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

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Summary

• 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 lifestyle and inability to escape ambient atmospheric conditions.

• Here, intraspecific variation in freezing tolerance was investigated in *Arabidopsis thaliana* by conducting freezing tolerance assays on 71 accessions collected from across the native range of the species. 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 evolutionary genetic analysis at multiple levels.

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

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Introduction

Species with broad geographic ranges often exhibit considerable intraspecific 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) are 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, 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, 2001; Xin & Browse, 2000; 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), which begin within minutes following exposure of plants to cold but nonfreezing 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 intraspecific 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, in this report natural phenotypic variation in freezing tolerance was examined among 71 *A. thaliana* accessions collected originally from geographically diverse regions of the native range of the species. Freezing tolerance was investigated over a series of minimum temperatures and following both cold-acclimation and non-cold-acclimation treatments. Considerable intraspecific variation was documented in freezing tolerance among accessions that was highly correlated with their latitude of origin. These patterns of variation are discussed in light of the genetic pathways and physiological mechanisms involved in plant freezing tolerance, and the suitability of this trait for studying the evolutionary genetics and physiology of an adaptive phenotype is highlighted.

Materials and Methods

Arabidopsis thaliana accessions and growing conditions

Freezing tolerance assays were conducted on 71 accessions of *Arabidopsis thaliana* (L.) Heynh. originally collected from diverse regions of the native range of the species (Supplementary Material, Table S1). Seeds of all accessions were obtained from The Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. Before 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 23°C growth room under short day conditions (10 h light: 14 h dark). Plants were grown in 54 cm × 27 cm rectangular flats with plastic inserts capable of accommodating 72 plants per 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 Horticulture, Bellevue, WA, USA): 1 part sand and subirrigated with house distilled H_2O.

Cold-acclimation treatment and freezing tolerance assays

Plants were cold-acclimated in a 4°C walk-in chamber for 7 d, where they experienced identical light and photoperiod conditions as in the 23°C growth room. Cold-acclimated plants were allowed to grow for 23 d in the growth room before a 7-d cold-acclimation treatment, followed by freezing stress. Non-cold-acclimated plants were allowed to grow for 23 d in the growth room before 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 (ESPEC North America, Hudsonville, MI, USA). Replicated sets of accessions were subjected to four minimum temperatures: –6, –8, –10 and –12°C, and –2, –4, –6 and –8°C, for cold-acclimation and non-cold-acclimation 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 4°C. During freezing trials, all plants experienced rates of temperature change of 2°C h^{-1} 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 h. To facilitate ice nucleation and prevent supercooling 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 four 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 4°C for 24 h and then returned to the 23°C growth room. After 2 wk, 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

$$y = \mu + L + T + B(T) + L \times T + L \times B(T) + E \qquad \text{Eqn 1}$$

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

$$y = \mu + L + T + B(T(A)) + A + L \times T + L \times B(T(A)) + L \times A + T \times A + L \times T \times A + E \qquad \text{Eqn 2}$$

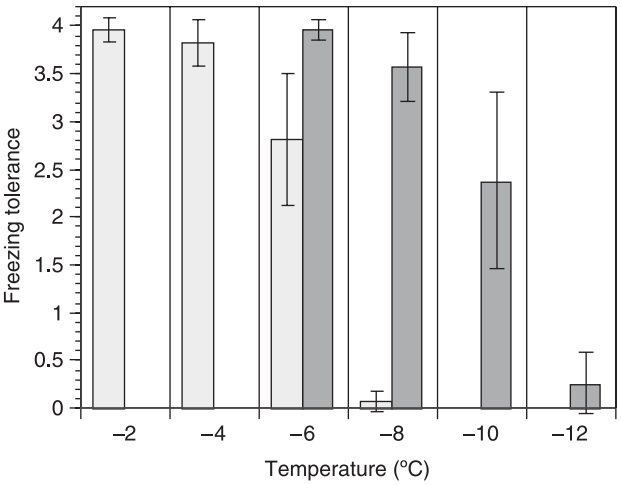

Fig. 1 Mean freezing tolerance scores for 71 *Arabidopsis thaliana* accessions assayed at different minimum temperatures both with and without cold-acclimation treatment. Cold-acclimated plants (dark gray columns) and non-cold-acclimated plants (light gray columns) were assayed at different (but partially overlapping) sets of minimum temperatures. Each histogram bar represents the global average of 71 least square means. Error bars, ± 1 SD.

