern regions, accessions of *A. thaliana* from the southern part of their geographic range exhibit significantly higher levels of nonsynonymous polymorphisms in coding regions of *CBF/DREB1* genes. Relaxed selection on the *CBF/DREB1*s in southern accessions also has resulted in mutations in regulatory regions that lead to abrogated expression. These mutations in coding and regulatory regions compromise the function of CBF/DREB1 transcriptional activators during the cold acclimation process, as determined by reductions in rates of induction and maximum levels of expression in the downstream genes they regulate. These mutations could be selective neutral or beneficial in southern accessions depending on whether there is an allocation cost associated with cold acclimation. The fitness benefit and possible allocation cost of cold acclimation was examined in freezing and freezing-free environments using natural accessions exhibiting contrasting abilities of cold acclimation as well as transgenic *CBF* gene over-expression or knockdown/knockout lines. The extent to which cold acclimation benefits the plant in presence of freezing temperature is revealed, but a cost of cold acclimation wasn't detected in the absence of freezing temperature under our experimental design, which suggests that these mutations in *CBF* genes in southern accessions might be neutral to natural selection.

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CHAPTER 1 - Introduction

One of the major goals in evolutionary biology is to understand the underlying genetic basis of adaptive phenotypic variation. An organism occupying a certain environment has to evolve to adapt to its local climate conditions, including the temperature condition. Due to the sessile lifestyle of plants and their inability to escape unpleasant environmental conditions, freezing temperature represents one of the major environmental challenges that limit plant growth, productivity and geographic distribution. Different plant species have specific temperature requirements for optimum growth, and different ability to tolerant freezing temperatures. The ability of freezing tolerance has been observed to vary considerably not only among different plant species, but also within the same plant species, especially for plant species with broad geographic distributions, where selective pressures for freezing tolerance are expected to be diverse for different local populations (Andaya and Mackill 2003; Hannah et al. 2006; Sackville Hamilton et al. 2002). In addition, for most temperate plant species, including the model species *Arabidopsis thaliana*, freezing tolerance can increase dramatically through cold acclimation, a process where a plant is pre-subjected to low, but non-freezing temperatures (Thomashow 1999; Xin and Browse 2000). Although the genetics of cold acclimation and freezing tolerance in plants has been studied extensively during recent years (Ruelland et al. 2009; Van Buskirk and Thomashow 2006; Xin and Browse 2000), the ecological and evolutionary dynamics of freezing tolerance within plant species hasn't been systematically investigated.

Arabidopsis thaliana: an excellent model

Arabidopsis thaliana (L.) Heynh., also known as thale cress or mouse-ear cress, belongs to the family Brassicaceae. It is a small weedy plant native to Europe, Asia and northwestern Africa. Due to human activity, it is now found world-wide. *Arabidopsis thaliana* has several characters that make it a great plant model organism. It has a small compact genome (2n = 10, 157Mb) that has been sequenced and annotated (The Arabidopsis Genome Initiative, 2000). It is

easy to grow and has a relatively short generation time, and it produces prolific seeds per generation. Tools have been developed for genetic manipulation, and mutant libraries saturating the whole genome are been developed (Alonso et al. 2003). Such advantages explain why *A. thaliana* has been extensively used as a model to study genetics, development, cellular, and molecular processes in flowering plants. In particular, the understanding of genetic mechanisms of cold acclimation and freezing tolerance is mostly facilitated by studies conducted in *A. thaliana*.

In addition to the fact that *A. thaliana* has the best understood cold responsive pathway among flowering plants, another great resource of *A. thaliana* is that there are hundreds of natural accessions that have been collected world-wide and their seeds are available from stock centers such as ABRC (The Arabidopsis Resource Center). These accessions have been found to exhibit considerable variation in all different aspects, such as seed size, flowering time, inflorescence architecture, and pathogen resistance (Koornneef et al. 2004). Moreover, several studies have investigated population structure and genome wide pattern of polymorphism among these natural accessions (Nordborg et al. 2005; Schmid et al. 2003). This species thus provides an especially useful experimental system to study natural variation and evolution of adaptive traits. While some evidences exists for differences in freezing tolerance among natural accessions of *A. thaliana*, a systematic survey of the variation has not been done, which gives us an excellent opportunity to study the ecological and evolutionary dynamics of freezing tolerance at the intra-specific level using this model species.

How does the ability of freezing tolerance vary among natural populations of *A. thaliana* with regard to the temperature variability across its native range? To address this question, we selected 71 natural accessions originally collected from across its native range to characterize the pattern of variation in freezing tolerance in *A. thaliana*. We conducted a large scale phenotypic assay to measure the freezing tolerance of each accession at a series of freezing temperatures both with and without a prior cold acclimation treatment. Considerable variation in freezing tolerance among these accessions was observed both with and without cold acclimation. A highly significant positive relationship also was revealed between freezing tolerance and latitude of origin of these accessions under both cold-acclimated and non-cold-acclimated conditions,

indicating that factors both intrinsic and associated with cold acclimation contribute to the clinal variation in freezing tolerance in *A. thaliana.* Since local temperature, which probably acts as selective agent for freezing tolerance, also follows a latitudinal cline, this suggests a role of natural selection in shaping the variation in this trait.

Freezing tolerance and cold acclimation

The primary location of freezing injury in plants is at cell membrane. The level of freezing injury is determined by the physiological state of a plant, as well as the process of freezing and thawing, *e.g.,* freezing injury increases with lower freezing temperatures, increased length of exposure to freezing temperature, repeated freezing and thawing (Burke et al. 1976), and an increased rate of temperature change during freezing. Freezing tolerance reflects the ability of plant to minimize or prevent deleterious consequences during freezing. Freezing tolerance is a multigenic, quantitative and multilevel trait. Plants subjected to direct, abrupt freezing temperature exhibit a minimum, intrinsic level of freezing tolerance, which is recognized as non-cold-acclimated freezing tolerance. In contrast, cold-acclimated freezing tolerance acquired by pre-subjection to low nonfreezing temperatures defines the maximum freezing tolerance of plant, and plays a major role in the winter survival of plant. Although these two levels of freezing tolerance are clearly distinguished, whether they share a common molecular basis is not well understood, mainly because of the lack of knowledge on the mechanisms of non-cold-acclimated freezing tolerance.

The genetic basis of non-cold-acclimated freezing tolerance is poorly understood. It has been reported that there may be independent genetic controls of non-cold-acclimated freezing tolerance and cold acclimation capacity by studying two potato species exhibiting extremes of these two traits (Stone et al. 1993). Transcriptional studies revealed differences in transcriptome response of Barley varieties to non-cold-acclimated freezing treatment comparing to cold-acclimated freezing treatment (Koo et al. 2008). Different sets of quantitative trait loci (QTL) were identified for freezing tolerance in cold-acclimated and non-cold-acclimated faba bean, with only one QTL for each colocalizing on the same linkage group (Arbaoui et al. 2008). However, *A. thaliana* accessions with high non-cold-acclimated freezing tolerance have been

shown to have a similar metabolic state as that of cold acclimated plants, with high baseline expression of cold responsive genes at normal temperature (Hannah et al. 2006), which suggests there might be some level of overlapping of genes that control both non-cold-acclimated freezing tolerance and cold acclimated freezing tolerance.

In contrast to non-cold-acclimated freezing tolerance, the cold acclimation process and the underlying genetic mechanisms that could contribute to the increase of freezing tolerance have been extensively studied (Chinnusamy et al. 2006; Hannah et al. 2005; Thomashow 1999; Xin and Browse 2000; Yang et al. 2005). During cold acclimation, numerous physiological and biochemical changes take place in the plant, including reductions or cessation in plant growth, reductions in tissue water content, transient increases in level of plant hormone abscisic acid, increased levels of antioxidants, and modification of cell wall (Thomashow 1999; Xin and Browse 2000). There are also distinct changes in membrane lipid composition (Welti et al. 2002) and rapid intracellular accumulation of compatible osmolytes such as proline, betaine, polyols and soluble sugars (Cook et al. 2004; Valluru et al. 2008). In addition, transcriptional profiling studies revealed that the expression patterns of hundreds of genes are changed during cold acclimation (Fowler and Thomashow 2002; Hannah et al. 2005). These genes are referred to as cold responsive genes.

A cis-acting CRT/DRE (C-repeat/dehydration responsive) element is found in one to multiple copies in the promoters of many plant cold responsive genes. This led to the discovery of the *CBF/DREB1* (C-repeat binding factor/dehydration responsive element binding factor 1) transcriptional activators (Stockinger et al. 1997), which are frequently referred to as the master switches of cold acclimation. The *CBF* pathway is the best understood genetic pathway that plays a major role in cold acclimation process. In *A. thaliana*, there are three *CBF* genes, *CBF1*, *CBF2*, and *CBF3*, also known as *DREB1b*, *DREB1c*, and *DREB1a* respectively. They lack introns, and lie in a tandem array spanning an 8.7kb region on chromosome four. They are about 85% identical at the nucleotide level in pairwise comparisons. Under low temperatures, all *CBF* genes are rapidly induced, their expression peaks at about two hours, and returns to base level after 24 hours. Over-expression of each individual *CBF* gene has a remarkable effect on the biochemical composition, morphology and development of the transgenic plants (Gilmour et al.

2004; Gilmour et al. 2000). These over-expression lines have increased freezing tolerance even without cold acclimation treatment, and increased intracellular levels of some metabolites at warm temperature, but also exhibit a dwarf phenotype with stunted growth and delayed flowering. However, *A. thaliana* transcriptome profiling indicates that in addition to the *CBF* cold responsive pathway, many other regulatory pathways are involved, although not as well understood (Fowler and Thomashow 2002).

Although *A. thaliana* has been used as a model system to study the genetic mechanisms of plant responses to low temperature, there is limited knowledge about the genetic basis of differences in freezing tolerance among different accessions of *A. thaliana.* One study took a QTL (quantitative trait loci) approach to address this question using an RIL population derived from *A. thaliana* accessions Landsberg erecta (Ler) and Cape Verde Islands (Cvi). Ler is a lab derivative of a natural accession with high freezing tolerance, and Cvi is a natural accession with low freezing tolerance. Seven QTLs were identified for cold-acclimated freezing tolerance at two different photoperiod conditions. The QTL with largest effect under both photoperiods co-located with the known *CBF* gene cluster. A 1.6kb deletion of the promoter region of Cvi *CBF2* allele was found to be the molecular basis of the low freezing tolerance of Cvi alleles, which was associated with low expression of *CBF2* and several *CBF* target genes (Alonso-Blanco et al. 2005). Moreover, a comparison of metabolome profiles of high freezing tolerant accession Wassilewskija-2 (Ws-2) and low freezing tolerant accession Cape Verde Islands-1 (Cvi-1) indicated that low-temperature-induced expression of *CBF1, CBF2, CBF3*, and *CBF* targeted genes was much lower in Cvi-1 than in Ws-2 plants (Cook et al. 2004). In addition, it has also been reported that transcript level of *CBF1* and *CBF2* show positive correlations with CA freezing tolerance (Hannah et al. 2006).

These pioneering studies suggest that functional difference of *CBF* genes could be associated with difference in freezing tolerance in some natural accessions of *A. thaliana.* Considering that *CBF* genes play a pivotal role in the ability of *A. thaliana* plants to undergo cold acclimation and achieve maximum freezing tolerance, and because they are positioned early in a genetic network such that improper functioning would have numerous and undoubtedly detrimental downstream consequences, they are excellent evolutionary candidate genes to

investigate the underlying genetic basis of natural variation in freezing tolerance in *A. thaliana* across its native range.

Our phenotypic assay revealed a latitudinal cline in freezing tolerance among natural accessions of *A. thaliana*. Is there any functional variation in *CBF* genes corresponding to this clinal variation among these accessions across the native range? To address this, I looked at the sequence and expression variation of *CBF* genes in 24 representative accessions with regards to their geographic origin. These 24 accessions were selected from the 71 natural accessions used in the phenotypic assay, and were categorized as northern and southern accessions according to their latitude of origin. All three *CBF* genes were sequenced from these 24 accessions, and the pattern of nucleotide variation was examined. The level of nonsynonymous polymorphism was found to be significantly higher in southern accessions than in northern accessions, which suggested that purifying selection on *CBF* genes was relaxed in southern range of *A. thaliana*, probably due to the warmer climate. Expression of each *CBF* gene in these 24 accessions was assayed, and four accessions exhibiting abrogated expression of either *CBF1* or *CBF2* were identified. All four accessions also originate from the species' southern range and exhibited low freezing tolerance. Sequencing the flanking regions of *CBF* genes in these accessions revealed that the abrogated expression was associated with independent indel mutations in regulatory regions. These mutations in coding and regulatory regions resulted from relaxed purifying selection and compromised the functionality of *CBF* genes in southern accessions, which contributed to the clinal pattern of variation in freezing tolerance we observed.

Fitness cost of cold acclimation

Tolerance traits against environmental stresses can be inducibly or constitutively expressed. Inducible defense are only expressed, or expressed to a higher degree, in response to specific cues indicating that a defense is needed. The full development of an inducible defense need extra time which compromises a faster defense when comparing with constitutive defense. A hypothesis of the evolution of inducible defenses is that it involves an allocation cost, whereby energy and resources allocated to stress defense cannot be used for growth or reproduction, thus is too costly to maintain in the absence of an environmental stress (Strauss et al. 2002; Walters

and Heil 2007). Such a cost should be evident if the defense is expressed in the absence of the stress, but the cost could be counterbalanced by the benefit of defense in stressed conditions. Most studies that empirically explored the existence of this allocation cost focused on induced defense against biotic stress, such as pathogen resistance and herbivore resistance. Allocation cost was found in many cases (Smedegaardpetersen and Stolen 1981). However, detecting the cost of inducible defense can be difficult because plant-environment interactions can be complex. A number of studies found no allocation cost of inducible defense on plant growth and reproduction (Bergelson and Purrington 1996; Walters and Heil 2007). Some studies even reported that plants can benefit from herbivory damage by overcompensation, ultimately achieving greater fitness (Paige and Whitham 1987; Tiffin 2000).

Cold acclimation represents a well-characterized plant inducible defense against freezing temperatures. Although the genetics of cold acclimation pathway have been studied intensively during recent years, the ecological consequences of cold acclimation in natural accessions of plants haven't been investigated. Possessing high cold acclimation ability is beneficial in environments where plants frequently encounter freezing stress. However, cold acclimation may be metabolically costly since it involves expression changes of hundreds of genes and intracellular accumulation of large amount of metabolites. High induced freezing tolerance acquired through cold acclimation may be a waste of energy if it is induced when there is no subsequent freezing. The cost of *CBF* over-expression has been investigated in transgenic *A. thaliana* plants (Jackson et al. 2004). A cost of over-expression was found to be transgene dependent. In the absence of freezing, a cost has been detected in *CBF2* and *CBF3* over-expressers with and without cold acclimation, while *CBF1* over-expressers showed no cost of tolerance without cold acclimation, and a benefit with cold acclimation (Jackson et al. 2004). However, the cost observed from *CBF* over-expression lines could be exaggerated by the fact that these transgenic plants have a constitutively up-regulated cold acclimation pathway. A more realistic way to examine a potential cost of cold-acclimation is to use natural accessions of *A. thaliana* with contrasting cold acclimation capacities, as well as *CBF* T-DNA insertion lines.

The finding of relaxed selection on the *CBF* genes in southern accessions raises interesting questions regarding the potential allocation cost of cold acclimation. Mutations in

CBF genes in southern accessions could be selectively neutral or beneficial depending on whether there is an allocation cost of cold acclimation. In the southern range of *A. thaliana*, temperature rarely drops to freezing, but it can be low enough to induce the cold acclimation pathway. If the allocation cost is significant and can't be compensated for by any other way, it will cause a reduction in growth and/or reproductive fitness. As such, the cold acclimation ability could be selected against by natural selection in warmer climates. Under such circumstance, mutations compromising the cold acclimation pathway such as those in the *CBF* genes might be favored by natural selection. However, if there isn't such an allocation cost, cold acclimation ability should be selectively neutral in southern accessions, and thus mutations in the *CBF* genes compromising their function would also be expected to be neutral.

To test these hypotheses and determine whether there is an allocation cost of cold acclimation, we used 6 northern accessions with high cold acclimation capacity, 6 southern accessions with low cold acclimation capacity and mutations in coding and/or regulatory regions of *CBF* genes and, as well as several *CBF* T-DNA insertion lines together with their background lines. Fruit number, as a direct measurement of fitness, and several other fitness related phenotypic traits were measured in plants both with and without cold acclimation in the absence of a subsequent freezing stress. If there is an allocation cost of cold acclimation, we would expect to see a reduction in fitness in cold acclimated plants compared with non-cold-acclimated plants, and the fitness reduction would be greater for northern accessions/background lines than southern accessions/T-DNA insertion lines because northern accessions/background lines have higher cold acclimation capacity. A cost of cold acclimation was not detected in set of comparisons under our experimental conditions, and thus mutations that compromise cold acclimation capacity in southern accessions might be selective neutral. Interestingly, in our experiment, cold acclimation increased the fruit number of all categories of plants. The possible reason of this phenomenon needs to be further addressed.

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CHAPTER 2 - Clinal variation in freezing tolerance among natural accessions of *Arabidopsis thaliana*

Abstract

Low temperature represents a form of abiotic stress that varies predictably with latitude and altitude and to which organisms have evolved multiple physiological responses. Plants provide an especially useful experimental system for investigating the ecological and evolutionary dynamics of tolerance to low temperature because of their sessile life style and inability to escape ambient atmospheric conditions. We investigated intra-specific variation in freezing tolerance in *Arabidopsis thaliana* by conducting freezing tolerance assays on 71 accessions collected from across the species' native range. Assays were performed at multiple minimum temperatures and on both cold-acclimated and non cold-acclimated individuals. Considerable variation in freezing tolerance was observed among accessions both with and without a prior cold-acclimation treatment, suggesting that differences among accessions in cold-acclimation capacity as well as differences in intrinsic physiology contribute to variation in this phenotype. A highly significant positive relationship was observed between freezing tolerance and latitude of origin of accessions, consistent with a major role for natural selection in shaping variation in this phenotype. Clinal variation in freezing tolerance in *A. thaliana* coupled with considerable knowledge of the underlying genetics and physiology of this phenotype should allow for evolutionary genetic analysis at multiple levels.

Key words: local adaptation, freezing tolerance, cold acclimation, inducible response, ecological diversification, clines.

Introduction

Species with broad geographic ranges often exhibit considerable intra-specific variation in morphology, physiology, and development. This variation is often most pronounced along

latitudinal or altitudinal gradients where differences in climatic factors can result in strong natural selection for local adaptation and ecological specialization (Endler, 1977). Traits that exhibit such clinal patterns of variation represent excellent phenotypes for studies of adaptive evolution, especially when phenotypic differences among populations can be linked functionally to diverse environments and selection pressures (Endler, 1977). In instances where the molecular genetic or physiological underpinnings of the focal trait(s) is known, functional variation in phenotype can be investigated concurrently with molecular variation in candidate genes and/or variation in physiological response (Crawford & Powers, 1989; Crawford *et al.*, 1990; Crawford *et al.*, 1999; Johanson *et al.*, 2000; Maloof *et al.*, 2001; Caicedo *et al.*, 2004; Stinchcombe *et al.*, 2004; Balasubramanian *et al.*, 2006).

Freezing tolerance in plants is an ecologically relevant phenotype for which there are predictable patterns of variation across latitudes and climates (Xin & Browse, 2000). For plants occurring outside the tropics, maximum freezing tolerance is achieved following a period of acclimation to low but non-freezing temperatures during which numerous physiological and biochemical changes occur (Guy, 1990; Xin & Browse, 2000). These changes can have pronounced effects on freezing tolerance, enabling individuals to withstand temperatures several degrees colder than non cold-acclimated controls. Cold acclimation is thus an inducible response and likely evolved as a mechanism by which plants could prepare physiologically for colder and potentially more damaging temperatures. The evolution of such inducible responses is especially relevant for plants given their sessile lifestyle and inability to otherwise escape potentially harmful abiotic conditions.

Our understanding of the physiological, molecular and developmental mechanisms underlying plant freezing tolerance and the enhancement of freezing tolerance via cold-acclimation has improved significantly in recent years (Thomashow, 1999; Xin & Browse, 2000; Thomashow, 2001; Van Buskirk & Thomashow, 2006). These advances have been driven in large measure by studies in the model plant species *Arabidopsis thaliana*. It is now known, for example, that freezing tolerance is a highly complex trait influenced by multiple factors, including quantitative variation in abundance of particular metabolites (Cook *et al.*, 2004;

Kaplan *et al.*, 2004; Hannah *et al.*, 2006), increased production of antioxidants and abscisic acid (Chen *et al.*, 1983; Mantyla *et al.*, 1995; Okane *et al.*, 1996; Tao *et al.*, 1998; Iba, 2002), compositional changes in membrane lipid molecular species (Uemura *et al.*, 1995; Uemura & Steponkus, 1999; Li *et al.*, 2004; Welti & Wang, 2004; Li *et al.*, 2006), and whole organism responses such as reductions or delays in growth and reproduction (Levitt, 1980). Coupled with, and presumably underlying many of these changes are large-scale alterations in gene expression (Fowler & Thomashow, 2002; Hannah *et al.*, 2005; Vogel *et al.*, 2005), that begin within minutes following exposure of plants to cold but non-freezing temperatures.

