Relaxed Selection on the *CBF/DREB1* Regulatory Genes and Reduced Freezing Tolerance in the Southern Range of *Arabidopsis thaliana*

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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 Y, Ungerer MC. 2008. Clinal variation in freezing tolerance among natural accessions of *A. thaliana*. New Phytol. 177:419–427). 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 et al. 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 et al. 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). Although 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, 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 et al. 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 h (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 cold-responsive (*COR*) genes that possess the C-repeat/dehydration responsive element (CRT/DRE) in their promoters (Van Buskirk and Thomashow 2006). Transgenic overexpression of individual *CBF* genes induces the cold acclimation pathway and results in enhanced freezing tolerance in the absence of a cold acclimation treatment (Gilmour et al. 2004). The *CBF* genes thus provide excellent candidates for evolutionary genetic analyses in the context of geographically structured variation in freezing tolerance because they 1) play a pivotal role in the ability

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Table 1
***Arabidopsis thaliana* accessions examined in this study**

Arabidopsis Biological Resource Center Stock Number	Accession	Origin	Latitude	Longitude	Mean January Temperature (°C)	Mean July Temperature (°C)
CS8580	Cvi-1	Cape Verde Islands	15	−23	21.6	24.7
CS22614	Cvi-0	Cape Verde Islands	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

of *A. thaliana* plants to undergo cold acclimation and thus achieve maximum freezing tolerance, 2) are positioned early in a genetic network such that improper functioning would have numerous and undoubtedly detrimental downstream consequences, and 3) 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 *A. thaliana* accessions (table 1) were obtained from the *Arabidopsis* Biological Resource Center 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 days 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). 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/h 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, Valencia, CA) 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 S1, Supplementary Material online). In some instances, more than one pair of primers was required to amplify each region from all accessions. Polymerase chain reaction (PCR) amplifications were conducted using GoTaq Flexi DNA Polymerase (Promega, Madison, WI) according to the manufacture'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, Carlsbad, CA) and aligned with ClustalW (Thompson et al. 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 J and Rozas R 1999).

Polymorphism at *CBF1–3* nonsynonymous and synonymous sites was determined in northern and southern accessions and compared with empirical data for 139 other *A. thaliana* genes in accessions from the same (or similar) geographic regions (Nordborg et al. 2005) (supplementary fig. S1*A*, Supplementary Material online). This set of 139 loci was selected based on the criteria that these sequence reads 1) be distributed across all five *A. thaliana* chromosomes (supplementary table S2, Supplementary Material online), 2) have uninterrupted open reading frames (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 nonindependent 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 data set allowed us to discriminate between patterns of polymorphism in northern and southern accessions attributable to selective versus nonselective evolutionary forces. Data were analysed 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 was compared between northern and southern accessions (supplementary fig. S1*B*, Supplementary Material online).

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 h for *CBF* expression assays and 0, 24, and 48 h 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′ untranslated regions (UTRs). The specificity of these primers was confirmed by 1) testing their efficacy via reverse-transcription PCR in individual (noncold acclimated) *CBF1*, *2*, and *3* overexpressing *A. thaliana* transgenic lines kindly provided by the laboratory of Michael Thomashow and 2) 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 S2, Supplementary Material online. Normalized expression was determined using the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPC*, GenBank accession # NM_111283) according to the equation, Normalized expression $= \frac{(E_{\mathrm{ref}})^{\mathrm{CT_{ref}}}}{(E_{\mathrm{GOI}})^{\mathrm{CT_{GOI}}}}$ (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, $\mathrm{CT_{ref}}$ is the cycle threshold of the reference gene, and $\mathrm{CT_{GOI}}$ 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 30 s, 55 °C for 30 s, and 72 °C for 1 min. 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 S2, Supplementary Material online, 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 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 42°N (mean = 32.68°N, standard deviation [SD] = 10.31) whereas northern accessions were designated as those from latitudes at or above 46.7°N (mean = 50.75°N, SD = 2.78) (table 1, supplementary fig. S1*A*, Supplementary Material online). 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 1). Southern and northern accessions exhibit drastically different survivorship following exposure to freezing stress (fig. 1*A*, 1*B*), with mean survivorships of 0.323 (standard error [SE] = 0.035) and 0.833 (SE = 0.035) for southern and northern accessions, respectively.