(A, acclimation treatment (fixed effect); all other variables are as described above.) ANOVA models were evaluated using the Proc GLM procedure of SAS 9.1 (SAS Institute, Cary, NC, USA).

Regression analyses of freezing tolerance scores with latitude of origin were conducted using the least square means of freezing tolerance scores measured at –10°C for cold-acclimated plants and –6°C for non-cold-acclimated plants (least square means derived from Eqn 1 above). These data were selected for analysis because the highest variance in freezing tolerance among accessions was observed at these temperatures (Fig. 1). All regression analyses were conducted using JMP IN software (SAS Institute). 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 *Arabidopsis 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 (Fig. 1). Mixed-model ANOVAs conducted separately on data derived from the cold-acclimation

Source	d.f.	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 (SS). Parentheses indicate the nested data structure.
d.f., degrees of freedom; MS, mean square.

Table 1 Mixed-model ANOVA results for 71 cold-acclimated *Arabidopsis thaliana* accessions assayed for freezing tolerance at –6, –8, –10 and –12°C

Source	d.f.	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 (SS). Parentheses indicate the nested data structure.
d.f., degrees of freedom; MS, mean square.

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

Source	d.f.	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 (SS). Parentheses indicate the nested data structure.
d.f., degrees of freedom; MS, mean square.

Table 3 Mixed-model ANOVA results for 71 *Arabidopsis thaliana* accessions assayed for freezing tolerance at –6 and –8°C both with and without a cold-acclimation treatment

and non-cold-acclimation treatments indicated highly significant effects of line, temperature, batch nested within temperature, and line × temperature ($P < 0.0001$; Tables 1, 2). The line × batch(temperature) interaction term was not significant in either analysis ($P = 0.9674$ and $P = 0.3574$, for the cold-acclimation and non-cold-acclimation treatments, respectively).

The partial overlap of temperatures at which cold-acclimated and non-cold-acclimated plants were assayed (i.e. –6 and –8°C) enabled the evaluation of a statistical model examining the additional effect of acclimation and its corresponding higher level interaction terms (Table 3). Highly significant effects of acclimation, temperature × acclimation, and line × temperature × acclimation were observed ($P < 0.0001$; Table 3). The significant interaction effect of temperature × acclimation results from the fact that a transition from –6 to –8°C 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 (Fig. 1). The enhancement of freezing tolerance by cold acclimation was especially evident for assays conducted at –8°C. 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

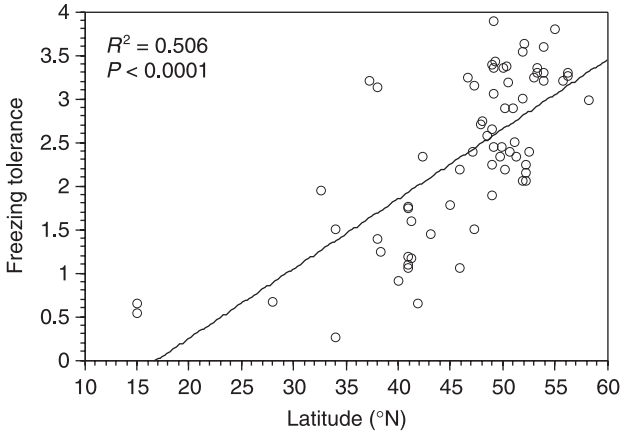

Fig. 2 Freezing tolerance scores plotted against the latitude of origin for 71 *Arabidopsis thaliana* accessions. Data are for cold-acclimated plants assayed for freezing tolerance at –10°C. Plotted are least square means of 20 replicates per accession.

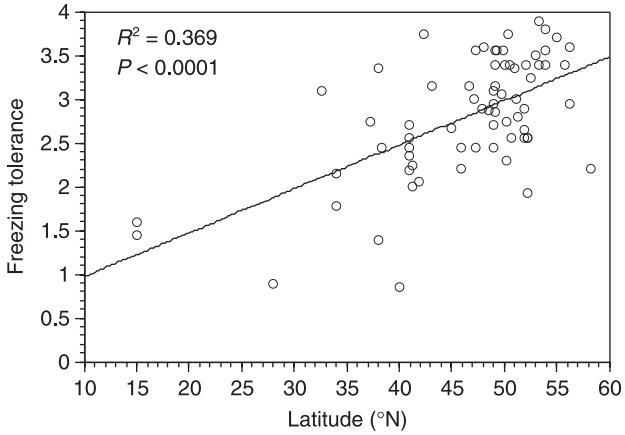

Fig. 3 Freezing tolerance scores plotted against the latitude of origin for 71 *Arabidopsis thaliana* accessions. Data are for non-cold-acclimated plants assayed for freezing tolerance at –6°C. Plotted are least square means of 20 replicates per accession.