While *A. thaliana* has served as an excellent experimental system in which to investigate many of the underlying genes, pathways, and physiological mechanisms involved in freezing tolerance and cold-acclimation, a systematic investigation of intra-specific variability in freezing tolerance along with its associated molecular basis and evolutionary dynamics has not been undertaken in this species, although interest in this area has spurred some recent investigation (Cook *et al.*, 2004; Alonso-Blanco *et al.*, 2005; Hannah *et al.*, 2006). The native geographic range of *A. thaliana* spans a broad spectrum of latitudes and climatic conditions (Koornneef *et al.*, 2004) where selection pressures for freezing tolerance are expected to be diverse. The broad geographic range of *A. thaliana* coupled with the availability through stock centers of accessions from across that range, provides an excellent opportunity to examine freezing tolerance in an ecological and evolutionary genetic context in this species. Towards this goal, we examine in this report natural phenotypic variation in freezing tolerance among 71 *A. thaliana* accessions collected originally from geographically diverse regions of the species' native range. We investigate freezing tolerance over a series of minimum temperatures and following both cold-acclimation and non cold-acclimation treatments. We document considerable intra-specific variation in freezing tolerance among accessions that is highly correlated with their latitude of origin. We discuss these patterns of variation in light of the genetic pathways and physiological mechanisms involved in plant freezing tolerance and highlight the suitability of this trait for studying the evolutionary genetics and physiology of an adaptive phenotype.

Materials and Methods

Arabidopsis thaliana *accessions and growing conditions*

Freezing tolerance assays were conducted on 71 accessions of *Arabidopsis thaliana* originally collected from diverse regions of the species' native range (Supplementary Table 2.1). Seeds of all accessions were obtained from The *Arabidopsis* Biological Resource Center (ABRC) at The Ohio State University. Prior to these experiments, plants of individual accessions were grown and allowed to self-fertilize in order to generate the necessary seed quantities. All plants were grown in a 23ºC growth room under short day conditions (10 hr light: 14 hr dark). Plants were grown in 54 cm × 27 cm rectangular flats with plastic inserts capable of accommodating 72 plants/flat. A complete set of 71 accessions could therefore be represented on a single flat and additional flats served as additional full replicates. Within each flat, assignments of individual plants to cell positions were randomized. All plants were grown in a mixture of 2 parts MetroMix 350 planting media (Sun Gro): 1 part sand and sub-irrigated with house distilled H_2O.

Cold-acclimation treatment and freezing tolerance assays

Plants were cold-acclimated in a 4ºC walk-in chamber for seven days where they experienced identical light and photoperiod conditions as in the 23ºC growth room. Cold-acclimated plants were allowed to grow for 23 days in the growth room prior to a seven-day cold-acclimation treatment, followed by freezing stress. Non cold-acclimated plants were allowed to grow for 23 days in the growth room prior to freezing stress. Because of greatly diminished growth of plants during cold-acclimation, this design resulted in cold-acclimated and non cold-acclimated plants experiencing freezing stress at similar stages of development (i.e., similar sized rosettes with approximately the same number of leaves).

Plants were subjected to freezing stress in an *ESPEC* ESU-3CA Platinous series environmental test chamber (Hudsonville MI, USA). Replicated sets of accessions were subjected to four minimum temperatures: (-6ºC, -8ºC, -10ºC, -12ºC) and (-2ºC, -4ºC, -6ºC, -8ºC), for cold-acclimated and non cold-acclimated treatments, respectively. These temperatures were

selected based on preliminary experiments exploring the full range of tolerances both with and without cold-acclimation. Freezing trials consisted of exposing 20 replicates of each accession (i.e., 20 flats) to the same minimum temperature for two consecutive nights, with the intervening day spent at 4ºC. During freezing trials, all plants experienced rates of temperature change of 2°C/hour during cooling and warming periods in order to mimic naturally encountered atmospheric cooling/warming rates and were subjected to minimum temperatures for a duration 2.5 hours. To facilitate ice nucleation and prevent super cooling of plant tissue during cooling periods, ice chips were added to flats when the chamber temperature reached -1°C. Because the 20 replicates of each accession (i.e., 20 flats) assayed at each temperature/acclimation combination exceeded the capacity of our environmental chamber, freezing trials were conducted in 4 groups (batches) of five flats. This design also enabled the estimation of an appropriate mean square (Batch nested within Temperature) over which to test the main effect of Temperature. In order to ensure that all plants experienced freezing stress after the same number of days post germination, planting dates for individual groups (batches) were staggered temporally. Overall, the full design of this experiment consisted of 71 accessions × 20 replicates × 2 acclimation treatments × 4 temperatures = 11,360 individuals.

Following the second consecutive night of freezing stress, plants were allowed to recover at 4ºC for 24 hours and then returned to the 23ºC growth room. After two weeks, plants were scored for above-ground (rosette) tissue damage using the following scale: 0, 100% tissue death; 1, >75% but <100% tissue death; 2, >50% but ≤75% tissue death; 3, >25% but ≤50% tissue death; and 4, ≤25% tissue death. This semi-quantitative measure of tissue damage enabled gradations of freezing tolerance to be assessed (as opposed to a binary 'alive' vs. 'dead').

Statistical analysis

All data were analyzed by mixed-model analysis of variance (ANOVA). Because cold-acclimated and non cold-acclimated accessions were assayed over different ranges of minimum temperatures, statistical analyses first were conducted separately on data within each acclimation treatment according to the model,

$$y = \mu + L + T + B(T) + L*T + L*B(T) + E, \quad (1)$$

where *L* represents accession (line) of *A. thaliana* (random effect), *T* represents temperature (fixed effect), *B*(*T*) represents replicate batch nested within temperature (see explanation above; random effect), *L*T* is the interaction between accession and temperature, *L*B*(*T*) is the interaction between accession and batch nested within temperature, and *E* represents residual error. Because of the partial overlap in temperatures at which cold-acclimated and non cold-acclimated plants were assayed (i.e., -6ºC and -8ºC), a second model was evaluated on freezing tolerance scores assessed inclusively at those temperatures,

$$y = \mu + L + T + B(T(A)) + A + L*T + L*B(T(A)) + L*A + T*A + L*T*A + E, \quad (2)$$

where *A* represents acclimation treatment (fixed effect) and all other variables are as described above. ANOVA models were evaluated using the Proc GLM procedure of SAS 9.1 (SAS Institute 1988).

Regression analyses of freezing tolerance scores with latitude of origin were conducted using the least square means of freezing tolerance scores measured at -10ºC for cold acclimated plants and -6ºC for non cold-acclimated plants (LS means derived from model 1 above). These data were selected for analysis because highest variance in freezing tolerance among accessions was observed at these temperatures (see Figure 2.1). All regression analyses were conducted using JMP IN® software (SAS Institute 2005). Climate data for the collection locations of accessions were obtained from the International Water Management Institute (IWMI) (http://www.iwmi.cgiar.org/WAtlas/AtlasQuery.htm) and the Integrated Database Information System (IDIS) [http://dw.iwmi.org/dataplatform/Home.aspx].

Results

Effects of temperature and cold-acclimation on freezing tolerance in A. thaliana

Cold-acclimated and non cold-acclimated plants exhibited similar patterns of decline in freezing tolerance with decreasing temperature, although the temperature range over which these declines were observed differed substantially between treatments, with cold-acclimated plants expectedly more tolerant at lower temperatures (Figure 2.1). Mixed model ANOVA conducted

separately on data derived from the cold-acclimated and non cold-acclimated treatments indicate highly significant effects of Line, Temperature, Batch nested within Temperature, and Line*Temperature ($P < 0.0001$; Tables 2.1 and 2.2). The Line*Batch(Temperature) interaction term was not significant in either analysis ($P = 0.9674$ and $P = 0.3574$, for the cold-acclimated and non cold-acclimated treatments, respectively).

The partial overlap of temperatures at which cold-acclimated and non cold-acclimated plants were assayed (i.e., -6ºC and -8ºC) enabled the evaluation of a statistical model examining the additional effect of Acclimation and its corresponding higher-level interaction terms (Table 2.3). Highly significant effects of Acclimation, Temperature*Acclimation, and Line*Temperature*Acclimation were observed ($P < 0.0001$; Table 2.3). The significant interaction effect of Temperature*Acclimation results from the fact that a transition from -6ºC to -8ºC had only minor effects on cold-acclimated plants but resulted in a steep decline in freezing tolerance scores for non cold-acclimated plants; this temperature transition defines the lower range of tolerance in the absence of a cold-acclimation treatment (Figure 2.1). The enhancement of freezing tolerance by cold-acclimation is especially evident for assays conducted at -8ºC. In the absence of a cold-acclimation treatment, most accessions exhibited high mortality at this temperature (mean freezing tolerance score = 0.074, SD = 0.111) whereas following cold-acclimation, mean freezing tolerance at this temperature was high (mean = 3.575, SD = 0.362). The highly significant three-way interaction of Line*Temperature*Acclimation suggests that accessions may have different acclimation capacities dependent upon temperature. To explore this possibility further, we examined Line*Acclimation interaction terms in statistical models evaluated separately at -6ºC and -8ºC. A significant Line*Acclimation interaction terms was detected at both temperatures (-6ºC: $F = 3.37$, $P < 0.0001$; -8ºC: $F = 1.49$, $P < 0.0103$). This result is consistent with a previous report of variation in cold-acclimation capacity among different accessions of *A. thaliana* (Hannah *et al.*, 2006).

Latitudinal cline in freezing tolerance

The highly significant effect of Line (accession) under both cold-acclimated and non cold-acclimated conditions (Tables 2.1 and 2.2), coupled with significant variation in acclimation

capacity among accessions indicates that attributes both related and unrelated to cold-acclimation contribute to differences in freezing tolerance among accessions. To investigate variation in freezing tolerance in light of the biogeographic origins of these accessions, we examined the relationship between freezing tolerance and latitude of origin under both cold-acclimated and non cold-acclimated conditions. A positive and highly significant linear relationship was observed between freezing tolerance and latitude of origin of accessions under cold-acclimated conditions (Figure 2.2) indicating the presence of a steep latitudinal cline in freezing tolerance in this species. Interestingly, a positive and significant relationship also was observed under non-cold-acclimated conditions (Figure 2.3), indicating that, in addition to differences in cold-acclimation capacity, these accessions differ physiologically for intrinsic factors influencing freezing tolerance. If individual freezing tolerance is measured as survivorship (i.e., number of individuals receiving non zero freezing tolerance scores divided by the total number of replicates), these regression analyses remain highly significant: cold-acclimated plants, $P < 0.0001$, $R^2 = 0.422$; non cold-acclimated plants, $P < 0.0001$, $R^2 = 0.338$. Plots of freezing tolerance versus latitude of origin for the remainder of temperatures at which plants were assayed are available as Supplementary material (Supplementary figures 2.1 and 2.2).

Because regression analyses were conducted at temperatures at which greatest variance among accessions was observed (i.e., -10ºC for cold-acclimated plants and -6ºC for non cold-acclimated plants), maximum freezing tolerance scores and cline steepness are not directly comparable between the two analyses. However, freezing tolerance scores under these two different treatments were highly correlated ($r = 0.774$, $P < 0.0001$), indicating that higher (lower) intrinsic tolerance is associated with higher (lower) tolerance following cold-acclimation.

To determine the extent to which latitude is a reasonable predictor of temperature across the geographic range of these accessions, we obtained data on mean monthly temperature (January and July) for the geographic coordinates of the 71 accessions and plotted these data against their latitude of origin (Supplementary figure 2.3). Both January and July mean temperatures demonstrate a negative and highly significant linear relationship with latitude

(mean January temperature, $P < 0.0001$, $R^2 = 0.690$; mean July temperature, $P < 0.0001$, $R^2 = 0.545$), lending further support to the observed latitudinal cline in freezing tolerance.

Discussion

Freezing temperatures represent a significant abiotic challenge to plants given their sessile lifestyle and inability to escape ambient atmospheric conditions. Many plant species are found over broad geographic ranges where selection pressures for freezing tolerance are expected to be diverse. We examined variation in freezing tolerance in a panel of *A. thaliana* accessions from different regions of the species' native range. Averaged across all accessions, maximum freezing tolerance decreased with decreasing temperature and, predictably, was enhanced following a period of acclimation to low but non-freezing temperature. Because a major aim of this study was to evaluate the degree of intra-specific variation in freezing tolerance in light of the biogeographic origins of populations of this species, accessions of *A. thaliana* were selected to be representative of a broad range of latitudes and geographic regions where selection pressures vary with respect to freezing stress. A highly significant linear relationship was observed between freezing tolerance and latitude of origin of the accessions, demonstrating the existence of a steep latitudinal cline in freezing tolerance. This cline was observed under both cold-acclimated and non cold-acclimated conditions. Significant Line*Acclimation interaction terms indicate that accessions differ in their cold-acclimation capacities; these differences clearly contribute to clinal variation in freezing tolerance under cold-acclimated conditions. The persistence of clinal variation in freezing tolerance under non cold-acclimation conditions, however, indicates that factors intrinsic to the un-acclimated physiologies of the 71 accessions also contribute to within-species variation in *A. thaliana*.

Latitudinal clines have been reported for other *A. thaliana* traits such as hypocotyl growth response (Maloof *et al.*, 2001; Stenoien *et al.*, 2002), length of the circadian period (Michael *et al.*, 2003), flowering time (Stinchcombe *et al.*, 2004), and sensitivity to vernalization (Stinchcombe *et al.*, 2005). These previously reported clines likely are the result of major environmental factors that vary with latitude such as light, temperature, and perhaps, precipitation (Stinchcombe *et al.*, 2004). In our own study, while latitude might be considered

only a crude predictor of temperature (consider seasonal climatic differences for coastal versus landlocked regions at the same latitude), a regression analysis of mean January and mean July temperatures on the latitude of the collection locations of accessions was highly significant (Supplementary figure 2.3), and thus likely explains the highly significant regression analyses observed in Figures 2.2 and 2.3. These results indicate a strong role for natural selection in shaping variation in freezing tolerance in *A. thaliana* and suggest that freezing tolerance may be an excellent candidate phenotype for evolutionary genetic and physiological analyses.

Genetic and physiological mechanisms of freezing tolerance variation

Given basic scientific interest in plant freezing tolerance and the obvious agricultural significance of this phenotype, the molecular and physiological mechanisms involved in plant freezing tolerance have been the subject of considerable investigation (Guy, 1990; Thomashow, 1999; Xin & Browse, 2000; Thomashow, 2001; Iba, 2002; Van Buskirk & Thomashow, 2006). While *A. thaliana* has been a focus of extensive work and progress in this area, examinations of natural variability in freezing tolerance in this species and its underlying genetic and physiological basis have been few in number. Recent studies, however, are providing a first glimpse into naturally occurring variability in this phenotype and indicate that intra-specific variation may be associated with differences of cold-induced metabolite production (Cook *et al.*, 2004; Hannah *et al.*, 2006), differences in global patterns of gene expression during cold-acclimation (Hannah *et al.*, 2006), and expression variation of key transcription factors in the cold-acclimation pathway (Cook *et al.*, 2004; Alonso-Blanco *et al.*, 2005).

The underlying molecular basis of freezing tolerance variation among accessions assayed in this study, though not addressed herein, is currently under investigation in our laboratory. Progress to date indicates that variation in freezing tolerance among these accessions is attributable to variation in multiple genes and/or pathways, including expression variation of members of the *CBF/DREB1* family of transcriptional activators (Zhen and Ungerer, unpublished data), genes which play a central role in the cold-acclimation pathway and which have been previously implicated in underlying natural variation among *A. thaliana* accessions (Alonso-Blanco *et al.*, 2005; Hannah *et al.*, 2006).

Non cold-acclimated variation in freezing tolerance

Because maximum freezing tolerance in most temperate plant species is achieved following a period of cold-acclimation, molecular and physiological studies of plant freezing tolerance have focused primarily on the genetic, metabolic and physiological changes that occur during the cold-acclimation period (Guy, 1990; Thomashow, 1999; Xin & Browse, 2000; Thomashow, 2001; Van Buskirk & Thomashow, 2006). While variation in cold-acclimation capacity clearly contributes to intra-specific variation in freezing tolerance in *A. thaliana* as demonstrated in this study and elsewhere (Hannah *et al.*, 2006), clinal variation in freezing tolerance also was observed in the absence of a cold-acclimation treatment, indicating that intrinsic biochemical and physiological factors also contribute to variation in this phenotype.

Clinal variation in freezing tolerance under non cold-acclimated conditions raises an interesting question regarding the extent to which non cold-acclimated and cold-acclimated freezing tolerance may share a common mechanistic basis. The molecular basis of non-cold-acclimated freezing tolerance is not well understood, with only a limited number of studies having addressed this subject (Stone *et al.*, 1993; Teutonico *et al.*, 1995; Hannah *et al.*, 2006). It has been reported that the underlying mechanisms of non cold-acclimated freezing tolerance and *cold-acclimation capacity* may differ (Stone *et al.*, 1993); this conclusion was based on a lack of phenotypic correlation between these traits in segregating inter-specific backcross populations of wild *Solanum* species. More recent studies in *Arabidopsis thaliana*, however, indicate that many of the same genes and metabolites exhibiting expression/abundance changes during cold-acclimation also exhibit variability among accessions under non cold-acclimated conditions (Hannah *et al.*, 2006). This would suggest that similar mechanisms might be involved in freezing tolerance under cold-acclimated and non cold-acclimated conditions in this species. Our own data based on 71 accessions of *A. thaliana* demonstrate a very strong correlation between non cold-acclimated and cold-acclimated freezing tolerance ($r = 0.774$, $P < 0.0001$) and thus also suggest the possibility of a considerable degree of shared mechanistic basis.

Costs of cold-acclimation and freezing tolerance?

Reduced freezing tolerance in accessions from milder climates coupled with their diminished acclimation capacity raises the question of whether there are costs associated with cold-acclimation in geographic regions that are unlikely to experience freezing stress. The costs of inducible responses of plants to stress have been the subject of considerable interest, although this subject is more commonly framed in terms of induced or acquired resistance to herbivores, herbicides, and/or pathogens (Bergelson & Purrington, 1996; Heil & Baldwin, 2002; Baucom & Mauricio, 2004). The cold-acclimation response is certain to be metabolically costly, with large numbers of genes up-regulated followed by substantial quantitative increases in several classes of metabolites (Cook *et al.*, 2004; Hannah *et al.*, 2005; Vogel *et al.*, 2005; Hannah *et al.*, 2006). In geographic regions that experience low but non-freezing temperatures, induction of the cold-acclimation pathway could be negatively selected in the absence of a subsequent freezing stress. In such regions, mutations that compromise the cold-acclimation pathway might thus be favored by natural selection.

The notion that a cold-acclimation response might have negative fitness consequences in the absence of freezing stress is supported by observations of transgenic *Arabidopsis* lines over-expressing members of the *CBF/DREB1* family of transcriptional activators. The *CBF/DREB1* genes have been described as "master switches" of the cold-acclimation pathway (Van Buskirk & Thomashow, 2006) because they are induced within minutes of placing plants at low temperature and regulate the expression of numerous downstream *COR* (cold-responsive) genes. Plants over-expressing individual members of this family tend to be diminutive in stature and have reduced reproductive output (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000), presumably because resources typically invested in growth and reproduction are diverted in order to sustain an upregulation of the *CBF/DREB1* -mediated cold-acclimation pathway. It should be noted, however, that costs associated with *CBF/DREB1* over-expression have not been observed universally (Jackson *et al.*, 2004). While analyses of over-expressing transgenic lines can in principle provide support for a cost of cold-acclimation, such a cost is likely to be exaggerated under a situation of constitutive over-expression and sustained upregulation of the cold-acclimation pathway. A more realistic assessment of the cost of cold-acclimation will require

analyses of natural accessions that exhibit a range of freezing tolerance capabilities and cold-acclimation capacities.

Conclusions

Surveys of freezing tolerance in 71 *A. thaliana* accessions demonstrate considerable differences among accessions and indicate clinal patterns of variation associated with latitude and temperature. These patterns are observed under both cold-acclimated and non cold-acclimated conditions, indicating the evolution of mechanisms associated both with an inducible response as well as intrinsic to the un-acclimated physiologies of plants. Given the emergence of *A. thaliana* as a model experimental system for studies of the underlying genetics and physiology of cold-acclimation and freezing tolerance in plants, many resources are currently available for detailed investigation of the molecular mechanisms underlying the phenotypic variation reported here.

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Figures and tables

Figure 2.1 Mean freezing tolerance at different minimum temperatures

Mean freezing tolerance scores for 71 *A. thaliana* accessions assayed at different minimum temperatures both with and without cold-acclimation treatment. Cold-acclimated and non cold-acclimated plants were assayed at different (but partially overlapping) sets of minimum temperatures. Each histogram bar represents the global average of 71 least square means. Error bars represent one SD.

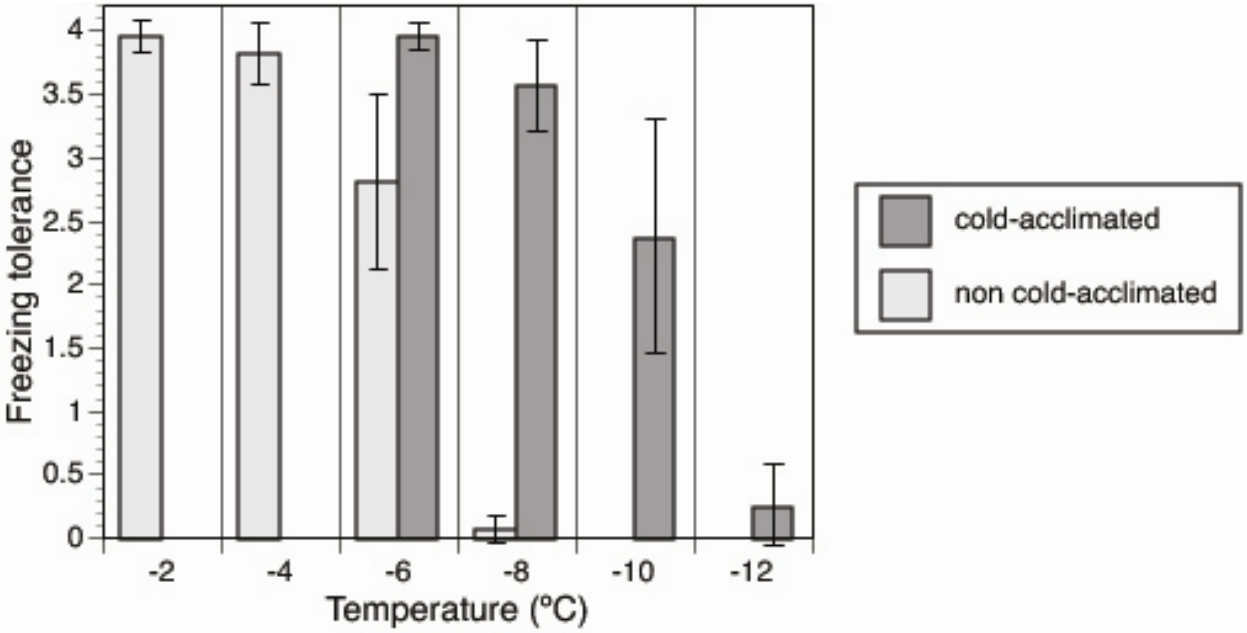


Figure 2.2 Cold-acclimated freezing tolerance against latitude of origin

Freezing tolerance scores plotted against the latitude of origin for 71 A. thaliana accessions. Data are for cold-acclimated plants assayed for freezing tolerance at –10°C. Plotted are least square means of 20 replicates/accession.