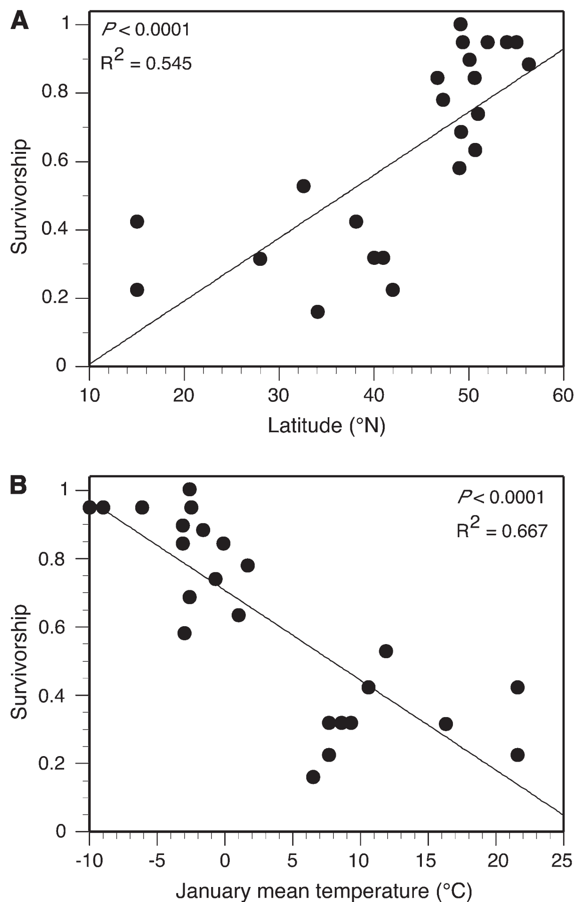

FIG. 1.—Survivorship plotted against latitude of origin (*A*) and January mean temperature (*B*), for 24 *Arabidopsis thaliana* accessions subjected to −10 °C for two consecutive nights. All plants were first cold acclimated for 7 days at 4 °C. Data are based on 20 replicates per accession. In panel *A*, two accessions overlap entirely (i.e., latitude = 41°N, survivorship = 0.316).

Table 2
Polymorphism within the *CBF* transcriptional activators in northern and southern accessions of *Arabidopsis thaliana*

Gene	Length (bp)	*n*	θSynonymous	θNonsynonymous
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

NOTE.—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 1). *CBF1–3* indicates a concatenated sequence including all three genes, with stop codons of two genes removed. θ, Watterson's theta (Watterson 1975).

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. S3, Supplementary Material online). Bootstrap support was high along branches defining the different subfamily members. Depending on which *CBF* copies are compared, 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. S3, Supplementary Material online). 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 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. 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 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 with 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