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, line × acclimation interaction terms were examined in statistical models evaluated separately at –6 and –8°C. A significant line × acclimation interaction term was detected at both temperatures (–6°C: $F = 3.37$, $P < 0.0001$; –8°C: $F = 1.49$, $P < 0.0103$). 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-acclimation and non-cold-acclimation conditions (Tables 1, 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, the relationship between freezing tolerance and latitude of origin under both cold-acclimation and non-cold-acclimation conditions was examined. A positive and highly significant linear relationship was observed between freezing tolerance and latitude of origin of accessions under cold-acclimation conditions (Fig. 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-acclimation conditions (Fig. 3), indicating that, in addition to differences in cold-acclimation capacity, these accessions differ physiologically for intrinsic factors influencing freezing tolerance. When individual freezing tolerance was measured as survivorship (i.e. number of individuals receiving nonzero freezing tolerance scores divided by the total number of replicates), these regression analyses remained 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 vs latitude of origin for the remainder of temperatures at which plants were assayed are available as Supplementary Material (Figs S1 and S2).

Because regression analyses were conducted at temperatures at which the greatest variance among accessions was observed (i.e. –10°C for cold-acclimated plants and –6°C 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, data were obtained on mean monthly temperature (January and July) for the geographic coordinates of the 71 accessions and plotted against their latitude of origin (Supplementary Material, Fig. S3). 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. Variation in

freezing tolerance was examined in a panel of *A. thaliana* accessions from different regions of the native range of the species. Averaged across all accessions, maximum freezing tolerance decreased with decreasing temperature and, predictably, was enhanced following a period of acclimation to low but nonfreezing temperature. Because a major aim of this study was to evaluate the degree of intraspecific 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-acclimation and non-cold-acclimation 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-acclimation conditions. The persistence of clinal variation in freezing tolerance under non-cold-acclimation conditions, however, indicates that factors intrinsic to the unacclimated 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 vs 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 Material, Fig. S3), and thus likely explains the highly significant regression analyses observed in Figs 2 and 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, 2001; Xin & Browse, 2000; 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 intraspecific 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, although 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* (C-repeat/dehydration-responsive element – binding factor) family of transcriptional activators (Y. Zhen and M. C. Ungerer, unpublished data), genes that play a central role in the cold-acclimation pathway and that have been previously implicated in underlying natural variation among *A. thaliana* accessions (Alonso-Blanco *et al.*, 2005; Hannah *et al.*, 2006).

Non-cold-acclimation 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, 2001; Xin & Browse, 2000; Van Buskirk & Thomashow, 2006). While variation in cold-acclimation capacity clearly contributes to intraspecific 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-acclimation 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 interspecific 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-acclimation conditions (Hannah *et al.*, 2006). This would suggest that similar mechanisms might be involved in freezing tolerance under cold-acclimation and non-cold-acclimation 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 overlap in the 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, 2006; Vogel *et al.*, 2005). In geographic regions that experience low but nonfreezing 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 *A. thaliana* 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 cold-responsive (*COR*) 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 up-regulation 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 up-regulation 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 were observed under both cold-acclimation and non-cold-acclimation conditions, indicating the evolution of mechanisms associated with an inducible response as well as intrinsic to the unacclimated 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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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Freezing tolerance scores plotted against latitude of origin for 71 *Arabidopsis thaliana* accessions under cold-acclimation conditions.

Fig. S2 Freezing tolerance scores plotted against latitude of origin for 71 *Arabidopsis thaliana* accessions under non-cold-acclimation conditions.

Fig. S3 Temperature plotted against latitude for the collection location of 71 *Arabidopsis thaliana* accessions used in this study.

Table S1 *Arabidopsis thaliana* accessions assayed for freezing tolerance

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