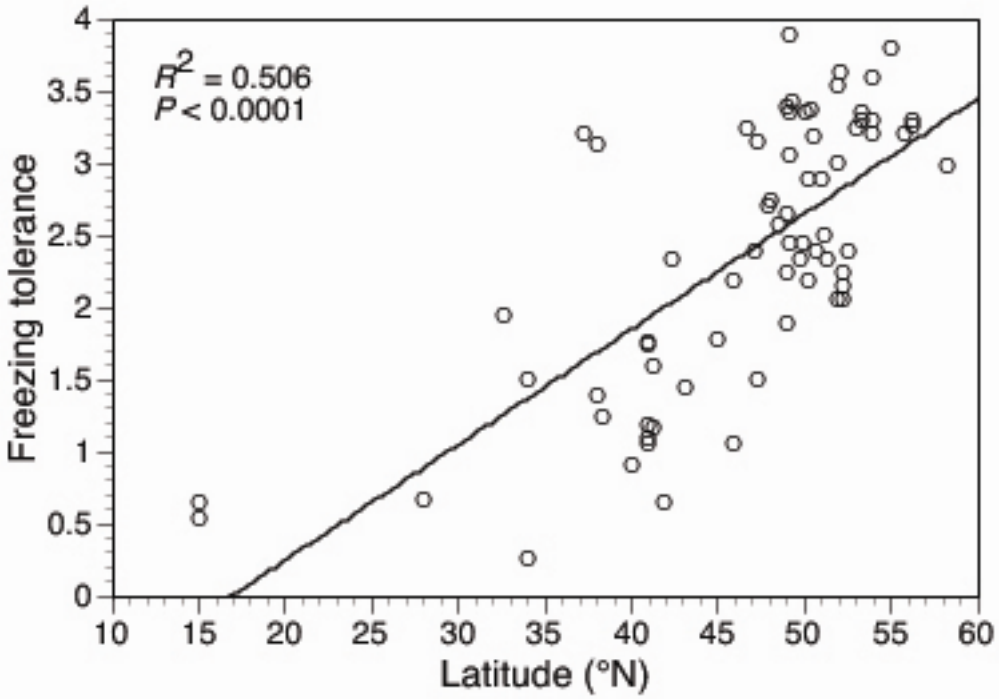


Figure 2.3 Non-cold-acclimated freezing tolerance against latitude of origin

Freezing tolerance scores plotted against the latitude of origin for 71 *A. thaliana* accessions. Data are for non cold-acclimated plants assayed for freezing tolerance at –6°C. Plotted are least square means of 20 replicates/accession.

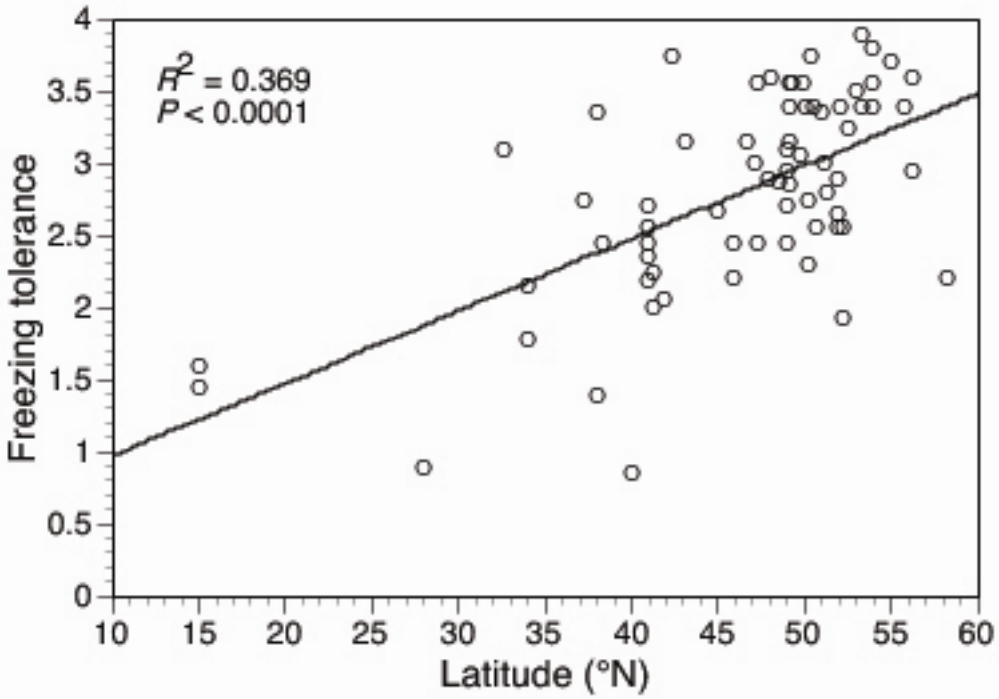


Table 2.1 Mixed-model ANOVA results for 71 cold-acclimated *A. thaliana* accessions assayed for freezing tolerance at –6°C, –8°C, –10°C, and –12°C

Source	df	SS	MS	*F*	*P*
Line	70	589.6012	8.4229	2.29	<0.0001
Temperature	3	10869	3622.8949	197.34	<0.0001
Batch(Temperature)	12	193.3579	16.1132	15.36	<0.0001
Line × Temperature	210	778.0933	3.7052	3.54	<0.0001
Line × Batch(Temperature)	835	873.4423	1.0460	0.90	0.9674
Error	4280	4949.9000	1.1565		

Twenty replicates per accession were measured at each temperature.

Presented are type III sums of squares. Parentheses indicate nested data structure.

Table 2.2 Mixed-model ANOVA results for 71 non cold-acclimated *A. thaliana* accessions assayed for freezing tolerance at –2°C, –4°C, –6°C, and –8°C

Source	DF	SS	MS	*F*	*P*
Line	70	304.2989	4.3471	2.08	<0.0001
Temperature	3	13204	4401.3619	358.96	<0.0001
Batch(Temperature)	12	132.5124	11.0427	14.20	<0.0001
Line × Temperature	207	434.7566	2.1003	2.70	<0.0001
Line × Batch(Temperature)	830	645.4477	0.7776	1.02	0.3574
Error	4384	3345.4833	0.7631		

Twenty replicates per accession were measured at each temperature.

Presented are type III sums of squares. Parentheses indicate nested data structure.

Table 2.3 Mixed-model ANOVA results for 71 *A. thaliana* accessions assayed for freezing tolerance at –6°C and –8°C both with and without a cold-acclimation treatment

Source	df	SS	MS	*F*	*P*
Line	70	299.3284	4.2761	2.76	0.0547
Temperature	1	3117.5160	3117.5160	265.78	<0.0001
Acclimation	1	7023.1860	7023.1860	569.33	<0.0001
Batch(Temperature(Acclimation))	12	136.5687	11.3807	11.13	<0.0001
Line × Temperature	70	134.8860	1.9269	0.65	0.9631
Line × Acclimation	70	180.8619	2.5837	0.87	0.7170
Line × Batch(Temperature(Acclimation))	832	849.8735	1.0215	0.97	0.7222
Temperature × Acclimation	1	1863.9225	1863.9225	141.86	<0.0001
Line × Temperature × Acclimation	69	206.0976	2.9869	2.92	<0.0001
Error	4376	4616.7167	1.0550		

Twenty replicates per accession were measured for each temperature/acclimation treatment.

Presented are type III sums of squares. Parentheses indicate nested data structure.

Supplementary materials

The following Supplementary Material is available for this article:

Supplementary Table 2.1 *Arabidopsis thaliana* accessions assayed for freezing tolerance

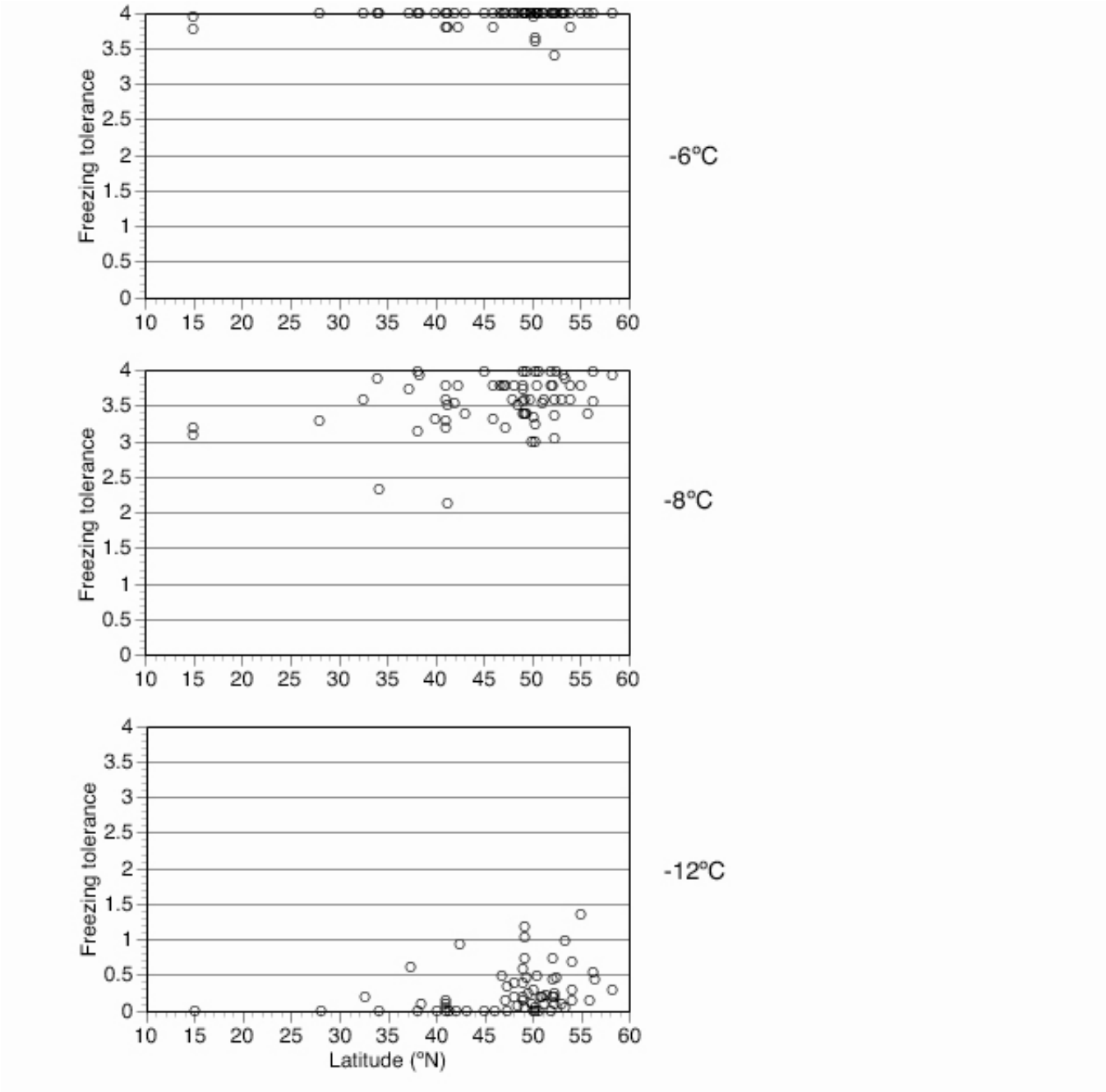
ABRC Stock #	Accession	Origin	Latitude	Longitude	Mean January temp. (°C)	Mean July temp. (°C)
CS910	Di-G	France	47.3	5.1	1.7	19.7
CS913	RLD-1	Russia	56.3	34.3	-10.7	17
CS924	Je54	Czechoslovakia	49.8	15.5	-3	16.6
CS1064	Can-0	Spain	28	-15	16.3	22.6
CS1072	Chi-0	Russia	54	34	-10	17.7
CS1084	Co-1	Portugal	40	-8	8.6	21.6
CS1244	Ita-0	Morocco	34.08	-4.2	6.5	24.1
CS1286	Kn-0	Lithuania	54	23	-5.4	17
CS1298	La-0	Germany	52	15	-1.9	18.8
CS1338	Ll-0	Spain	42	3	7.7	23.1
CS1364	Me-0	Germany	51.9	10.2	-0.7	16.1
CS1380	Mt-0	Libya	32.6	22.8	11.9	24.5
CS1516	Sf-2	Spain	41	3	7.7	23.1
CS1538	Stw-0	Russia	52	36	-9	18.8
CS1595	Wil-1	Russia	55	25	-6.1	17
CS1636	Nd-1	Germany	51	10	-0.7	17
CS6182	Wei-0	Switzerland	47.25	8.26	-0.6	17.4
CS6604	An-2	Belgium	51.2	4.4	2.5	17.8
CS6609	Bch-1	Germany	53.4	10.6	0.3	17
CS6626	Br-0	Czech Republic	49.2	16.6	-2.3	18.7
CS6659	Cal-0	United Kingdom	53.3	-1.6	2.8	15
CS6665	Chi-1	Russia	54	34	-10	17.7
CS6703	Fe-1	Germany	48	7.9	-1.6	15.9

CS6714	Ga-0	Germany	50.4	8	0	17.3
CS6720	Gie-0	Germany	50.6	8.7	-0.1	17.5
CS6751	Kas-2	India	34	74	1.6	19.8
CS6752	Ka-0	Austria	46.7	13.9	-3.1	16.8
CS6780	Lip-0	Poland	50.1	19.4	-3.1	17.7
CS6797	Ms-0	Russia	55.8	37.6	-9.5	18.3
CS6818	Ob-2	Germany	50	9	0	18.3
CS6825	Pa-1	Italy	38.1	13.4	10.6	24.8
CS6827	Pa-3	Italy	38.1	13.4	10.6	24.8
CS6832	Pi-0	Austria	47.1	10.9	-6.2	9.4
CS6834	Pla-0	Spain	41	2	9.3	23.2
CS6835	Pla-1	Spain	41	2	9.3	23.2
CS6839	Po-0	Germany	50.7	7.1	1	17.6
CS6855	Sf-1	Spain	41	3	7.7	23.1
CS6856	Sav-0	Czech Republic	49	15.4	-3	17.1
CS6864	Ste-0	Germany	53	12	-0.2	17.5
CS6867	Ta-0	Czech Republic	49.4	14.7	-2.5	17.3
CS6924	Ws-3	Russia	52.5	30	-7	18.3
CS8580	Cvi-1	Cape Verde Isl.	15	-23	21.6	24.7
CS22582	Spr1-2	Sweden	56.32	14.29	-1.6	15.9
CS22588	Zdr-1	Czech Republic	49.12	16.37	-2.6	18.3
CS22590	Bor-1	Czech Republic	49.12	16.37	-2.6	18.3
CS22592	Pu2-7	Croatia	42.38	18.07	4.3	22.9
CS22594	Lp2-2	Czech Republic	49.22	16.39	-2.6	18.3
CS22606	Kz-1	Kazakhstan	49.5	73.1	-14.7	21
CS22610	Ren-1	France	48.5	-1.41	4.9	17.8
CS22612	Uod-1	Austria	48.07	14.53	-3.1	16.8
CS22614	Cvi-0	Cape Verde Isl.	15	-23	21.6	24.7
CS22615	Lz-0	France	46	3.3	2.9	19
CS22616	Ei-2	Germany	50.3	6.3	-0.3	15.9
CS22626	An-1	Belgium	51.3	4.3	2.8	17.6

CS22629	Est-1	Estland	58.3	25.3	-6.4	16.5
CS22630	Ag-0	France	45	1.3	4.5	19.8
CS22631	Gy-0	France	49	2	3.6	18.5
CS22632	Ra-0	France	46	3.3	2.9	19
CS22633	Bay-0	Germany	49	11	-2	17
CS22635	Mrk-0	Germany	49	9.3	0.4	18.6
CS22636	Mz-0	Germany	50.3	8.3	-0.3	17.2
CS22637	Wt-5	Germany	52.3	9.3	0.1	16.7
CS22639	Ct-1	Italy	37.3	15	10	24.8
CS22643	Nok-3	Netherlands	52.3	4	3	16.6
CS22644	Wa-1	Poland	52.3	21	-4	18.4
CS22645	Fei-0	Portugal	41	-8	10.6	23.3
CS22646	Se-0	Spain	41.3	2.3	9.5	23.2
CS22647	Ts-1	Spain	41.3	3	7.5	22.7
CS22649	Pro-0	Spain	43.15	-6	1	15.3
CS22651	Kondara	Tajikistan	38.35	68.48	0.4	25
–	Hague	Netherlands	52.1	4.3	3	16.6

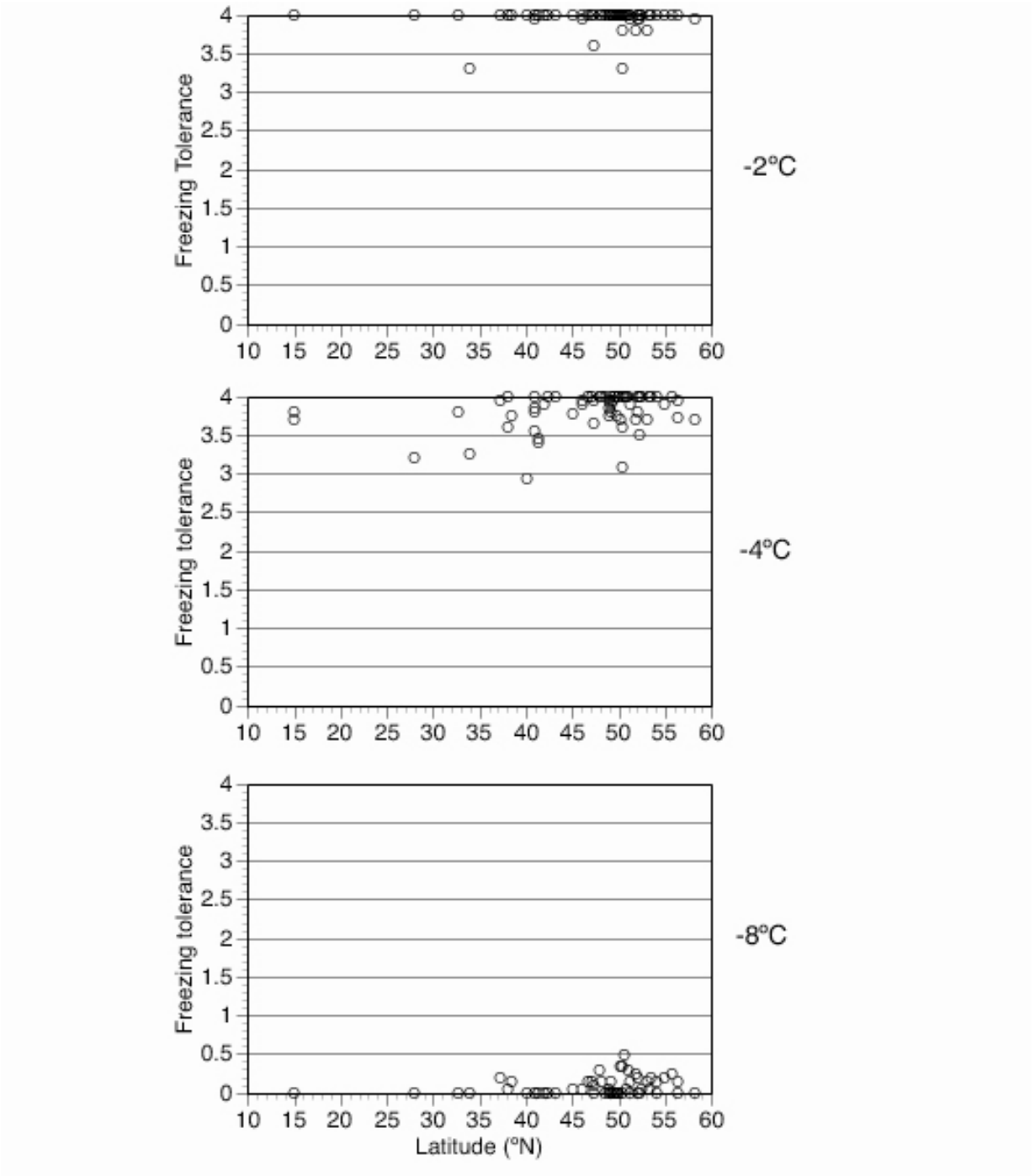
Supplementary Figure 2.1 Cold-acclimated freezing tolerance against latitude of origin

Freezing tolerance scores plotted against latitude of origin for 71 *A. thaliana* accessions under cold-acclimated conditions. Data are cold-acclimated plants assayed at -6, -8, and -12°C. Plotted are lease square means of 20 replicates per accession.



Supplementary Figure 2.2 Non cold-acclimated freezing tolerance against latitude of origin

Freezing tolerance scores plotted against latitude of origin for 71 *A. thaliana* accessions under non cold-acclimated conditions. Data are for non cold-acclimated plants assayed at -2, -4, and -8°C. Plotted are least square means of 20 replicates per accession.