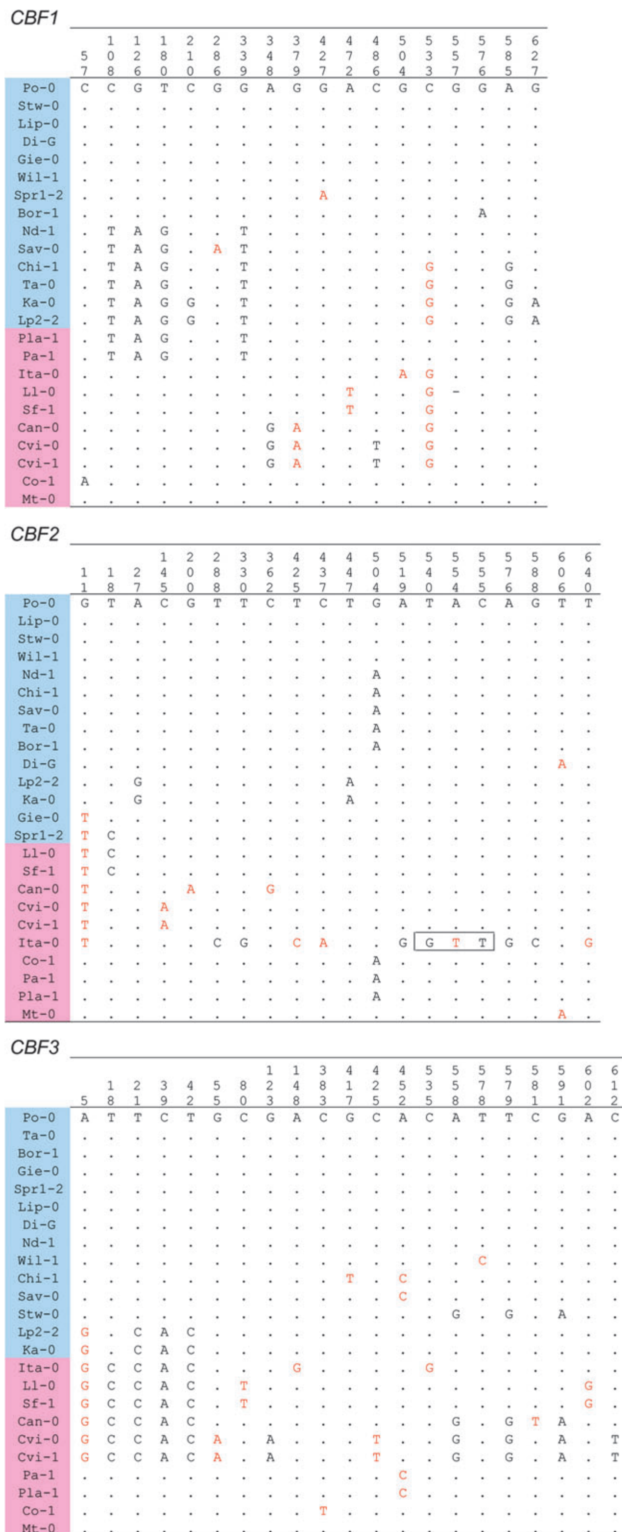

FIG. 2.—Sequence variation of *CBF1–3* in 24 accessions of *Arabidopsis thaliana*. Sequences for the accession Po-0, from Germany, are given as a reference. The locations of polymorphic positions are given at the top for each gene. Nucleotide polymorphisms are indicated by black (synonymous) and red (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.

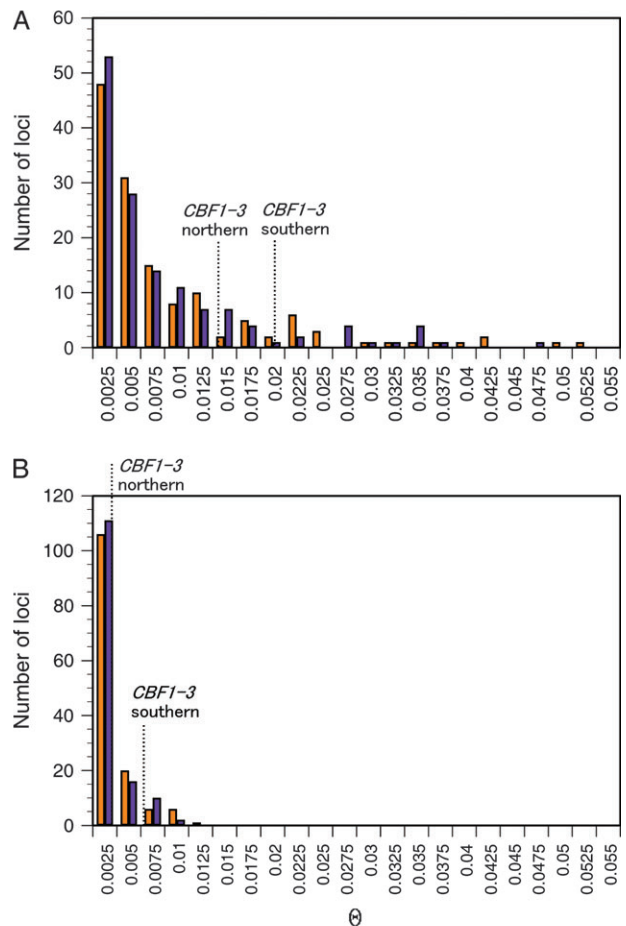

FIG. 3.—Distribution of polymorphism at synonymous (*A*) and nonsynonymous (*B*) sites for 139 loci in the *Arabidopsis thaliana* genome (supplementary table S2, Supplementary Material online). Orange and purple bars indicate polymorphism levels for southern and northern accessions, respectively (see supplementary fig. S1*A*, Supplementary Material online). Polymorphism values for *CBF1–3* concatenated sequences in northern and southern accessions are indicated by dotted lines.