CHAPTER 3 - Relaxed selection on the *CBF/DREB1* regulatory genes and reduced freezing tolerance in the southern range of *Arabidopsis thaliana*

Abstract

Elucidating the molecular basis of adaptive phenotypic variation represents a central aim in evolutionary biology. Traits exhibiting patterns of clinal variation represent excellent models for studies of molecular adaptation, especially when variation in phenotype can be linked to organismal fitness in different environments. Natural accessions of the model plant species *Arabidopsis thaliana* exhibit clinal variation in freezing tolerance that follows a gradient of temperature variability across the species' native range [Zhen & Ungerer, (2008) *New Phytol* 177: 419]. Here, we report that this pattern of variation is attributable, at least in part, to relaxed purifying selection on members of a small family of transcriptional activators (the *CBF/DREB1*s) in the species' southern range. These regulatory genes play a critical role in the ability of *A. thaliana* plants to undergo cold acclimation and thereby achieve maximum freezing tolerance. Relative to accessions from northern regions, accessions of *A. thaliana* from the southern part of their geographic range exhibit levels of nonsynonymous nucleotide polymorphism that are approximately 2.8-fold higher across this small gene subfamily. Relaxed selection on the *CBF/DREB1*s in southern accessions also has resulted in multiple mutations in regulatory regions resulting in abrogated expression of particular subfamily members in particular accessions. These coding-region and regulatory mutations compromise the ability of these genes to act as efficient transcriptional activators during the cold acclimation process, as determined by reductions in rates of induction and maximum levels of expression in the downstream genes they regulate. This study highlights the potential role of regulatory genes in underlying adaptive phenotypic variation in nature.

Introduction

Populations within species often exhibit adaptive phenotypic differences resulting from local selection pressures that vary across environments. Understanding the genetic basis of these adaptive differences requires linking variation in fitness-related phenotype to functional polymorphisms at individual genes (Nachman, Hoekstra, and D'Agostino 2003; Stinchcombe et al. 2004; Storz et al. 2007). An increasingly feasible approach for elucidating the genetic basis of molecular adaptation involves searching for functional variation in genes previously reported to control a given phenotype or physiological response of adaptive significance– a so called ecological or evolutionary 'candidate' gene. Because genes controlling variation in fitness are targets of natural selection, population-level analyses of DNA sequences can reveal the strength and/or type of selection that has acted (Nachman 2006). Population-level analyses then can be combined with functional genetic assays to determine the extent and geographic patterning of functional allelic diversity.

Investigating the genetic underpinnings of natural variation in freezing tolerance in the model plant species *Arabidopsis thaliana* provides an excellent opportunity to study how ecologically relevant and geographically structured phenotypic variation has been shaped by functional variation at specific loci. Low temperature represents a strong agent of natural selection in plants due to their sessile lifestyle and inability to escape ambient atmospheric conditions. Natural accessions of *A. thaliana* are distributed over a broad geographic range where selection pressures for tolerance to low temperature are diverse (Koornneef, Alonso-Blanco, and Vreugdenhil 2004). Previous work has documented a steep latitudinal cline in freezing tolerance in this species that is consistent with climatic variability across its native range (Hannah et al. 2006; Zhen and Ungerer 2008).

In *A. thaliana* and numerous other temperate plant species, maximum freezing tolerance is achieved following a period of cold-acclimation during which extensive biochemical and physiological changes take place (Thomashow 1999; Xin and Browse 2000; Smallwood and

Bowles 2002). While the mechanisms that underlie these changes are complex and involve many genes and multiple pathways, the *CBF/DREB1* subfamily of transcriptional activators plays a critical role in the cold-acclimation process and thus the ability of plants to achieve maximum freezing tolerance (Shinwari et al. 1998; Thomashow 1999; Thomashow 2001; Van Buskirk and Thomashow 2006). This subfamily consists of three members known alternatively as *CBF1*, *CBF2*, and *CBF3* or *DREB1b*, *DREB1c*, and *DREB1a*, respectively. The members of this subfamily are arrayed in tandem triplicate within a 8.7 kb region on chromosome four (Shinwari et al. 1998) and are thought to have largely redundant functions (Gilmour, Fowler, and Thomashow 2004).

The *CBF/DREB1* genes (hereafter referred to as *CBF*s) are induced within minutes of exposure of plants to cold temperatures and reach peak expression after approximately 2 hours (Gilmour et al. 1998; Shinwari et al. 1998; Cook et al. 2004). The transcription factors encoded by these genes are members of the AP2 family of DNA-binding proteins and regulate the expression of approximately 100 *co*ld-*r*esponsive (*COR*) genes that possess the C-repeat/dehydration responsive element (CRT/DRE) in their promoters (Van Buskirk and Thomashow 2006). Transgenic over-expression of individual *CBF* genes induces the cold acclimation pathway and results in enhanced freezing tolerance in the absence of a cold acclimation treatment (Gilmour, Fowler, and Thomashow 2004). The *CBF* genes thus provide excellent candidates for evolutionary genetic analyses in the context of geographically structured variation in freezing tolerance because they (*i*) play a pivotal role in the ability of *A. thaliana* plants to undergo cold-acclimation and thus achieve maximum freezing tolerance, (*ii*) are positioned early in a genetic network such that improper functioning would have numerous and undoubtedly detrimental downstream consequences, and (*iii*) have been implicated previously in underlying natural variation in freezing tolerance among different accessions of *A. thaliana* (Cook et al. 2004; Alonso-Blanco et al. 2005; Hannah et al. 2006).

In this report we examine patterns of nucleotide, expression, and functional variation of the *CBF* transcriptional activators in the context of geographically structured variation in freezing tolerance in *A. thaliana*. We show that relatively strong purifying selection on these

genes persists among accessions from northern regions of the species' range, but that these genes are undergoing relaxed purifying selection in the warmer, southern range of the species. Relaxed purifying selection in the southern range has resulted in multiple independent mutations in both regulatory and coding regions that compromise proper functioning of the *CBF* subfamily of genes in southern accessions.

Materials and Methods

Plant materials and freezing tolerance assays

Seeds of 24 *Arabidopsis thaliana* accessions (table 3.1) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at The Ohio State University. For phenotypic assays of freezing tolerance, plants were grown for 23 days before receiving a cold acclimation treatment of 7 d at 4°C. Following cold acclimation, plants (20 replicates/accession) were subjected to freezing stress for two consecutive nights at –10°C in an ESPEC ESU-3CA Platinous Series programmable environmental test chamber (Hudsonville, MI, USA). While in the chamber, plants were subjected to –10°C for a duration of 2.5 h and experienced a rate of temperature change of 2°C/hr during cooling and warming periods. To facilitate ice nucleation during periods of cooling, ice chips were added to flats when the chamber temperature reached –1°C. Following the second consecutive night of freezing stress, plants were transferred to a 4°C cold room for a duration of 24 h and then returned to the 23°C growth room for recovery. Survivorship of each accession (based on 20 replicates) was determined after 2 weeks recovery time. Additional details of plant growing conditions and freezing tolerance assays are described in Zhen and Ungerer (2008).

Isolation, sequencing and analysis of CBF *alleles*

Genomic DNA from individual accessions was isolated using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. Primers were designed with the program Primer3 (http://frodo.wi.mit.edu/) to amplify the coding region of each member of the *CBF* subfamily (supplementary table 3.1). In some instances, more than one pair of primers was

required to amplify each region from all accessions. PCR amplifications were conducted using GoTaq® Flexi DNA Polymerase (Promega) according to the manufactor's protocols, with a final concentration of 2.0 mM $MgCl_2$ and 0.5 μM of each primer. PCR conditions were optimized individually for each pair of primers. PCR products were purified and then sequenced (both forward and reverse reads) on an ABI 3730xl automated sequencer. When necessary, additional internal sequencing primers were designed and utilized in order to obtain full reads in both directions. Sequence polymorphisms were rechecked visually from chromatograms and confirmed by comparing forward and reverse reads of the same region. Sequences generated by this study are available from GenBank (accession numbers FJ169255-FJ169326).

Sequences were assembled using Vector NTI Advance 10 (Invitrogen Corporation) and aligned with ClustalW (Thompson, Higgins, and Gibson 1994). Phylogenetic analyses of aligned members of the *CBF* subfamily were conducted using the Neighbor Joining method (Saitou and Nei 1987) in PAUP* 4.0b10 (Swofford 2002) with the Kimura 2-Parameter model of sequence evolution. Branch support was determined with 1000 bootstrap replications. Nucleotide polymorphism, θ (Watterson 1975), at synonymous and nonsynonymous positions was determined using the software package DnaSP (Rozas and Rozas 1999).

Polymorphism at *CBF1-3* nonsynonymous and synonymous sites was determined in northern and southern accessions and compared to empirical data for 139 other *A. thaliana* genes in accessions from the same (or similar) geographic regions (Nordborg et al. 2005) [supplementary fig. 3.1A]. This set of 139 loci was selected based on criteria that these sequence reads (1) be distributed across all five *A. thaliana* chromosomes (supplementary table 3.2), (2) have uninterrupted ORFs [based on annotation information in Nordborg et al. (2005)], and (3) be spaced at distances of at least 0.2cM so as to avoid loci in linkage disequilibrium and thus with non-independent evolutionary histories (Ramos-Onsins et al. 2008). This set of 139 loci comprises approximately 70 kb of coding DNA. Comparison of *CBF1-3* polymorphism data to empirical distributions based on this large dataset allowed us to discriminate between patterns of polymorphism in northern and southern accessions attributable to selective versus non-selective evolutionary forces. Data were analyzed by 2 × 2 contingency analyses using Fisher's exact test

where, at both synonymous and nonsynonymous sites, the proportion of loci with θ values greater than and less than that for a concatenated *CBF1-3* sequence were compared between northern and southern accessions (supplementary fig. 3.1B).

Gene expression assays

Plants were allowed to grow for 23 days in a 23°C growth room (see above) prior to transfer to a cold room at 4°C. After different durations of cold acclimation (2 hr for *CBF* expression assays and 0, 24, and 48 hr for *COR6.6, COR15A,* and *COR78* expression assays), all above ground tissue was harvested and immediately flash frozen in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen) and purified with a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Purified RNA samples were treated with RQ1 RNase-Free DNase (Promega) and tested by PCR to confirm the absence of DNA contamination. RNA samples were reverse-transcribed with ImProm-II™ Reverse Transcriptase (Promega).

Gene expression assays were conducted by quantitative PCR on a Bio-Rad Real-Time PCR Detection System using the Bio-rad iQ™ SYBR Green Supermix kit. For assays of *CBF* expression, *CBF1*, *2*, and *3* specific primers were designed that amplify fragments in the range of 116-147 bp, with all reverse primers anchored in the 3' UTRs. The specificity of these primers was confirmed by (*i*) testing their efficacy via RT-PCR in individual (non cold-acclimated) *CBF1*, *2*, and *3* over-expressing *A. thaliana* transgenic lines kindly provided by the laboratory of Michael Thomashow, and (*ii*) confirming that quantitative PCR melt curves for the different primer pairs had single and unique peaks. For assays of *COR15A*, *COR6.6*, and *COR78* expression, primers were designed to amplify fragments in the range of 83-128 bp. All primer sequences used in quantitative PCR assays were designed with the program Primer3 (http://frodo.wi.mit.edu/) and are listed in supplementary table 3.2. Normalized expression was determined using the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPC,* Genbank accession # NM_111283) according to the equation, $NE = \frac{(E_{ref})^{CT_{ref}}}{(E_{GOI})^{CT_{GOI}}}$, (Muller et al. 2002), where E_{ref} is the PCR amplification efficiency of the reference gene, E_{GOI} is the PCR

amplification efficiency of the gene of interest, CT_{ref} is the cycle threshold of the reference gene, and CT_{GOI} is the cycle threshold of the gene of interest. Amplification conditions for quantitative PCR assays consisted of 94°C for 2 min, followed by 40 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1min. Three biological replicates and two technical replicates were assayed for each accession at each time-point, with the exception of data reported in supplementary figure 3.2, where only one biological replicate, but two technical replicates, were performed for each accession at each time point.

Results/Discussion

Geographic variation in freezing tolerance in A. thaliana

A steep latitudinal cline in freezing tolerance among natural accessions of this species has been documented previously (Hannah et al. 2006; Zhen and Ungerer 2008). We report here on 24 accessions of *A. thaliana* (table 3.1) that represent a subset of accessions examined in Zhen and Ungerer (2008) and that display a wide range of freezing tolerance capability. The 24 accessions examined herein were categorized as from southern or northern regions based on their latitudinal origins. Southern accessions were designated as those from latitudes at or below 42ºN (mean = 32.68 ºN , SD = 10.31) whereas northern accessions were designated as those from latitudes at or above 46.7ºN (mean = 50.75ºN, SD = 2.78) [table 3.1, supplementary fig. 3.1A]. The significant latitudinal break separating southern and northern accessions is paralleled by a complementary break in mean January and mean July temperature for the collection locations of accessions (table 3.1). Southern and northern accessions exhibit drastically different survivorship following exposure to freezing stress (fig. 3.1A and 3.1B), with mean survivorships of 0.323 (SE = 0.035) and 0.833 (SE = 0.035) for southern and northern accessions, respectively.

CBF *subfamily variation and divergence*

Sequences of the *CBF* genes were obtained for all 24 *A. thaliana* accessions under investigation. Phylogenetic analysis of aligned sequences revealed three major clades corresponding to the three *CBF* members (supplementary fig. 3.3). Bootstrap support was high

along branches defining the different subfamily members. Between 28 and 29 fixed nonsynonymous changes differentiate the members of this small subfamily. The *CBF* domains involved in DNA binding versus transcriptional activation have been characterized previously (Wang et al. 2005) and thus the locations of nonsynonymous mutations can be examined in the context of the structural organization of these genes. A large bias was observed in the numbers of nonsynonymous changes found in the transcriptional activation domains versus the DNA-binding domains. In the three different pairwise comparisons of *CBF* members, between 18 and 21 fixed nonsynonymous changes were observed among the transcriptional activation domains whereas only 2 or 3 fixed nonsynonymous changes were observed among the DNA-binding domains (all other nonsynonymous substitutions were outside these two domains). These patterns could indicate functional variation in activation although there is evidence that the transcriptional activation domain of one of these genes (*CBF1*) may be somewhat tolerant of amino acid substitutions (Wang et al. 2005). Within each of the three major *CBF* clades, there was only limited evidence of accessions grouping by their southern or northern latitudinal designations (supplementary fig. 3.3). A single 16 bp region of *CBF2* was identified as a potential gene conversion tract in one accession (Ita-0). This tract was excluded from all further analyses of nucleotide polymorphism.

Southern and northern accessions exhibit contrasting patterns of nucleotide polymorphism in the *CBF* subfamily. At nonsynonymous sites, nucleotide polymorphism is from 1.5 to 4.6-fold higher in southern accessions versus northern accessions in comparisons of the individual *CBF* genes (table 3.2). In an analysis of concatenated *CBF1-3* sequences, θ_{nonsyn} was 2.8-fold higher in southern versus northern accessions. All nonsynonymous substitutions found exclusively in southern accessions were present at low frequency (in 1-3 accessions only; fig. 3.2), indicating that these mutations arose and have persisted in local populations and thus are derived. Interestingly, nucleotide polymorphism at synonymous sites also is elevated in southern accessions, albeit to a lesser extent (from 1.7 to 2-fold) and for only two of the three *CBF* genes (table 3.2). In an analysis of concatenated *CBF1-3* sequences, θ_{syn} was 1.5-fold higher in southern versus northern accessions.

Nonsynonymous and synonymous CBF1-3 *polymorphism compared to empirical data from the* A. thaliana *genome*

Elevated nonsynonymous polymorphism in southern accessions suggests that purifying selection on these genes may be relaxed in the southern range of *A. thaliana* where plants experience warmer climates. Relaxed selection cannot explain elevated synonymous polymorphism in southern accessions, however, given that synonymous substitutions are not visible to natural selection. To evaluate these patterns of polymorphism in greater detail, we compared levels of *CBF1-3* nonsynonymous and synonymous polymorphism in northern and southern accessions to distributions of polymorphism obtained from a set of 139 loci from the *A.thaliana* nuclear genome (Nordborg et al. 2005)[fig. 3.3]. Sequence data for the 139 loci were obtained for twenty-three accessions with the same (or similar) latitudinal coordinates as those accessions examined in this study (supplementary fig. 3.1A). At nonsynonymous sites, polymorphism is significantly elevated in southern accessions in comparison to northern accessions (Fisher's exact test, $P = 0.0007$; supplementary figure 3.1B). At synonymous sites, however, there is no significant difference between southern and northern accessions (Fisher's exact test, $P = 0.6158$, supplementary figure 3.1B). These results indicate that this small gene family currently is undergoing relaxed purifying selection in the species' southern range and that patterns of nonsynonymous polymorphism cannot be attributed to aspects of population demography. Relaxed selection on the *CBF* genes in southern accessions is additionally supported by findings of a frameshift mutation in *CBF1* in an accession from southern Spain (Ll-0) (fig. 3.2) and mutations outside of coding regions in several additional southern accessions that are associated with abated expression of *CBF 1* and *2* (see below).

CBF *expression variation*

Relaxed selection on the *CBF* subfamily in the species' southern range also could result in mutations that compromise regulation. Such mutations would not be detectable in analyses of coding regions. To explore the possibility and frequency of mutations affecting regulation of the *CBF* subfamily, we conducted expression assays via quantitative PCR for each of the *CBF* members in our panel of 24 *A. thaliana* accessions. The *CBF* genes are induced within minutes of exposing *A. thaliana* plants to cold temperatures and reach peak expression after

approximately two hours of cold acclimation (Shinwari et al. 1998; Cook et al. 2004). Fig. 3.4 depicts normalized expression of the three *CBF* genes after two hours of exposure to 4°C as a function of their phenotypic freezing tolerance (see fig. 3.1). While considerable variation among accessions was revealed, four accessions exhibiting the lowest normalized expression (for *CBF1* and *CBF2*) also exhibited low survivorship in freezing tolerance assays and originate from southern locations of the species' range.

Mutations responsible for expression changes can be more difficult to identify than those altering protein function via amino acid substitutions because expression changes typically result from mutations outside of coding regions, such as in *cis*-acting regulatory regions or in genes encoding *trans*-acting DNA-binding factors. Previously, a 1.6 kb deletion of the *CBF2* promoter region was reported in an accession from the Cape Verde Islands (Cvi) (Alonso-Blanco et al. 2005). This deletion was confirmed in our own Cvi-0 and Cvi-1 samples. For the remaining two accessions exhibiting reduced expression, one (Co-1, from Portugal) possesses a 465 bp insertion in the *CBF1* promoter region that is 10 bp upstream of the transcriptional start site, and the second (Ita-0, from Morocco), possesses a 1.3 kb insertion in the 3' untranslated region (UTR) of *CBF2*. Thus, these four instances of abated expression are associated with indel mutations in *cis* regions with regulatory function.

Downstream consequences of mutations in the CBF *subfamily*

In order to explore the functional consequences of regulatory and coding region mutations in the *CBF* subfamily, we compared the ability of northern and southern accessions (via their *CBF* transcriptional activators) to induce three *co*ld-*r*esponsive genes (*COR15a*, *COR6.6*, and *COR78*). *COR* genes possess the CRT/DRE regulatory element in their promoters and are induced by the *CBF* transcription activators (Jaglo-Ottosen et al. 1998; Fowler and Thomashow 2002; Gilmour, Fowler, and Thomashow 2004). Time-course expression assays of three *COR* genes were conducted for three northern accessions and five southern accessions at three time points over a 48 hour period of cold acclimation at 4°C (fig. 3.5). The three northern accessions (blue lines in fig. 5) exhibited the highest induction rates and maximum expression levels for each of the three *COR* genes assayed. Southern accessions possessing regulatory

and/or non-synonymous mutations (green and red lines in fig. 3.5) exhibited quantitative reductions in both rates of induction and maximum levels of expression for each *COR* gene over the same period (fig. 3.5). Southern accessions possessing a combination of regulatory mutations and nonsynonymous mutations in the *CBF* genes (red lines in fig. 3.5) exhibited the lowest levels of *COR* gene induction, indicating a likely synergistic effect of multiple mutations on the ability of the products encoded by these genes to act as effective transcriptional activators.

The finding of quantitative reductions in *COR* gene expression for accessions possessing mutations in their *CBF* genes is consistent with previous studies documenting that *A. thaliana* accessions exhibiting reduced freezing tolerance (some of the same examined herein) also exhibit quantitative reductions in global gene expression patterns and metabolite changes during cold acclimation (Cook et al. 2004; Hannah et al. 2006). Moreover, the fact that combinations of mutations in the *CBF* genes reduce but do not abrogate *COR* gene expression is consistent with reports of functional redundancy in these genes (Gilmour, Fowler, and Thomashow 2004) as well as their potential ability to tolerate some degree of mutation (Wang et al. 2005).

Biogeographic patterns of selection

Arabidopsis thaliana is native to Europe and central Asia (Al-Shehbaz and O'Kane 2002; Koornneef, Alonso-Blanco, and Vreugdenhil 2004) with suggestions of the Caucasus as a potential ancestral area (Beck, Schmuths, and Schaal 2008). Thus, the species' wider current day distribution that includes Mediterranean regions and subtropical oceanic islands is a result of historical range expansion southward. (Several recent studies have examined genetic diversity in this species in a geographic context (Sharbel, Haubold, and Mitchell-Olds 2000; Nordborg et al. 2005; Bakker et al. 2006; Schmid et al. 2006; Beck, Schmuths, and Schaal 2008)). Our data suggest that, following initial range expansion into warmer climates, relaxed purifying selection on the *CBF* subfamily resulted in multiple mutations that arose independently in both regulatory and coding regions, and that these mutations persisted in local populations. These mutations have resulted in diminished freezing tolerance among populations in southern regions of the species' range. This relaxed selection is likely to have occurred in recent evolutionary time, as evidenced

by nonsynonymous polymorphism that is elevated in southern accessions compared to northern accessions, but still lower than synonymous polymorphism levels in southern accessions.

Whether mutations compromising *CBF* function were selectively neutral or selectively beneficial as populations colonized warmer climates remains to be determined. The cold acclimation pathway is certain to be metabolically costly as it involves global changes in gene expression patterns, metabolite profiles, and major changes in plant growth and physiology (Cook et al. 2004; Hannah et al. 2006). In climatic regions where plants might experience low temperatures but would be unlikely to experience freezing stress, mutations compromising the cold-acclimation pathway might be favored by natural selection because resources normally involved in the cold-acclimation process could be channeled more efficiently towards growth and reproductive output. Determining whether there is such a cost of cold-acclimation and freezing tolerance can be addressed in the laboratory and experiments testing these ideas are currently underway.

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Figures and Tables

Figure 3.1 Survivorship against latitude of origin and January mean temperature

Survivorship plotted against latitude of origin (A), and January mean temperature (B), for 24 *Arabidopsis thaliana* accessions subjected to -10°C for two consecutive nights. All plants were first cold-acclimated for 7 days at 4°C. Data are based on 20 replicates per accession. In panel A, two accessions overlap entirely (i.e., latitude = 41°N, survivorship = 0.316).

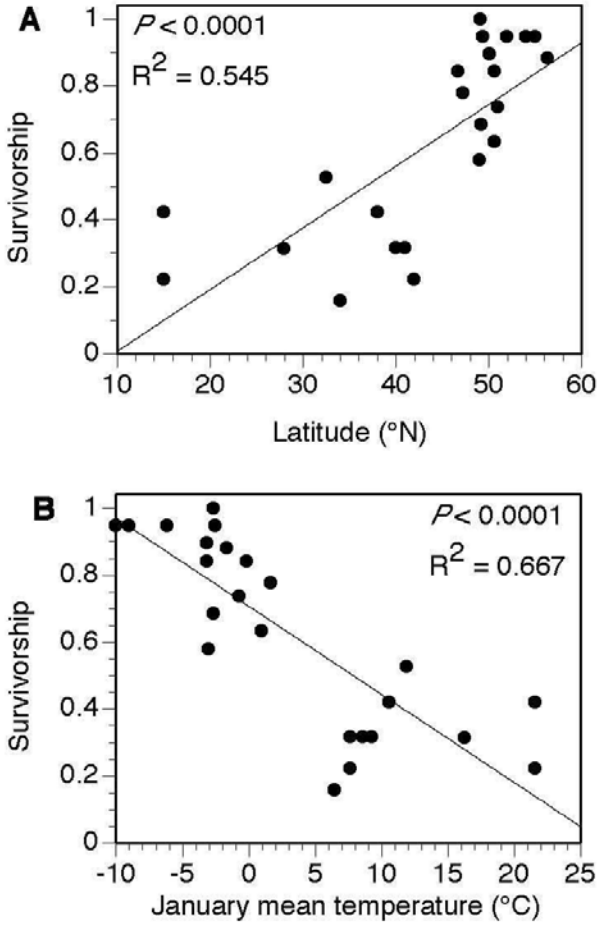


Figure 3.2 Sequence variation of *CBF1-3* in 24 accessions of *A. thaliana*

Sequences for the accession Po-0, from Germany, are given as a reference. The locations of polymorphic positions are given at the top for each gene. Nucleotide polymorphisms are indicated by black (synonymous) and red (nonsynonymous) letters; periods indicate identity to the reference allele and dashes indicate single base pair deletions. Accessions with northern and southern designations are indicated by blue and red, respectively. The box encompassing positions 540, 554, and 555 in *CBF2* indicates a potential gene conversion tract in accession Ita-0. The *CBF* transcriptional activators lack introns.

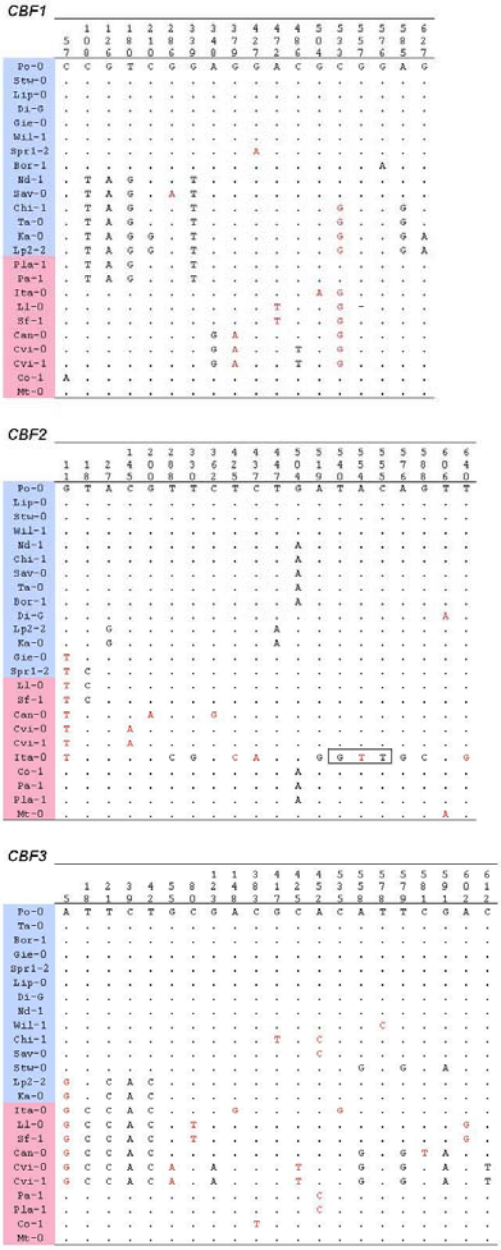


Figure 3.3 Distribution of polymorphism at synonymous and nonsynonymous sites in the *A. thaliana* genome

Distribution of polymorphism at synonymous (A) and nonsynonymous (B) sites for 139 loci in the *A. thaliana* genome (supplementary table 3.2). Orange and purple bars indicate polymorphism levels for southern and northern accessions, respectively (see supplementary fig3.1A). Polymorphism values for *CBF1-3* concatenated sequences in northern and southern accessions are indicated by dotted lines.

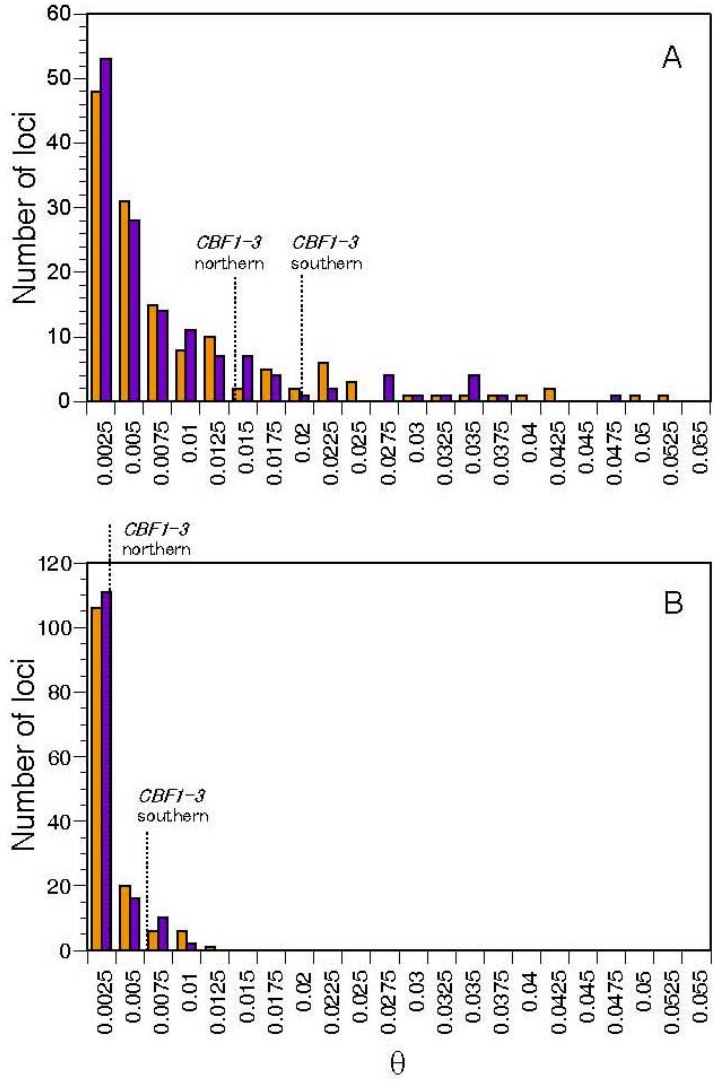


Figure 3.4 *CBF1-3* expression in 24 accessions of *A. thaliana*

Normalized *CBF1-3* expression in 24 accessions of *A. thaliana* following 2 hours of cold acclimation at 4°C. Normalized expression is plotted as a function of survivorship following two nights of freezing stress (see Methods and fig. 3.1). Vertical lines separate accessions with southern designations (left) and northern designations (right). Normalization scores are in reference to the housekeeping gene *GAPC* (see Methods). Four accessions with abrogated expression (for *CBF1* and *2*) are indicated. Error bars indicate one SE.

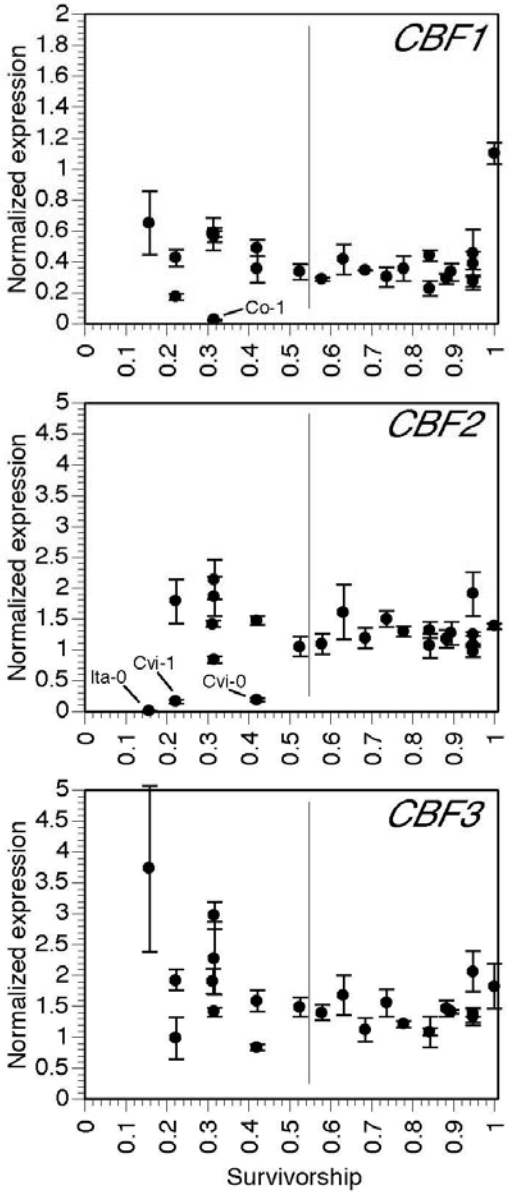


Figure 3.5 Expression of the three *COR* genes in 8 accessions of *A. thaliana*

Normalized expression of three cold-responsive (*COR*) genes in 8 accessions of *A. thaliana* following 0, 24, and 48 hours of cold acclimation at 4°C. Blue lines indicate northern accessions with normal levels of *CBF* expression; green lines indicate southern accessions with with multiple nonsynonymous/frameshift mutations in one or more of the *CBF* members; red lines indicate southern accessions with nonsynonymous mutations in one or more of the *CBF* members as well as with mutations in regulatory regions. Normalization scores are in reference to the housekeeping gene *GAPC* (see methods). Error bars indicate one SE.

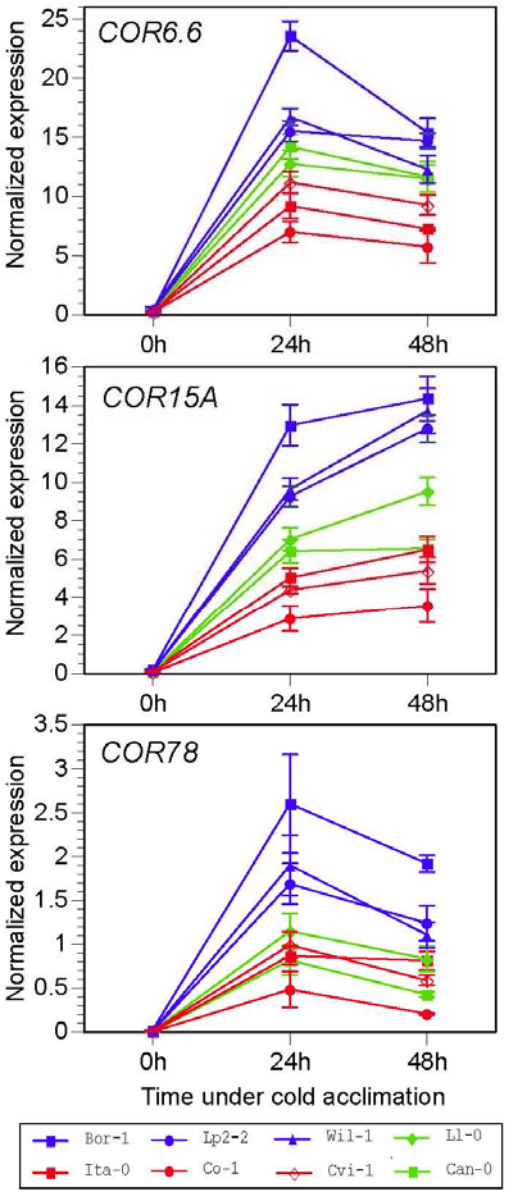


Table 3.1 *Arabidopsis thaliana* accessions examined in this study

ABRC Stock #	Accession	Origin	Latitude	Longitude	Mean January temp. (°C)	Mean July temp. (°C)
CS8580	Cvi-1	Cape Verde Isl.	15	-23	21.6	24.7
CS22614	Cvi-0	Cape Verde Isl.	15	-23	21.6	24.7
CS1064	Can-0	Spain	28	-15	16.3	22.6
CS1380	Mt-0	Libya	32.6	22.8	11.9	24.5
CS1244	Ita-0	Morocco	34.08	-4.2	6.5	24.1
CS6825	Pa-1	Italy	38.1	13.4	10.6	24.8
CS1084	Co-1	Portugal	40	-8	8.6	21.6
CS6835	Pla-1	Spain	41	2	9.3	23.2
CS6855	Sf-1	Spain	41	3	7.7	23.1
CS1338	Ll-0	Spain	42	3	7.7	23.1
CS6752	Ka-0	Austria	46.7	13.9	-3.1	16.8
CS910	Di-G	France	47.3	5.1	1.7	19.7
CS6856	Sav-0	Czech Republic	49	15.4	-3	17.1
CS22590	Bor-1	Czech Republic	49.12	16.37	-2.6	18.3
CS22594	Lp2-2	Czech Republic	49.22	16.39	-2.6	18.3
CS6867	Ta-0	Czech Republic	49.4	14.7	-2.5	17.3
CS6780	Lip-0	Poland	50.1	19.4	-3.1	17.7
CS6720	Gie-0	Germany	50.6	8.7	-0.1	17.5
CS6839	Po-0	Germany	50.7	7.1	1	17.6
CS1636	Nd-1	Germany	51	10	-0.7	17
CS1538	Stw-0	Russia	52	36	-9	18.8
CS6665	Chi-1	Russia	54	34	-10	17.7
CS1595	Wil-1	Russia	55	25	-6.1	17
CS22582	Spr1-2	Sweden	56.32	14.29	-1.6	15.9

Table 3.2 Polymorphism within the *CBF* transcriptional activators in northern and southern accessions of *A. thaliana*.

gene	Length (bp)	n	$\theta_{synonymous}$	$\theta_{nonsynonymous}$
CBF1-3 northern	1938	14	0.01308	0.00188
CBF1-3 southern	1938	10	0.01898	0.00523
CBF1 northern	642	14	0.01730	0.00191
CBF1 southern	642	10	0.01714	0.00288
CBF2 northern	651	14	0.00880	0.00125
CBF2 southern	651	10	0.01774	0.00577
CBF3 northern	651	14	0.01306	0.00250
CBF3 southern	651	10	0.02200	0.00702

Accessions collected at or below 42°N are designated as southern accessions whereas those collected at or above 46.7°N are designated as northern accessions (see table 3.1).

CBF1-3 indicates a concatenated sequence including all three genes, with stop codons of two genes removed.

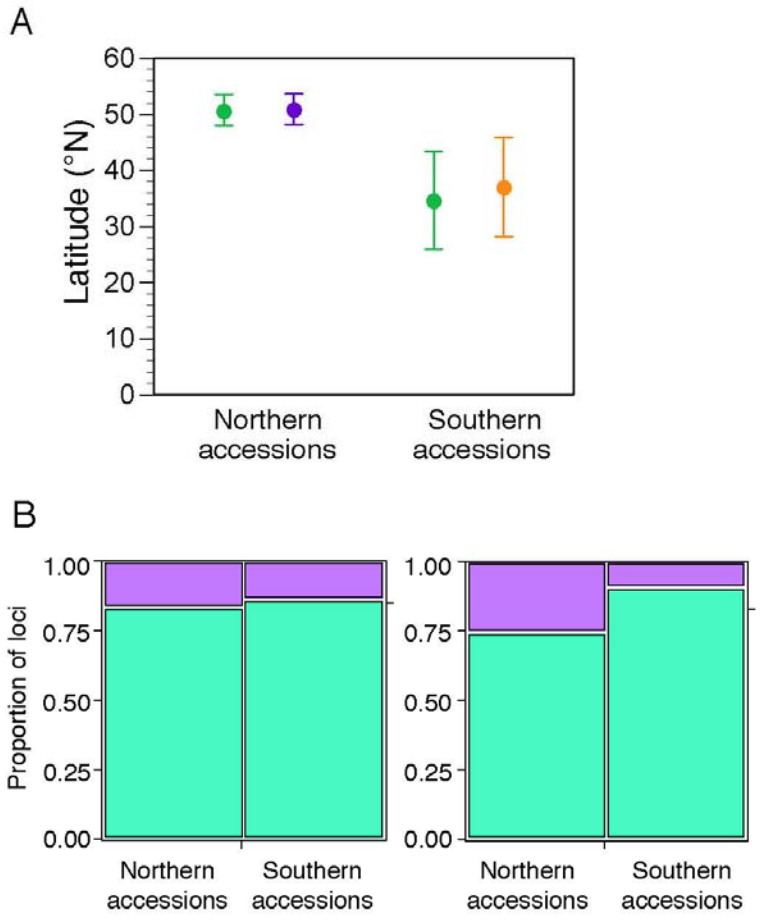
θ, Watterson's theta (Watterson,1975)

Supplementary materials

The following Supplementary Material is available for this article:

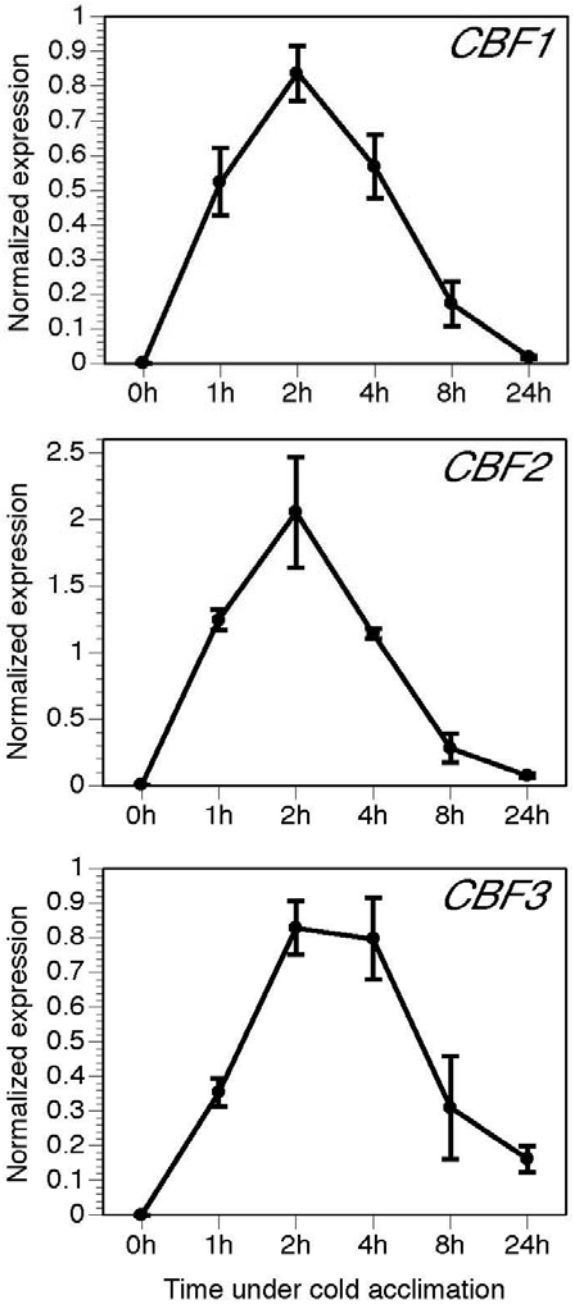
Supplementary Figure 3.1 Mean latitudes of natural accessions and mosaic plots

(A) Mean latitude (±SD) for northern and southern *A. thaliana* accessions examined in this study (green) and mean latitude (±SD) for northern (purple) and southern (orange) *A. thaliana* accessions used to generate empirical distributions of polymorphism based on 139 loci from the *A. thaliana* genome (data from Nordborg et al. 2005). Southern accessions from Nordborg et al. include: Cvi-0, Mt-0, Ll-0, Pu2-7, Ts-1, Ct-1, C24, Se-0, and Ts-5. Northern accessions from Nordborg et al. include: Bor-1, Lp2-2, Spr1-2, Nd-1, Uod-1, Wei-0, Br-0, Zdr-6, Wa-1, Gu-0, Ler-1, Ws-2, and Ms-0. (B) Mosaic plots indicating the proportion of 139 loci with polymorphism (θ) values less than (green) and greater than (purple) that estimated for a concatenated *CBF1-3* sequence. Left panel, synonymous sites; right panel, nonsynonymous sites.