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). Sequence data for the 139 loci were obtained for 23 accessions with the same (or similar) latitudinal coordinates as those accessions examined in this study (supplementary fig. S1*A*, Supplementary Material online). At nonsynonymous sites, polymorphism is significantly elevated in southern accessions in comparison to northern accessions (Fisher's exact test, $P = 0.0007$; supplementary fig. S1*B*, Supplementary Material online). At synonymous sites, however, there is no significant difference between southern and northern accessions (Fisher's exact test, $P = 0.6158$, supplementary fig. S1*B*, Supplementary Material online). 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

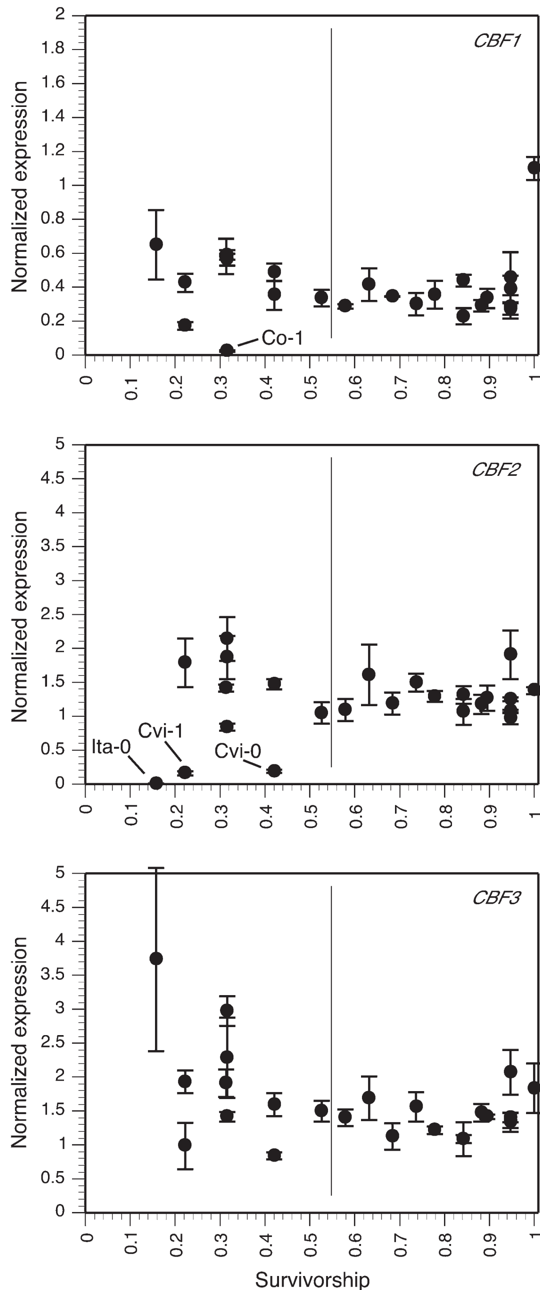

FIG. 4.—Normalized *CBF1–3* expression in 24 accessions of *Arabidopsis thaliana* following 2 h of cold acclimation at 4 °C. Normalized expression is plotted as a function of survivorship following two nights of freezing stress (see Materials and Methods and fig. 1). Vertical lines separate accessions with southern designations (left) and northern designations (right). Normalization scores are in reference to the housekeeping gene *GAPC* (see Materials and Methods). Four accessions with abrogated expression (for *CBF1* and *2*) are indicated. Error bars indicate one SE.