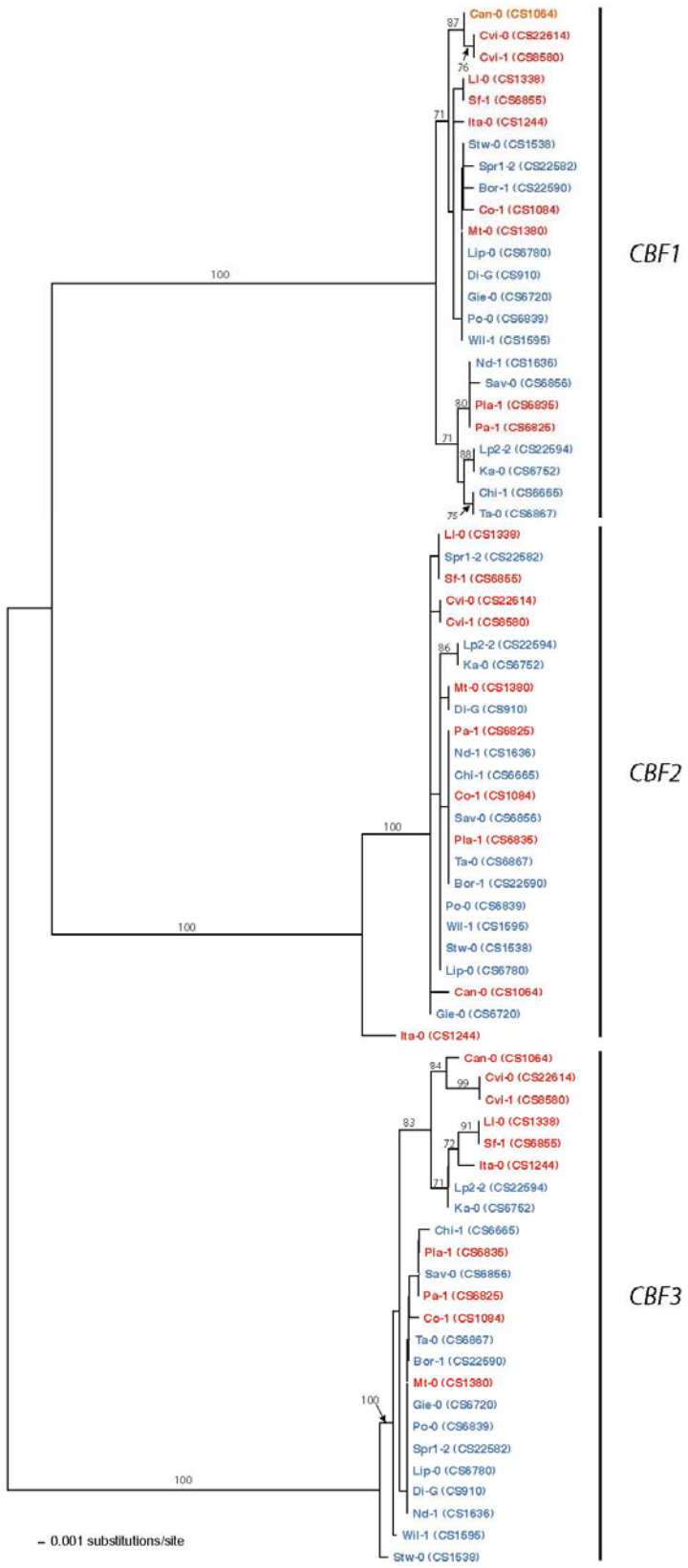
Supplementary Figure 3.2 Time-course expression of *CBF* genes

Time-course expression of *CBF1*, *2*, and *3* following transfer of plants from 23°C to 4°C. Data depict mean normalized expression for three *A. thaliana* accessions (Wil-1, Chi-1, and Ta-0) that exhibit high freezing tolerance (Zhen and Ungerer, 2008). Expression was normalized to *ACTIN 8* (NM_103814) as described in the Methods. Error bars depict one SE.



Supplementary Figure 3.3 NJ tree of *CBF* genes

Midpoint-rooted Neighbor-Joining tree of aligned sequences of the *CBF* subfamily of transcriptional activators. Sequences were obtained for 24 accessions of *A. thaliana* (Table 3.1). Accessions with southern and northern designations are indicated with red and blue colors, respectively. Numbers associated with branches indicate bootstrap support where greater than 70%.


Can-0 (CS1064)
Cvi-0 (CS22614)
Cvi-1 (CS8580)
LI-0 (CS1338)
Sf-1 (CS6855)
Ita-0 (CS1244)
Stw-0 (CS1538)
Spr1-2 (CS22582)
Bor-1 (CS22590)
Co-1 (CS1084)
Mt-0 (CS1380)
Lip-0 (CS6780)
Di-G (CS910)
Gie-0 (CS6720)
Po-0 (CS6839)
Wil-1 (CS1595)
Nd-1 (CS1636)
Sav-0 (CS6856)
Pla-1 (CS6835)
Pa-1 (CS6825)
Lp2-2 (CS22594)
Ka-0 (CS6752)
Chi-1 (CS6665)
Ta-0 (CS6867)
CBF1
CBF2
CBF3
100
0.001 substitutions/site

Supplementary Table 3.1 Primers used

Primers used for sequencing			
Gene	Forward primer(s) (5'→3')	Reverse primer (5'→3')	Additional sequencing primers (5'→3')
CBF1	TAACCGTCCATCGAAATTGA	CAAAAATGGAAACGACTATCGAATA	CTCCGATTACGAGCCTCAAG TCAGAAAAAAGCTGAAAAATGAGTTC
CBF2	TCTCATAAACCTTATCCAGTTTCTTG	TGCACTCAAAAACATTTGCAT	
CBF3	CGTCTTCGCCTTTTCTTTTG TTTCCGCCAAAACTACTTGG	GGAAGCTACGGACAGTGCTC	GACATGGAGGAGACGTTGGT GATCCGTCGTCGCATCACAC

Primers used for assays of gene expression via quantitative PCR			
Gene	Forward primer (5'→3')	Reverse primer (5'→3')	
CBF1	TGAAGGCATGCTTTTACCG	CAAAAATGGAAACGACTATCGAATA	
CBF2	CGATTTTTATTTCCATTTTTGGTAT	CAAAAACATTTGCATTTGACA	
CBF3	ACGTATCGTTATGGAGTTATTAAAAAC	CAATTTAATTTACACTCGTTTCTCAG	
COR15A	AGAGTCGGCCAGAAAACTCA	ATGTTGCCGTCACCTTTAGC	
COR6.6	CTGGCAAAGCTGAGGAGAAG	TGTTCAGGCCGGTCTTGT	
COR78	GAGGAGCCAAAACAGAGCAC	CCGCCACTTGAGTTTGATCT	
GAPC	ATGTCTTTCCGTGTCCCAACC	GATTCCCTTGAGTTTGCCTTC	

Supplementary Table 3.2 Loci used to generate empirical distribution of polymorphism

Loci (from Nordborg et al., 2005) used to generate empirical distributions of polymorphism. Map positions (in cM) obtained from Ramos-Onsins et al. (2008)

Chrom1	Position	cM	Chrom2	Position	cM	Chrom3	Position	cM	Chrom4	Position	cM	Chrom5	Position	cM
1	112445	2.60257	2	172277	0.168734	3	444578	5.22639	4	747636	10.25781	5	342177	2.708069
1	394740	3.069406	2	1149580	14.01915	3	690939	6.049992	4	2510259	22.1995	5	476340	4.339345
1	592464	3.431714	2	1610686	15.23294	3	2485139	12.14963	4	7077771	41.1208	5	657920	6.434382
1	3645216	12.28754	2	8041692	34.74313	3	2967832	13.83667	4	8177364	49.56293	5	872097	8.74543
1	3797953	12.87105	2	8212721	35.51959	3	3242782	14.80935	4	8788890	53.43133	5	947444	9.51877
1	4175290	14.36117	2	8370574	36.24193	3	3991563	17.50789	4	9877844	59.18273	5	1445887	14.14775
1	4631921	16.25224	2	8799503	38.23006	3	4355883	18.85026	4	10482038	61.89371	5	2084671	18.97366
1	6832887	26.49737	2	8957827	38.97245	3	6036261	25.34486	4	11984495	67.78493	5	4515967	29.91357
1	7095889	27.82351	2	9360787	40.88008	3	6810901	28.53668	4	12279689	68.87991	5	5103425	31.75163
1	7590430	30.36242	2	9653034	42.2778	3	7917372	33.35399	4	12978424	71.49888	5	5198066	32.04816
1	7735392	31.11681	2	10400180	45.8922	3	9747158	42.099	4	13680078	74.27179	5	5725918	33.763
1	8077027	32.91092	2	11691317	52.20444	3	10055557	43.6805				5	6523001	36.77012
1	8485357	35.08182	2	12296677	55.15682	3	11190073	49.79556				5	7543818	41.99915
1	8612871	35.76493	2	12955478	58.33935	3	15526753	55.15048				5	7742135	43.26664
1	9496666	40.55172	2	13326009	60.10795	3	16676839	60.96259				5	8791781	51.77804
1	9615268	41.19937	2	15645793	70.58279	3	17478329	64.65746				5	13614231	72.96469
1	9709142	41.71261	2	15801112	71.23431	3	17882280	66.42892				5	15964782	84.86562
1	10719833	47.25609	2	15936957	71.79778	3	18449959	68.83255				5	18258413	95.8867
1	11465886	51.33904	2	16080080	72.38482	3	18779784	70.1897				5	18666458	97.48831
1	11553447	51.81608	2	16352229	73.48162	3	18866920	70.54404				5	19917554	101.9684
1	11655171	52.36949	2	16437369	73.81932	3	18980171	71.00215				5	20128253	102.6763
1	14162569	61.62914	2	17050977	76.17178	3	19282780	72.2139				5	20723166	104.6319
1	17879944	69.69996	2	17375325	77.35383	3	19429153	72.79424				5	21038817	105.6533
1	18040681	70.33347	2	17859576	79.03295	3	20233050	75.93079				5	21115349	105.9001
1	18433043	71.88939	2	18304029	80.4776	3	20428108	76.68279				5	21318883	106.5561
1	20738912	81.3238	2	18652671	81.542	3	20728533	77.83743				5	21517341	107.1961
1	21140811	83.02226	2	19104081	82.82493	3	20927355	78.6004				5	22714241	111.145
1	25094209	100.6982				3	21528272	80.90987				5	23614758	114.3464
1	25314068	101.7369				3	21815226	82.01937				5	24316680	117.0731
1	27495891	112.396										5	24996339	119.9707
1	27734407	113.6013										5	25301036	121.3671
1	27959121	114.7445										5	25819581	123.9011
1	28034216	115.1282										5	26220285	126.0089
1	28190123	115.9273												
1	28302177	116.5039												
1	28774664	118.9556												
1	28897502	119.5984												
1	29393096	122.2154												
1	29496328	122.7652												

CHAPTER 4 - Fitness benefits and costs of cold acclimation in *Arabidopsis thaliana*

Abstract

In the face of limited resources, there is a tradeoff between growth/reproduction and stress defense in plants. Most temperate plant species, including *Arabidopsis thaliana*, could enhance their freezing tolerance through cold acclimation, a process that the plants are pre-subjected to low but non-freezing temperatures. Cold acclimation involves many genes and pathways, and CBF transcriptional activators play an important role. Induction of cold acclimation to acquire maximum freezing tolerance should be beneficial in environments where plants frequently encounter freezing stress, while induction of cold acclimation could be a waste of energy and resources in absence of freezing stress. This study utilizes naturally collected accessions of *A. thaliana* possessing contrasting abilities of cold acclimation, as well as transgenic lines with *CBF* gene over-expression or knockdown/knockout, to examine the fitness benefits and costs of cold acclimation in freezing and freezing-free environments. Benefit of cold acclimation is evident in presence of freezing, but cost of cold acclimation isn't detected in absence of freezing under our experimental conditions. Previous studies revealed relaxed purifying selection on *CBF* genes in accessions originated from warmer climate, and identified mutations in both regulatory and coding region. Our result from current study suggests that these mutations are neutral to natural selection.

Introduction

Phenotypes conveying tolerance to and/or defense against environmental stress can be inducibly or constitutively expressed. Inducible phenotypes are only expressed, or expressed to a higher degree, in response to specific cues indicating that a defense is needed. The time lag associated with the full development of an inducible defense compromises a faster or more immediate defense. The most common explanation for the evolution of inducible defenses is that

a constitutive defense is too costly to maintain in the absence of an environmental stress because it imposes an allocation cost (Strauss et al. 2002; Walters and Heil 2007), whereby energy and resources allocated to stress defense cannot be used for growth or reproduction. Such a cost should be evident if the defense phenotype is expressed in the absence of the stress.

Cold acclimation represents a well-characterized plant inducible defense against freezing temperatures. Low temperature exhibits a major environmental challenge that limits plant growth, productivity and geographic distribution. Most temperate plant species, including the model species *Arabidopsis thaliana*, can significantly increase their freezing tolerance via cold acclimation, a process whereby a plant is pre-subjected to low but non-freezing temperature (Gilmour et al. 1988; Xin and Browse 2000). Cold acclimation involves extensive physiological and biochemical changes that could be metabolically costly, including distinct changes in membrane lipid composition (Welti et al. 2002), global gene expression patterns and intracellular accumulation of compatible osmolytes (Cook et al. 2004; Hannah et al. 2005). Although the genetics and physiology of plant cold-acclimation has been studied intensively in recent years, the ecological and evolutionary consequences of cold-acclimation as an inducible response has received far less attention, especially as it relates to population-level variation in freezing tolerance across diverse temperature environments.

While the molecular mechanisms underlying cold acclimation are complex and involve many genes and pathways, the CBF (C-repeat binding factor) transcriptional activators are known to play a critical role in initiating the cold-acclimation response (Thomashow 2001; Fowler and Thomashow 2002; Hannah et al. 2005; Van Buskirk and Thomashow 2006). *Arabidopsis thaliana* has three *CBF* genes, *CBF1*, *CBF2*, and *CBF3*, also known as *DREB1b*, *DREB1c* and *DREB1a*, respectively. These genes are induced rapidly and transitorily at low, nonfreezing temperature, and encode transcriptional activators that regulate the expression of over one hundred downstream *co*ld *r*esponsive (*COR*) genes. Transgenic over-expression of individual *CBF* genes in *A. thaliana* induces the cold acclimation pathway and enhances freezing tolerance in the absence of a low temperature treatment. *CBF* over-expression lines exhibit a dwarf phenotype and retarded growth, indicating a likely cost of constitutive *CBF* expression

(Gilmour et al. 2000; Gilmour et al. 2004). The cost of *CBF* over-expression, however, appears to be subfamily-member-specific. Jackson et al. (2004) documented fitness costs of *CBF2* and *CBF3* over-expression but failed to document a consistent fitness cost associated with *CBF1* over-expression. It currently is unknown, however, the extent to which natural induction of the cold acclimation pathway via the *CBF* transcription activators is costly in the absence of a subsequent freezing stress, and whether such costs may influence evolutionary dynamics of cold-acclimation capacity in environments where selection pressures for freezing tolerance are diverse.

We previously documented a steep latitudinal cline in freezing tolerance in *Arabidopsis thaliana* that follows a gradient of temperature variability across the species' native range (Zhen and Ungerer 2008a). This pattern of clinal variation was shown to be attributable, at least in part, to relaxed purifying selection on the *CBF* subfamily of transcription activators in the species' southern, warmer range. Southern accessions were found to harbor an approximate 3-fold increase in nonsynonymous substitution rates in their *CBF* genes as well as possess a number of regulatory mutations leading to abrogated expression of particular *CBF* subfamily members in particular southern accessions. Mutations arising in the *CBF* subfamily in southern accessions could be selectively neutral or possibly selectively beneficial depending on the extent to which there is an allocation cost associated with cold-acclimation. For example, if cold-acclimation is metabolically costly and if the cold-acclimation pathway is induced in the species' southern range but temperatures rarely drop to levels where plants experience freezing-induced damage or death, mutations that compromise proper functioning of the cold-acclimation pathway might be favored by natural selection. In contrast, in the absence of a cost, cold-acclimation capacity is unlikely to be selected against in southern accessions, and thus mutations compromising *CBF* function would be selectively neutral in southern accessions. In the current report, we test these alternative hypotheses by quantifying the fitness benefits and the potential fitness costs of cold-acclimation in natural *A. thaliana* accessions from both northern and southern regions of the species' native range as well as in *CBF* T-DNA insertion and *CBF* over-expression transgenic lines.

Materials and Methods

Plant materials

Natural accessions

Seeds of 12 accessions of *Arabidopsis thaliana* (L.) Heynh were obtained from The Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. These accessions represent wild populations originally collected from the species' native range. Accessions were categorized as northern (N1-N6, table 4.1a) and southern (S1-S6, table 4.1a) reflecting differences in both geographic origin and maximum freezing tolerance (Zhen and Ungerer 2008a; 2008b). Northern accessions are derived from latitudes at or above 49.4° where mean January temperatures are below 0°C. Northern accessions possess functional *CBF* copies with normal expression level as well as higher cold-acclimation ability. In contrast, southern accessions are derived from latitudes at or below 42° northern latitude where mean January temperatures exceed 7.7°C. Southern accessions possess multiple coding and/or regulatory mutations in their *CBF* genes resulting from relaxed purifying selection on these genes in warmer climates (Zhen and Ungerer 2008b).

Transgenic over-expression lines

Seeds of 4 *Arabidopsis* transgenic lines over-expressing the individual *CBF* genes plus a null vector insertion line (B6) were graciously provided by Dr. Michael Thomashow at Michigan State University (Table 4.1b), including two *CBF1* over-expression lines (G5, G6), one *CBF2* over-expression line (E24) and one *CBF3* over-expression line (A40). These transgenic lines each have a single inserted copy of *CBF* gene driven by a CaMV 35S promoter in the background of *A. thaliana* accession Ws-2, an accession with northern origin and high cold acclimation capacity (Gilmour et al. 2000; Gilmour et al. 2004).

T-DNA insertion lines

Seeds of individual T-DNA insertion lines with insertions in or near the three *CBF* genes were obtained through the ABRC. The presence and homozygosity of insertions was determined

using the "iSct Primers" tool available at the SALK website (http://signal.salk.edu/tdnaprimers.2.html). Individuals homozygous for T-DNA insertions within, or near, any of the three *CBF* genes were tested via RT-PCR for expression of the relevant *CBF* copy. Lines lacking (or with greatly reduced) expression of the *CBF* copy target were retained. Seeds generated by these individual plants were used as *CBF* T-DNA insertion lines in our study (Table 4.1c). Three *CBF2* and two *CBF3* T-DNA insertion lines were identified by these methods; no *CBF1* T-DNA insertion line was identified from our screen.

Experimental design, cold acclimation and freezing treatment

Plants were subjected to four different combinations of cold-acclimation and freezing stress (Table 4.2). Comparisons of fitness differences among genotypes (i.e. natural accessions and transgenic lines) across treatments 1 and 2 allow quantification of the benefit of cold acclimation when plants experience freezing stress. Comparisons of fitness differences among genotypes across treatments 3 and 4 allow quantification of the cost of cold acclimation in the absence of freezing stress.

Each genotype had 20 replicate individuals for each treatment. All seeds were sowed in planting media (2 parts Sun Gro MetroMix 350 planting media: 1 part sand) in 72-well plastic flats (54cm×27cm) and sub-irrigated throughout the experiment. Each flat consisted of 2 complete replicated sets of all genotypes, and their positions on the flat were randomized. After sowing, the flats were kept at 4°C in dark for 3 days for stratification and to promote uniform germination. Then all plants were transferred to a growth room at 21°C and grown under a short day photoperiod (10 h light: 14 h dark). Twenty-five days after sowing, plants at the rosette stage were subjected to cold acclimation and/or freezing treatments depending on treatment group.

Cold acclimation was conducted in a 4°C walk-in cold chamber where plants experienced the same photoperiod as in the growth room. Plants were subjected to three rounds of 4°C for 3 days, with the intervening days in the 21°C growth room. The rationale of repeated exposure to 4°C was to increase the potential cost associated with cold-acclimation pathway by inducing the

CBF genes multiple times. After the third round of cold acclimation, plants were either returned to the growth room (treatments 3 and 4, see table 4.2), or subjected to freezing treatment immediately (treatments 1 and 2, see table 4.2).

Freezing treatments were conducted in an ESPEC ESU-3CA Platinous series environmental test chamber (Hudsonville, MI, USA). All plants receiving a freezing treatment were exposed to two nights of freezing stress (-10°C for the first night and -14°C for the second night) with the intervening day at 4°C. During freezing treatments, the temperature changed at a rate of 2°C per hour during cooling and warming, and stayed at the minimum temperature for 2.5 hours. During cooling periods, ice chips were added to flats when temperatures reached -1°C to induce ice nucleation and prevent supercooling. Following the freezing treatments, plants were transferred to the 21°C growth room, and freezing damage for each genotype was assayed after 3 weeks.

Phenotypic measurements

Because of high mortality associated with experimental treatments involving freezing stress (treatments 1 and 2), survivorship was used as a surrogate for fitness. For plants not experiencing freezing stress (treatments 3 and 4), reproductive output was used as the most direct measure of fitness. Here for each individual plant we recorded fruit number on the main inflorescence, and fruit number on axillary stems and basal shoots. Total fruit number was acquired by summing the above two. Mean seed number per fruit was averaged from counting seeds of 5 normal-looking fruits. Total seed number was estimated by multiplying total fruit number and mean seed number per fruit. Several additional fitness-related morphological traits also were measured, including bolting time, rosette leaf number at bolting, early flower number (the cumulative number of flowers produced 10 days after bolting), rosette diameter and plant height at the ending of the experiment.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence data were collected as a potential physiological component of fitness variation in response to cold-acclimation. The dark-adapted chlorophyll fluorescence parameter F_v/F_m ratio measures the potential quantum efficiency of photosystem II (F_v: the total amount of variable fluorescence; F_m: the maximum fluorescence yield), which is negatively affected by stress-induced photoinhibition of photosystem II reaction centers. Predawn dark-adapted F_v/F_m ratio was measured on northern and southern accessions in treatment 4 using a photosynthesis yield analyzer MINI-PAM (Heinz Walz GmbH). Measurements were taken on 4-6 replicates individuals per accession at several time points prior to, during, and following the cold-acclimation treatment: e.g., just prior to cold acclimation treatment (CA), first day and third day of the first CA, the intervening day at room temperature after first CA, third day of second round CA, the intervening day after second CA, the first and third day of the third round of CA, and everyday for seven days after third CA.

Data analysis

Mean survivorship of different plant categories (i.e., northern accessions, southern accessions, over-expression lines, T-DNA insertion lines) from treatment 2 were compared using one way analysis of variance (ANOVA) and Tukey's HSD test. Phenotypic data of northern and southern accessions from treatment 3 and 4 were analyzed using ANOVA according to the model

$$y = \mu + \text{line (origin)} + \text{origin} + \text{acclimation} + \text{line (origin)} \times \text{acclimation} + \text{acclimation} \times \text{origin} + E \quad \text{(Eqn 1)}$$

where line is a random effect and origin represents either the northern or south accession designation. Repeated-measures ANOVA was used to compare chlorophyll florescence data of northern and southern accessions in treatment 4.

For both treatments 3 and 4, over-expression lines were compared to the control line B6 using one-way ANOVA and Dunnett's test. T-DNA insertion line CBF3a was compared with its background line CS8846 using student's t test. All other T-DNA insertion lines were compared with their appropriate background line Col-0 using Dunnett's test. All data analyses were performed using JMP7.0.1.

Results and discussions

Cold acclimation enhances freezing tolerance

The relatively severe freezing stress imposed on plants in this experiment (one night at -10°C and a second night at -14°C), resulted in high plant mortality. Survivorship was thus used as a surrogate for fitness in comparisons of non-cold-acclimated and cold-acclimated plants subjected to freezing stress. In the absence of a cold-acclimation treatment, mortality was 100% for all individuals of northern accessions, southern accessions and *CBF* T-DNA insertion lines (Fig 4.1). The only plants surviving freezing stress under non cold-acclimated conditions were *CBF* over-expression lines, albeit at relatively low frequency (survivorship = 20.4%). This finding is consistent with previous reports demonstrating that transgenic *CBF* over-expression induces the cold-acclimation pathway even in the absence of a low temperature treatment (Gilmour et al. 2000; Gilmour et al. 2004).

Cold-acclimation treatment increased survivorship for all accessions/transgenic lines with the exception of southern accessions, for which mortality remained 100% (Fig 4.1). This result also in consistent with previous reports demonstrating that natural accessions of *A. thaliana* from the species' southern range exhibit reduced cold-acclimation capacity and maximum freezing tolerance relative to accessions from the species' northern range (Zhen and Ungerer 2008a). This reduction in freezing tolerance was shown to be associated with relaxed purifying selection on the *CBF* gene subfamily and a subsequent accumulation of mutations in both coding and regulatory regions that compromises proper functioning of these transcriptional activators (Zhen and Ungerer 2008b). Relaxed selection on freezing tolerance in southern accessions may have also impaired the function of other components in the cold acclimation pathway.

CBF over-expression lines exhibited the highest survivorship under cold-acclimated conditions (63.4%), following by northern accessions (27.7%) and the *CBF* T-DNA insertion lines (20.2%) (Fig 4.1). The increase in survivorship of the *CBF* over-expression lines may result from additional cold-acclimation capacity gained naturally via their native *CBF* copies and/or

additional pathways induced by low temperature but unrelated to the *CBF* transcription activators. While no significant difference was detected between northern accessions and *CBF* T-DNA insertion lines, the latter demonstrated a trend of lower survivorship. *CBF* T-DNA insertion lines were developed in Col-0 or CS8846 genetic backgrounds, both of which exhibit high cold acclimation capacity. A trend of lower survivorship in these lines (as compared to other northern accessions) is thus likely associated with disruption of individual *CBF* copies.

Evaluating the cost of cold acclimation

Northern versus southern accessions

In the absence of freezing stress, survivorship of all plants was 100% and thus total fruit number was used as a measure of fitness. If there is cost associated with cold acclimation, we predict a decrease in fruit number in cold acclimated plants (treatment 4) when compared to non-cold acclimated plants (treatment 3), and the decrease is expected to be greater for northern accessions versus southern accessions because northern accessions have a higher cold-acclimation capacity (Zhen and Ungerer 2008b). A cost of cold-acclimation should thus be revealed by a significant origin by acclimation interaction in our ANOVA model. Fruit number data from Northern and Southern accessions was analyzed using ANOVA according to the model in Eqn 1. A significant origin by acclimation interaction was not revealed (Table 4.3; F = 1.0126, P = 0.3374), indicating no difference among northern and southern accessions in their response to cold-acclimation and thus no evidence of a higher cost of cold-acclimation among northern accessions. A significant effect was detected for Line (F = 7.4306, P = 0.002), but there was no significant line by acclimation interaction (F = 0.7917, P = 0.6369).

Interestingly, while a significant effect of Acclimation was detected (F = 21.4238, P = 0.0009), cold acclimation treatment resulted in an increase in total fruit number (Student's t, P = 0.001 for northern accessions; P = 0.0154 for southern accessions). ANOVAs on average seed number per fruit and estimated total seed number showed similar patterns as total fruit number (supplementary fig 4.1 and table 4.2c-d). Although not designed to look at the effects of cold acclimation on fitness, there are reports in the agriculture literature demonstrating that low

temperature treatment increases seed number in lettuce (Toledo et al. 1981), Chinese cabbage (Linwattana et al. 1997) and onion (Reghin et al. 2005). In addition, the fruit number increase in our study is disproportionately attributable to increases of fruit number on axillary and basal shoots (Fig 4.2b; Student's t, $P = 0.0017$), which suggests an effect of cold acclimation on modifying plant architecture. This is not the sole explanation for these observations, however, as fruit number increases on the main inflorescence in response to cold-acclimation also are statistically significant (Student's t, $P = 0.0133$). It would be interesting to determine whether genes affecting architecture are cold responsive or whether genes in cold acclimation pathway have pleiotropic effect on plant architecture.

Several additional fitness-related traits (i.e. height, bolting time, rosette leaf number at bolting, maximum rosette diameter, and early flower number) were also measured and analyzed using the same statistical model. We failed to detect a significant Origin by Acclimation interaction for any of these additional traits (supplementary fig 4.1 and table 4.2e-i). However, a significant effect of Line was detected for all traits, a significant effect of Origin (i.e., northern or southern accessions) was detected for rosette leaf number at bolting and early flower number, a significant effect of Acclimation was detected for rosette leaf number at bolting, maximum rosette diameter and early flower number, and a significant Line by Acclimation interaction was detected for bolting time.

To explore a potential physiological component of fitness, we measured dark-adapted F_v/F_m ratio, which is the maximum quantum efficiency of photosystem II. This physiological measure is sensitive to environmental stress induced photoinhibition of PSII reaction centers (Maxwell and Johnson 2000). The more stressful the condition, the lower the F_v/F_m ratio. If there is a measurable physiological cost of cold acclimation in natural accessions, we expect a greater reduction in F_v/F_m ratio in northern accessions than southern accessions during cold acclimation and/or a slower recovery in days following the cold-acclimation treatment. We observed declines of F_v/F_m ratio during each cold acclimation treatment for both northern and southern accessions, with largest declines during the first cold acclimation treatment. Our data, however, revealed no significant difference between northern and southern accessions in these patterns (Repeated

measures ANOVA: $F_{1,53} = 0.0127$, $P = 0.9107$; Fig 4.3). These findings are consistent with results of our fitness and morphological data indicating no detectable cost of cold acclimation in natural accessions. We note that F_v/F_m ratios increased over the duration of the experiment. This pattern may be attributable to the fact that measurements were begun when plants were small and leaves were not fully expanded. A similar trend of increase was observed from an additional control experiment where plants were grown under normal growth room conditions without a cold-acclimation treatment (data not shown).

T-DNA insertion lines

To evaluate changes in the potential cost of cold-acclimation arising from mutations in individual *CBF* copies, we compared fitness of several *CBF* T-DNA insertion lines (for *CBF2* and *3*) with their genetic background control lines under non cold-acclimated and cold-acclimated conditions. Total fruit number of T-DNA insertion line CBF3a is comparable to its background line CS8846 in both cold acclimated (Student's t: $t = -0.7806$, $P = 0.44$) and non-acclimated (Student's t: $t = 0.4398$, $P = 0.6626$) conditions. T-DNA insertion lines CBF2b, CBF2d, CBF2f, and CBF3d also had similar total fruit number as their background line Col-0 with and without cold acclimation (Fig 4.4; Supplementary table 4.2k). No *CBF1* T-DNA insertion lines were recovered in our screen and thus mutations in this *CBF* copy were not evaluated. These findings are consistent with result found for the natural accessions in that no cost associated with the induction of cold acclimation was detected. Results from CBF T-DNA insertion lines also are consistent with results from the natural accessions in that cold-acclimation treatment actually resulted in more fruits produced (higher fitness).

Cost associated with CBF *gene over-expression*

The CBF transcription activators are thought to have largely redundant functions with regard to inducing the cold-acclimation pathway in the presence of low temperature (Gilmour et al. 2004). To explore potential variation among *CBF* copies to function in this capacity, we utilized available *CBF1-3* over-expression lines and measured fitness variation under both non cold-acclimated and cold-acclimated conditions. Two *CBF1* over-expression lines, one *CBF2*

over-expression line, and one *CBF3* over-expression lines were evaluated. *CBF1* over-expression lines G5 and G6 had fitness comparative to the null vector control line B6 both with and without cold acclimation treatment, indicating no cost of *CBF1* over-expression. Over-expression lines *CBF2* (E24) and *CBF3* (A40) exhibited significantly lower fitness both with and without cold acclimation treatment (Fig 4.5; Supplementary table 4.2j), suggesting a cost of *CBF2* and *CBF3* over-expression. These results are consistent with previous reports evaluating these same over-expression lines under similar experimental conditions (Jackson et al. 2004).

Cost of inducible defenses

Most studies that empirically explored the existence of this allocation cost focused on induced defense against biotic stress, such as pathogen resistance and herbivore resistance. A cost has been revealed in many cases. Barley cultivar inoculated with avirulent race of the powdery mildew pathogen remained entirely free of any symptom, but had significant reduction in grain yield (Smedegaardpetersen and Stolen 1981). *Nicotiana attenuata* treated with MeJA to induce defense against herbivore produced less seed than did their uninduced counterparts if plants had not been attacked (Heil and Baldwin 2002). Wheat plants treated with fungicides with BION® induced pathogen resistance, but also resulted in lower biomass, and developed fewer shoots and ears and therefore produced fewer seeds than untreated controls (Heil and Baldwin 2002). Assuming that induced defenses are metabolically costly, the detection of allocation cost is also dependent on whether the available resources or energy are limited. The nutrient level and existence of competition thus should affect the magnitude of cost. It has been reported that a cost was most pronounced when plants suffered from nitrogen shortage (Heil and Baldwin 2002). However, plant-environment interaction is complex, and existence of costs is not universal. A number of studies found no cost of inducible defense on plant growth and reproduction (Bergelson and Purrington 1996; Walters and Heil 2007). Induction of systemic resistance to rust infection using saccharin had no significant effect on broad bean growth and yield (Boyle and Walters 2005). In winter barley, induction of resistance by application of inducer was not associated with reduction in yield (Kehlenbeck et al. 1994). Possible reasons are not addressed, and could be linked to some reports that photosynthesis rates increased in response to pathogen (Rooney and Hoad 1989; Murray and Walters 1992), insect herbivory (Thomson et al. 2003) and

simulated insect defoliation (Macedo et al. 2006), which provides sufficient resources to compensate for the cost. Overcompensation has also been reported in mammalian herbivory (Paige 1992), that plants can benefit from being eaten by stimulating much more new inflorescences from dormant lateral buds, although how prevalence this phenomena is under herbivory damage and other stresses is not clear.

Conclusions

A benefit of cold acclimation in the presence of freezing stress was evident. In absence of freezing stress, by comparing fitness and physiology of natural accessions with better cold acclimation capacity with natural accessions with compromised cold acclimation capacity, cost of cold acclimation wasn't found under our experimental conditions, which was consistent with our results using *CBF* T-DNA insertion lines. However, turning on cold acclimation pathway constitutively by over-expressing *CBF* gene was costly (except *CBF1*), which helped us to understand the evolution of freezing tolerance as an inducible defense via cold acclimation.

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Tables and figures

Figure 4.1 Survivorships of different plant categories after freezing

Survivorships of different plant categories after freezing in treatment 1 and 2. *CBF* over-expression lines include two *CBF1* over-expressers, one *CBF2* over-expresser and one *CBF3* over-expresser. *CBF* T-DNA insertion lines include three *CBF2* T-DNA insertion lines and two *CBF3* T-DNA insertion lines. Grey bars, cold acclimated plants; white bars, non-cold acclimated plants. Each category should have one white bar at left and one grey bar at right. Some bars are missing because the survivorship is zero. Letters indicate significant pairwise differences among different categories of cold acclimated plants (Tukey-Kramer HSD test).

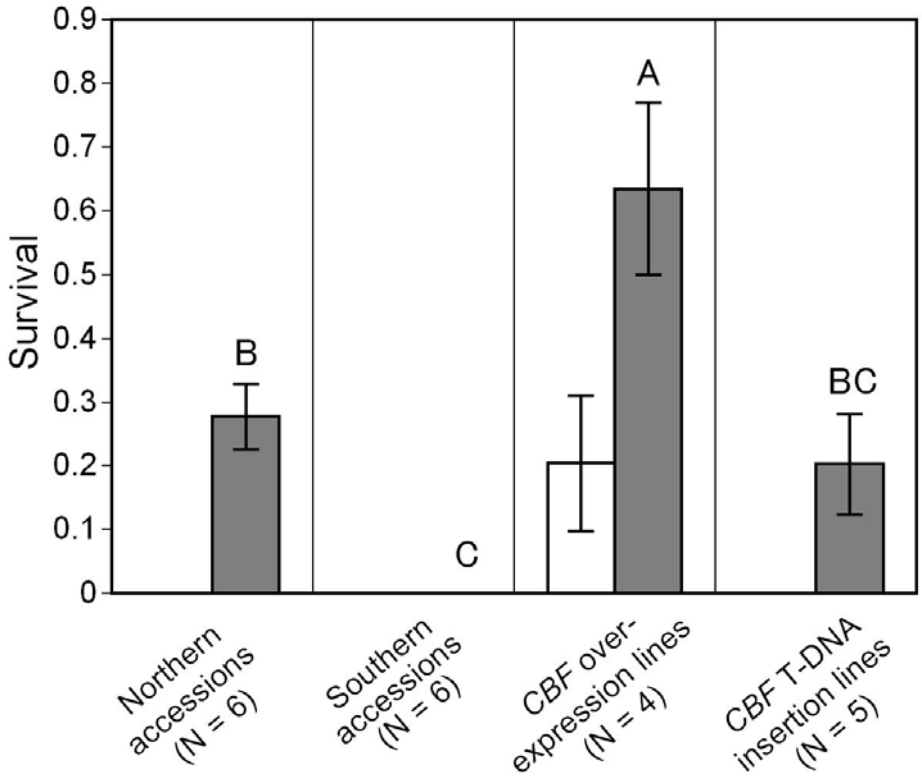


Figure 4.2 Fruit numbers of natural accessions in absence of freezing stress

a Total fruit number of natural accessions in absence of subsequent freezing stress. No significant interaction of cold acclimation and origin (ANOVA: $P = 0.3374$). **b** Fruit number from main stem (diamond) and secondary stems (triangle) in absence of freezing stress. Northern accessions, solid line; southern accessions, broken line. CA, cold acclimated plants; NA, non-cold acclimated plants.

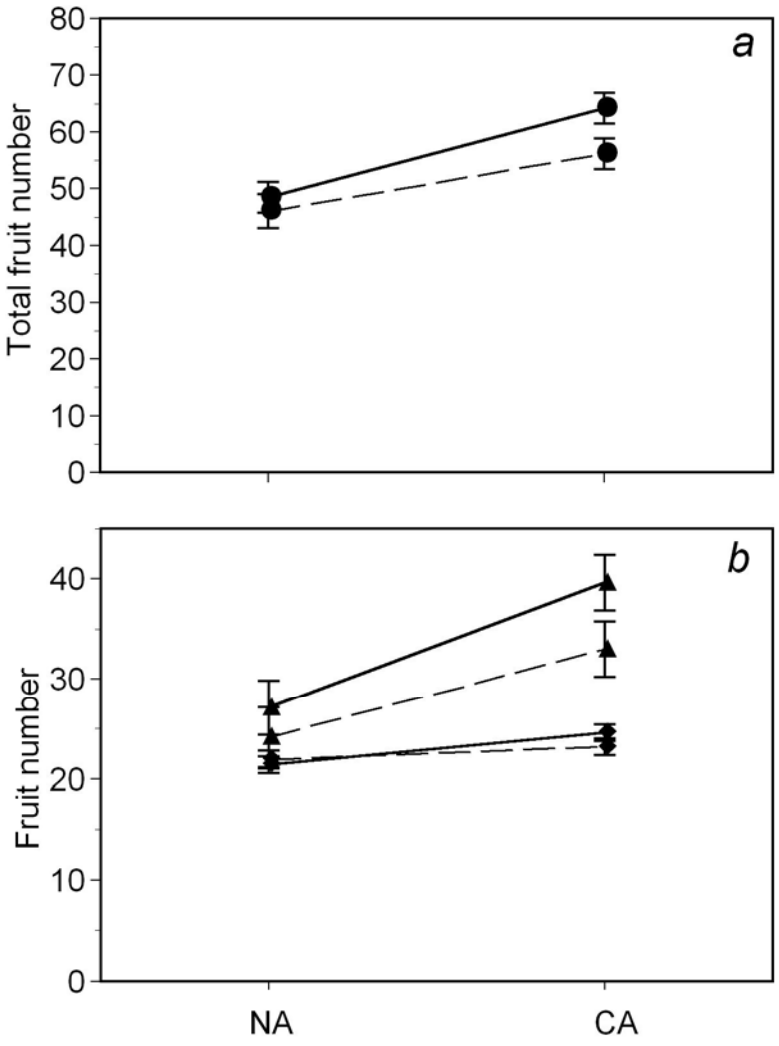


Figure 4.3 *Fv/Fm* ratio of natural accessions in treatment 4.

Pre-dawn *Fv/Fm* ratio of northern (black) and southern (grey) accessions in treatment 4. X-axis indicates days after seed sowing. Plants were subjected to cold acclimation starting from Day 25 after sowing. Bar above x-axis shows the treatment across time: dark portions indicate the three rounds of cold acclimation, open portion indicates time at room temperature.

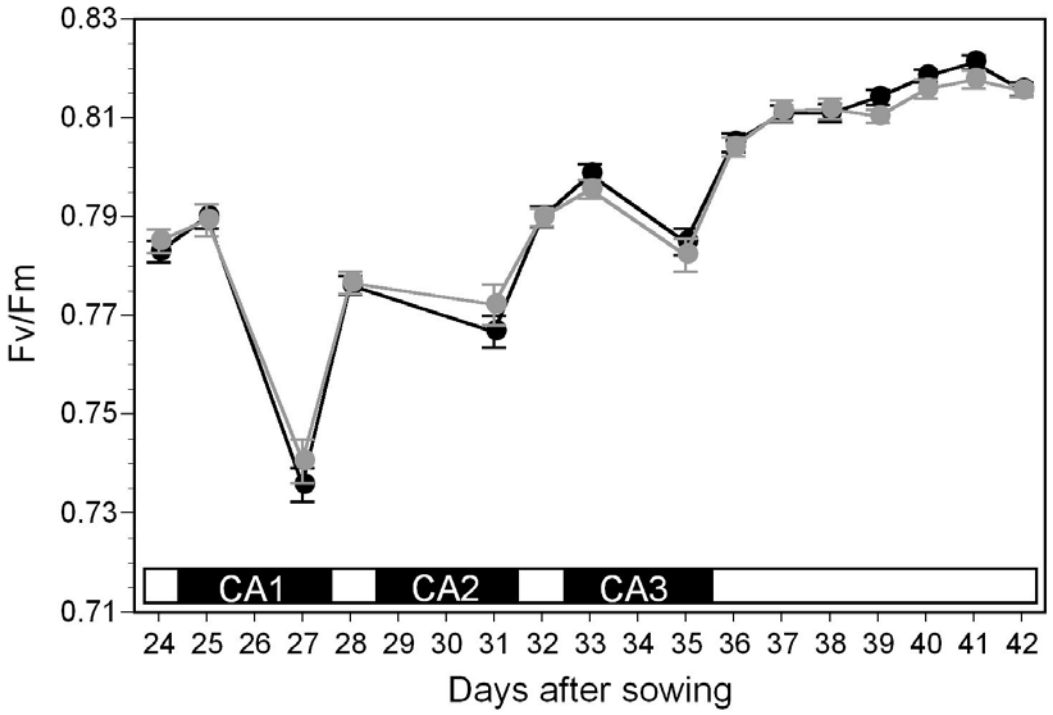


Figure 4.4 Total fruit number of T-DNA insertion lines

Total fruit number of three *CBF2* T-DNA insertion lines (CBF2b, CBF2d, and CBF2f) and two *CBF3* T-DNA insertion lines (CBF3d and CBF3a). CS8846 is background line for CBF3a, Col-0 is background line for all other transgenic line. White bars: non-cold acclimated plants; grey bars: cold acclimated plants.