demography. Relaxed selection on the *CBF* genes in southern accessions is additionally supported by findings of a frameshift mutation at position 557 in *CBF1* in an accession from southern Spain (Ll-0) (fig. 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 2 h of cold acclimation (Shinwari et al. 1998; Cook et al. 2004). Figure 4 depicts normalized expression of the three *CBF* genes after 2 h of exposure to 4 °C as a function of their phenotypic freezing tolerance (see fig. 1). Although 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′ 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 *COR* 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 et al. 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-h period of cold acclimation at 4 °C (fig. 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 nonsynonymous mutations (green and red lines in fig. 5) exhibited quantitative reductions in both rates of induction and maximum levels

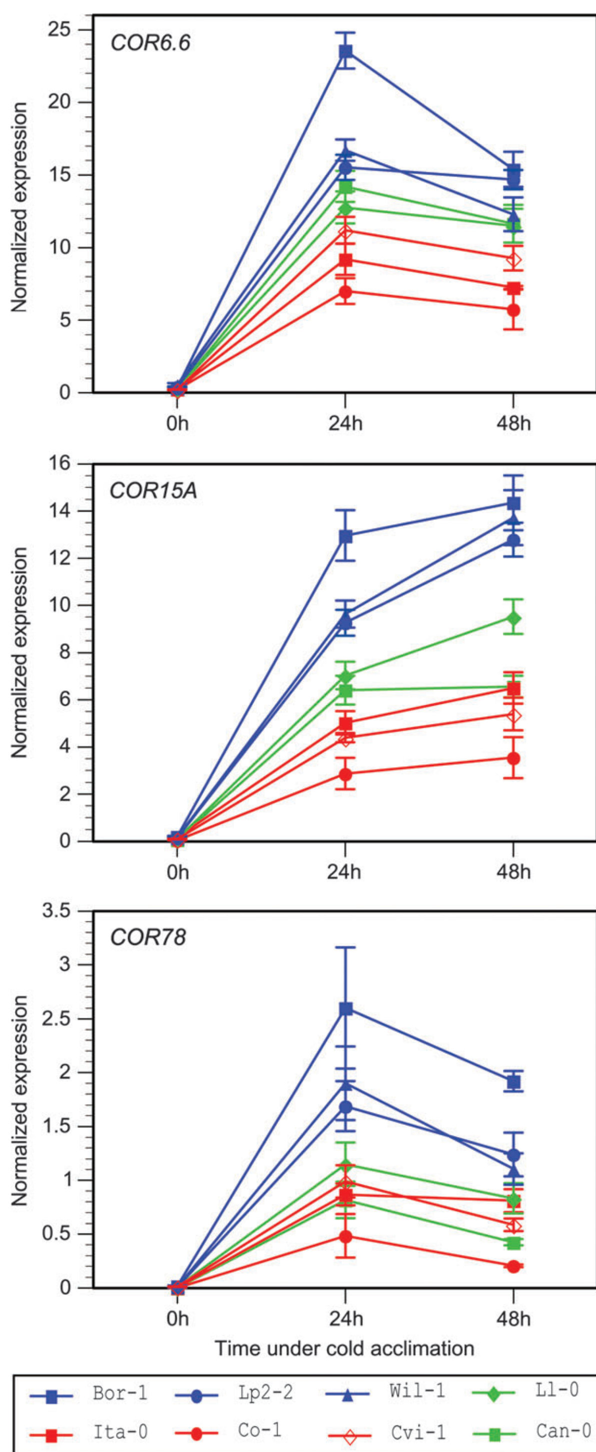

FIG. 5.—Normalized expression of three *COR* genes in eight accessions of *Arabidopsis thaliana* following 0, 24, and 48 h of cold acclimation at 4 °C. Blue lines indicate northern accessions with normal levels of *CBF* expression; green lines indicate southern accessions with multiple nonsynonymous/frameshift mutations in one or more of the *CBF* members; and 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 Materials and Methods). Error bars indicate one SE.

of expression for each *COR* gene over the same period (fig. 5). Southern accessions possessing a combination of regulatory mutations and nonsynonymous mutations in the *CBF* genes (red lines in fig. 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 et al. 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 et al. 2004) with suggestions of the Caucasus as a potential ancestral area (Beck et al. 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 et al. 2000; Nordborg et al. 2005; Bakker et al. 2006; Schmid et al. 2006; Beck et al. 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 with 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 toward 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.

Supplementary Material

Supplementary tables and figures are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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