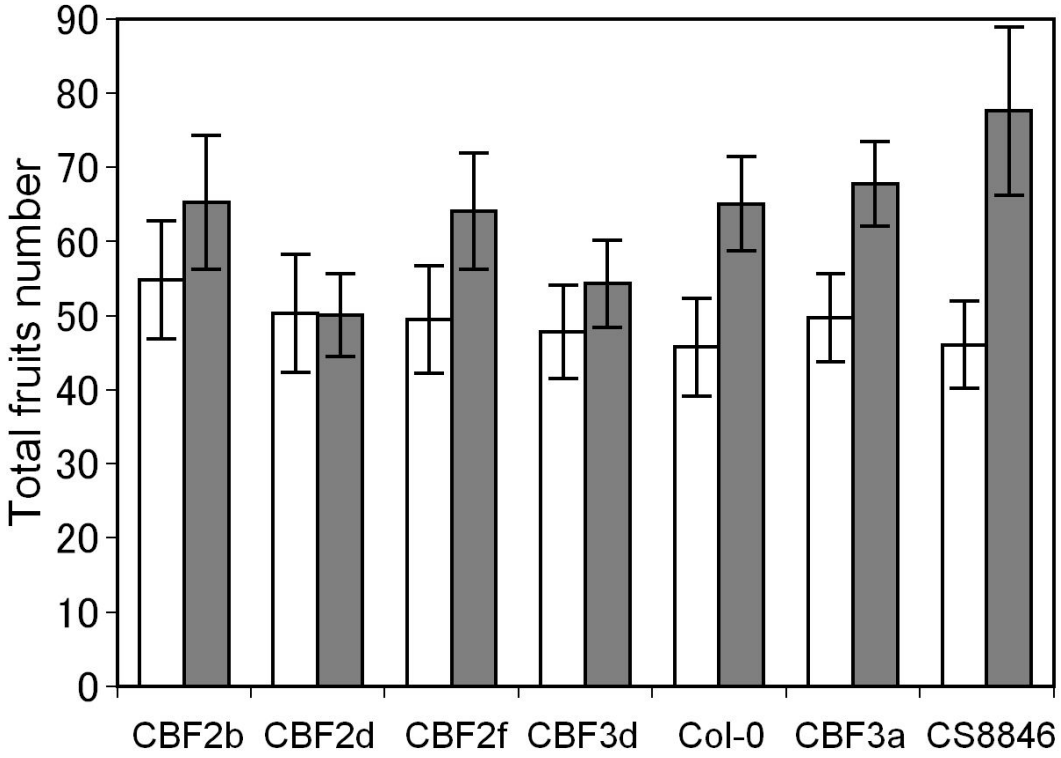


Figure 4.5 Total fruit number of *CBF* over-expression lines

Total fruit number of *CBF1* over-expressers (G5, G6), *CBF2* over-expresser E24, *CBF3* over-expresser A40, and null vector line B6. Background line Ws-2 was also shown but not included in data analysis. White bars: non-cold acclimated plants; grey bars: cold acclimated plants; *: significant different from B6 with same cold acclimation treatment (Dunnett's test).

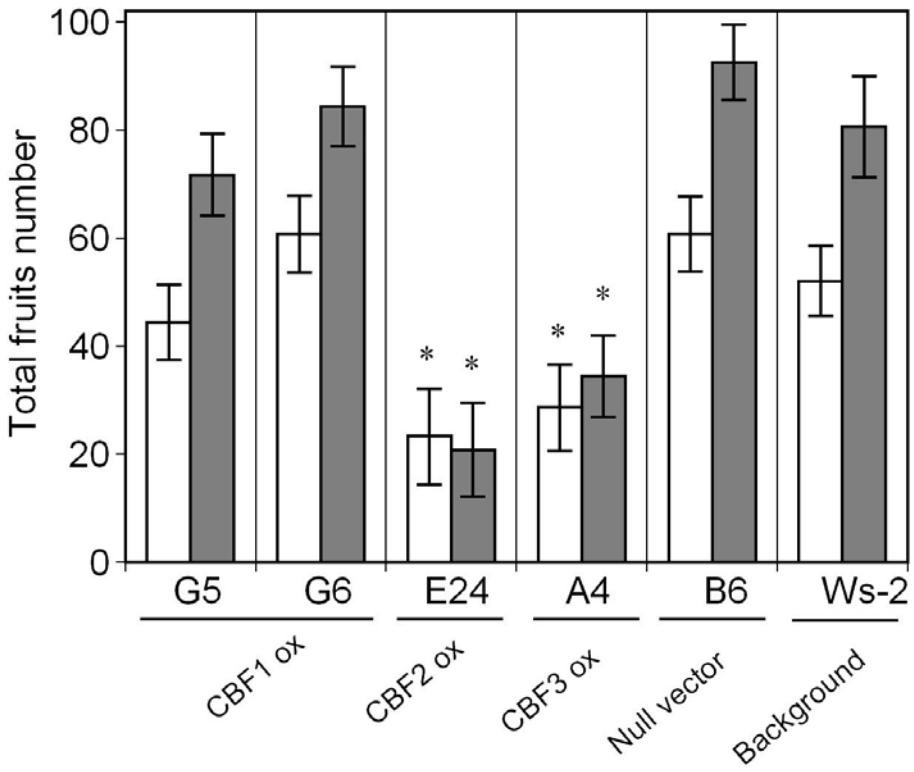


Table 4.1 All *A. thaliana* natural accessions and transgenic lines used in the study

a. *Arabidopsis thaliana* natural accessions used in the phenotypic assay.

Population	ABRC Stock #	Accession	Origin	Latitude (°)	Longitude (°)	Mean January temp. (°C)	Mean July temp. (°C)
S1	CS1084	Co-1	Portugal	40	-8	8.6	21.6
S2	CS1338	Ll-0	Spain	42	3	7.7	23.1
S3	CS1380	Mt-0	Libya	32.6	22.8	11.9	24.5
S4	CS6825	Pa-1	Italy	38.1	13.4	10.6	24.8
S5	CS6855	Sf-1	Spain	41	3	7.7	23.1
S6	CS8580	Cvi-1	Cape Verde Isl.	15	-23	21.6	24.7
N1	CS1538	Stw-0	Russia	52	36	-9	18.8
N2	CS1595	Wil-1	Russia	55	25	-6.1	17
N3	CS1636	Nd-1	Germany	51	10	-0.7	17
N4	CS6665	Chi-1	Russia	54	34	-10	17.7
N5	CS6720	Gie-0	Germany	50.6	8.7	-0.1	17.5
N6	CS6867	Ta-0	Czech Republic	49.4	14.7	-2.5	17.3

b. *Arabidopsis thaliana* transgenic over-expression lines used in this study.

lines	Transgenes
A40	CBF3 over-expression
E24	CBF2 over-expression
G5	CBF1 over-expression
G6	CBF1 over-expression
B6	null vector

c. *Arabidopsis thaliana* T-DNA insertion lines used in this study.

Lines	ABRC Stock #	Transgene	Genetic background
CBF2b	SALK_067966	CBF2 TDNA	Col-0
CBF2d	SALK_073208	CBF2 TDNA	Col-0
CBF2f	SALK_025203	CBF2 TDNA	Col-0
CBF3a	SAIL_244_D02	CBF3 TDNA	CS8846
CBF3d	SALK_007722	CBF3 TDNA	Col-0

Table 4.2 Four treatments conducted in this study.

Treatment	Cold acclimation	Freezing
1	−	+
2	+	+
3	−	−
4	+	−

Table 4.3 ANOVA results for total fruit number

ANOVA results for total fruits number of six northern and six southern accessions both with and without cold acclimation in absence of freezing.

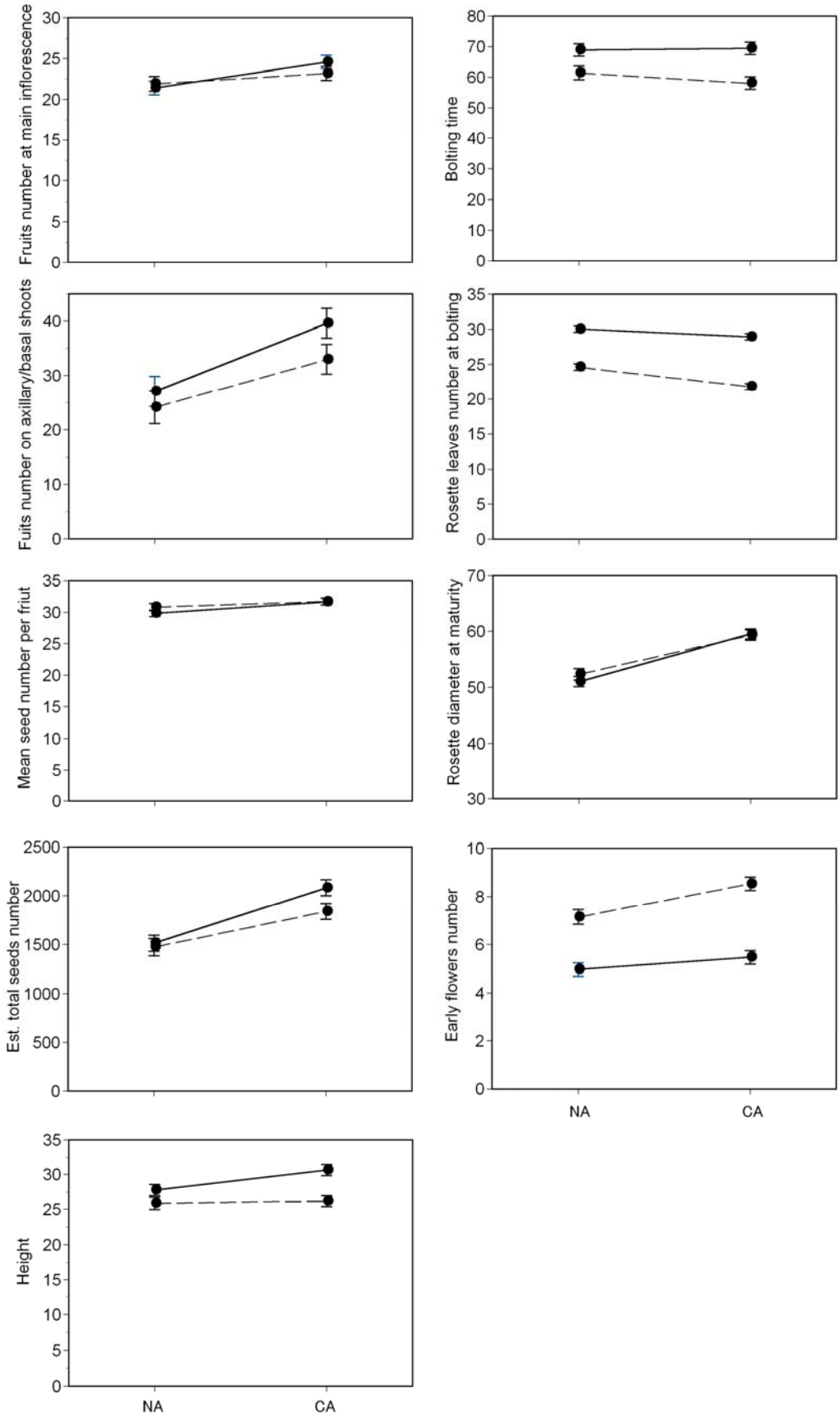
Source	SS	MS	DF	F Ratio	P
Line(origin)	64683.2	6468.32	10	7.4306	0.002
Origin	3089.86	3089.86	1	0.4817	0.5034
Acclimation	18698.4	18698.4	1	21.4238	0.0009
Line(origin)×acclimation	8704.94	870.494	10	0.7917	0.6369
Origin×acclimation	883.825	883.825	1	1.0126	0.3374
Error	477204.16	1099.55	434		

Supplementary materials

The following Supplementary Material is available for this article:

Supplementary Figure 4.1 All other phenotypic traits

Phenotypic traits of northern (solid lines) and southern (broken lines) accessions under cold acclimated (CA) and non-cold acclimated (NA)


Fruits number at main inflorescence
Bolting time
Fuits number on axillary/basal shoots
Rosette leaves number at bolting
Mean seed number per friut
Rosette diameter at maturity
Est. total seeds number
Early flowers number
Height
NA
CA
NA
CA

Supplementary Table 4.1 ANOVA tables and Dunnett's test

ANOVA tables for all other phenotypes for northern and southern accessions, and Dunnett's test for over-expresion lines

a. Fruit number on main inflorescence

Source	SS	MS	DF	F Ratio	P
Line(origin)	6440.25	644.025	10	7.8434	0.0016
Origin	24.4255	24.4255	1	0.0383	0.8488
Acclimation	550.818	550.818	1	6.7123	0.0265
Line(origin)×acclimation	821.106	82.1106	10	1.0648	0.3882
Origin×acclimation	105.78	105.78	1	1.289	0.2822
Error	33467.411	77.114	434		

b. Fruit number on axillary and basal shoots

Source	SS	MS	DF	F Ratio	P
Line(origin)	40563.6	4056.36	10	4.5839	0.0122
Origin	2564.85	2564.85	1	0.6374	0.4431
Acclimation	12830.7	12830.7	1	14.5104	0.0033
Line(origin)×acclimation	8849.15	884.915	10	1.0822	0.3744
Origin×acclimation	378.079	378.079	1	0.4276	0.5277
Error	354895.55	817.73	434		

c. Mean seed number per fruit

Source	SS	MS	DF	F Ratio	P
Line(origin)	2135.33	213.533	10	6.4318	0.0035
Origin	30.1483	30.1483	1	0.1424	0.7138
Acclimation	209.909	209.909	1	6.3249	0.0302
Line(origin)×acclimation	331.994	33.1994	10	1.036	0.4118
Origin×acclimation	24.3906	24.3906	1	0.7349	0.411
Error	13843.165	32.044	432		

d. Estimated total seed number

Source	SS	MS	DF	F Ratio	P
Line(origin)	80000000	8001354	10	10.315	0.0005
Origin	2253190	2253190	1	0.2839	0.6057
Acclimation	24400000	24400000	1	31.1865	0.0002
Line(origin)×acclimation	7757023	775702	10	0.5174	0.8781
Origin×acclimation	1159828	1159828	1	1.4814	0.2505
Error	647630689	1499145	432		

e. Height

Source	SS	MS	DF	F Ratio	P
Line(origin)	8331.52	833.152	10	11.049	0.0004
Origin	1138.42	1138.42	1	1.3795	0.2674
Acclimation	285.893	285.893	1	3.8082	0.0792
Line(origin)×acclimation	754.054	75.4054	10	1.7905	0.0601
Origin×acclimation	179.821	179.821	1	2.3953	0.1524
Error	18277.728	42.115	434		

f. Bolting time

Source	SS	MS	DF	F Ratio	P
Line(origin)	43155.1	4315.51	10	8.8011	0.001
Origin	10002.4	10002.4	1	2.3409	0.157
Acclimation	214.501	214.501	1	0.4412	0.5215
Line(origin)×acclimation	4903.39	490.339	10	6.5718	<.0001
Origin×acclimation	420.574	420.574	1	0.8651	0.3742
Error	32605.882	74.61	437		

g. Rosette leaves number at bolting

Source	SS	MS	DF	F Ratio	P
Line(origin)	8570.95	857.095	10	36.6551	<.0001
Origin	4442.39	4442.39	1	5.2346	0.0452
Acclimation	429.709	429.709	1	18.4191	0.0015
Line(origin)×acclimation	233.827	23.3827	10	1.2916	0.2325
Origin×acclimation	84.8331	84.8331	1	3.6363	0.0852
Error	7911.468	18.104	437		

h. Maximum rosette diameter

Source	SS	MS	DF	F Ratio	P
Line(origin)	22824.3	2282.43	10	23.0697	<.0001
Origin	33.2778	33.2778	1	0.0148	0.9057
Acclimation	6600.66	6600.66	1	66.0704	<.0001
Line(origin)×acclimation	989.36	98.936	10	0.5828	0.8283
Origin×acclimation	62.1542	62.1542	1	0.6221	0.4477
Error	72486.83	169.76	427		

i. Early flower number

Source	SS	MS	DF	F Ratio	P
Line(origin)	757.89	75.789	10	8.3379	0.0012
Origin	758.058	758.058	1	10.0887	0.0099
Acclimation	98.5607	98.5607	1	10.8618	0.0079
Line(origin)×acclimation	90.8967	9.08967	10	1.2208	0.2752
Origin×acclimation	21.602	21.602	1	2.3806	0.1534
Error	3171.7785	7.4455	426		

j. Dunnett's test_over-expression line_total fruit number (alpha = 0.05, control B6)

Line	Cold acclimated |d| = 2.50365		Non-cold acclimated |d| = 2.050283	
	Abs(Dif)-LSD	P	Abs(Dif)-LSD	P
G6	-20.8	0.8994	-20.6	1
G5	-8.64	0.246	-4.08	0.1555
A40	28.66	<.0001	10.14	0.0018
E24	39.95	<.0001	13.96	0.0006

k. Dunnett's test_T-DNA insertion line_total fruit number (alpha = 0.05, control Col-0)

Line	Cold acclimated |d| = 2.48473		Non-cold acclimated |d| = 2.48461	
	Abs(Dif)-LSD	P	Abs(Dif)-LSD	P
CBF2b	-24.7	1	-16.5	0.7893
CBF2d	-9.55	0.3635	-21	0.9775
CBF2f	-23.5	0.9999	-22.2	0.9898
CBF3d	-13.8	0.6507	-24.2	0.999

Positive values show pairs of means that are significantly different.

CHAPTER 5 - Conclusions and future directions

Conclusions

A major goal in evolutionary biology is to understand the underlying genetic basis of adaptive phenotypic variation. Towards this goal, I studied the intra-specific variation in freezing tolerance in the model plant species *Arabidopsis thaliana*. We measured freezing tolerance in 71 natural accessions of *A. thaliana* selected across its native range. Considerable variation was observed both with and without a prior cold acclimation treatment, suggesting that both differences in cold acclimation capacity as well as in intrinsic physiology contribute to this variation. A steep latitudinal cline in freezing tolerance was revealed among these natural accessions across the species' native range, indicating a role for natural selection in shaping the variation in this trait. Taking a candidate gene approach to understand the underlying genetic basis of this variation, I found evidence of relaxed purifying selection on the *CBF* genes in the species' southern range, which likely contributed to the observed clinal pattern of variation. These *CBF* genes encode transcriptional activators that play a critical role in the ability of *A. thaliana* plants to undergo cold acclimation and thereby achieve maximum freezing tolerance. Relaxed purifying selection resulted in significantly higher levels of nonsynonymous polymorphism in coding regions of *CBF* genes in southern accessions than in northern accessions, and also resulted in multiple independent indel mutations in regulatory regions of *CBF* genes that affect their expression. These mutations in coding and regulatory regions compromise the function of *CBF* genes and thus the plant's ability of cold acclimation. These mutations could be selective neutral or beneficial depending on whether there is fitness cost associated with cold acclimation, since in southern environments where freezing stress is rare, temperatures can still drop to levels low enough to induce cold acclimation. Fitness benefits and potential allocation costs of cold acclimation were examined using *A. thaliana* natural accessions exhibit contrasting abilities of cold acclimation as well as in *CBF* T-DNA insertion and *CBF* over-expression lines. An allocation cost of cold acclimation wasn't detected in the absence of

freezing stress, suggesting that these *CBF* mutations compromising cold acclimation pathway in southern accessions might be neutral to natural selection.

Relaxed selection

Relaxed selection is a common phenomenon and can be found under many circumstances (Lahti et al. 2009). For example, the power of selection is reduced by repeated bottlenecks or prolonged period of small population size (Burch et al. 2007). Gene duplication and polyploidy events which create extra gene copies could also relieve a subset of them from selection (Roth et al. 2007). Plastic trait expression can likewise shield genetic changes from selection since it doesn't make a phenotypic difference (Rutherford and Lindquist 1998). Moreover, the strength of selection is also affected by the reproductive potential of an individual, so it declines with age (Williams 1957). In addition and with the highest relevance to the current study, natural populations typically experience heterogeneous and changing environments across space and time, and when a source of selection pressure on an adaptive trait is removed or weakened, relaxation of former selection will affect the maintenance of a former adaptive trait. Understanding the consequences of such relaxed selection can yield more insight in trait evolution, and the outcome has been revealed by several interesting studies in natural populations (Lahti, Johnson et al. 2009). The Mexican blind cavefish lost its pigmentation and eyes due to the relaxed selection in a dark cave environment (Jeffery 2005). The threespine stickleback lost it armor quickly after invasion from ocean to fresh water habitat escaping from previous predators (Barrett et al. 2008). Our study provides another great example of relaxed selection for freezing tolerance in *A. thaliana* as the species spread historically into warmer climates.

Future directions

In addition to the work described here, two additional labs recently have investigated sequence variation of the *CBF* genes in natural accessions of *A. thaliana* (Lin et al. 2008; McKhann et al. 2008). With more and more *CBF* genes sequences information available, it would be interesting to examine the signature of relaxed purifying selection in a much broader

geographic range. Besides, relaxed selection probably acts not only on *CBF* genes but also on other components of the cold acclimation pathway in southern accessions. How much functional divergence exists in the *CBF* genes and how much this contributes to the difference in freezing tolerance could be estimated by reciprocal introgression of *CBF* genes from representative northern and southern accessions, and comparing of the freezing tolerance of introgression lines. In addition, accumulation of certain metabolites and changes in lipid profile are signature events during cold acclimation process, and are directly related to the ability of plant freezing tolerance (Xin and Browse 2000; Welti et al. 2002). Thus, examining and comparing lipid profiles and metabolite profiles between northern and southern accessions with and without cold acclimation could help us understand the difference in freezing tolerance among them from another angle.

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