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Natural variation in the C-repeat binding factor cold response pathway correlates with local adaptation of Arabidopsis ecotypes

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SUMMARY

The natural range of *Arabidopsis thaliana* (Arabidopsis) encompasses geographical regions that have greatly differing local climates, including harshness of winter temperatures. A question thus raised is whether differences in freezing tolerance might contribute to local adaptation in Arabidopsis. Consistent with this possibility is that Arabidopsis accessions differ in freezing tolerance and that those collected from colder northern latitudes are generally more tolerant to freezing than those collected from warmer southern latitudes. Moreover, recent studies with Arabidopsis genotypes collected from sites in Sweden (SW) and Italy (IT) have established that the two accessions are locally adapted, that the SW ecotype is more tolerant of freezing than the IT ecotype, and that genetic differences between the two ecotypes that condition local adaptation and freezing tolerance map to a region that includes the C-repeat binding factor (*CBF*) locus. The *CBF* locus includes three genes – *CBF1*, *CBF2* and *CBF3* – that are induced by low temperature and encode transcription factors that regulate a group of more than 100 genes, the CBF regulon, which impart freezing tolerance. Here we show that cold induction of most CBF regulon genes is lower in IT plants compared with SW plants, and that this is due to the IT *CBF2* gene encoding a non-functional CBF2 protein. The non-functional IT CBF2 protein also contributes to the lower freezing tolerance of the IT plants compared with the SW plants. Taken together, studies on the SW and IT ecotypes provide evidence that natural variation in the CBF pathway has contributed to adaptive evolution in these Arabidopsis populations.

Keywords: C-repeat binding factor regulatory pathway, natural variation, freezing tolerance, *Arabidopsis thaliana*.

INTRODUCTION

Freezing temperatures limit the geographical distribution of plants and cause losses in crop productivity on an annual basis (Sakai and Larcher, 1987). Thus, from both basic and applied science perspectives, there is considerable interest in identifying genes that have roles in freezing tolerance. Significant progress towards this goal has been made by studying *Arabidopsis thaliana* (hereafter Arabidopsis). It is well established that the C-repeat binding factor (CBF) regulatory pathway of Arabidopsis has a prominent role in cold acclimation, the process whereby plants increase their tolerance to freezing in response to low temperatures (Thomashow, 1999, 2010; Knight and Knight, 2012). The pathway includes action of three *CBF* genes that are arranged in tandem array on chromosome 4. These genes encode closely related AP2/ERF family DNA-binding proteins that recognize the CRT/DRE DNA regulatory element present in the promoters of CBF-regulated genes (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al.*, 1997). Within minutes of exposing Arabidopsis to low non-freezing temperatures, *CBF1*, *CBF2* and *CBF3* (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Medina *et al.*, 1999), also referred to as *DREB1b, DREB1c* and *DREB1a*, respectively (Liu *et al.*, 1998), are induced, followed by altered expression of the

CBF-targeted cold-regulated (COR) genes (Thomashow, 2010; Knight and Knight, 2012). Constitutive overexpression of *CBF1*, *CBF2* or *CBF3* in transgenic plants results in changes in the transcript levels for about 100 COR genes, known as the CBF regulon (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Vogel *et al.*, 2005; Park *et al.*, 2015), and an increase in freezing tolerance without the plants being exposed to low temperature (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2004). The mechanisms by which the CBF regulon increases freezing tolerance are not completely understood, but involve the action of genes that encode cryoprotective proteins (Steponkus *et al.*, 1998; Sror *et al.*, 2003; Hughes *et al.*, 2013) and enzymes involved in the biosynthesis of low-molecular-weight cryoprotectants (Cook *et al.*, 2004; Gilmour *et al.*, 2004; Kaplan *et al.*, 2007).

The natural range of Arabidopsis extends from North Africa, including the Canary and Cape Verde islands, to northern Europe, and east to Central Asia encompassing geographical locations that differ greatly in annual temperature (Hoffmann, 2002; Koornneef *et al.*, 2004). Natural variation in freezing tolerance has been observed among Arabidopsis accessions, with those collected from cooler northern latitudes generally being more tolerant of freezing than those collected from warmer southern latitudes (McKhann *et al.*, 2008; Zhen and Ungerer, 2008; Gery *et al.*, 2011). A fundamental question raised is whether natural variation in the CBF pathway contributes to this clinal variation in freezing tolerance. The results of Hannah *et al.* (2006) provide evidence in support of this possibility; they found a positive correlation between the relative freezing tolerance of seven accessions and the transcript levels for *CBF1*, *CBF2* and *CBF3* regulon genes in plants exposed to low temperature for 15 days. However, results from other studies using different accessions and growth conditions failed to provide a clear correlation between freezing tolerance and *CBF* gene expression (Le *et al.*, 2008; Gery *et al.*, 2011).

The results of Ågren and Schemske (2012) provide direct evidence for local adaptation in natural populations of Arabidopsis and implicate freezing tolerance as an adaptive trait. Over the course of 5 years, these investigators conducted reciprocal transplant experiments using Arabidopsis populations that they had collected from sites in Italy (IT) and Sweden (SW). Their results indicated that the IT genotype was fitter – measured as survival and total fruit production – than the SW genotype when both genotypes were grown at the Italian site, and that the reverse was true when both genotypes were grown at the Swedish site. Thus, the IT and SW genotypes were locally adapted and were true ecotypes. Moreover, they found that the relative survival of the IT ecotype at the Swedish site was highly positively correlated with the minimum soil freezing temperature (Ågren and Schemske, 2012). For instance, in a year when the winter was relatively warm (minimum soil temperature −1°C), the relative survival of the IT ecotype compared with the SW ecotype was about 0.9, but in a year when the winter was relatively cold (a minimum soil temperature −6°C), the relative survival of the IT ecotype was only about 0.1.

As a first step to determine the genetic basis for the observed local adaptation of the IT and SW ecotypes, Ågren *et al.* (2013) mapped quantitative trait loci (QTL) for fitness (total fruit number) using 398 recombinant inbred lines (RILs) that they had developed from a genetic cross between the IT and SW parent ecotypes. An analysis of the relative fitness of the RILs, quantified at both the IT and SW sites for three consecutive years, resulted in the identification of 15 QTL that contributed to fitness. One of these QTL mapped to chromosome 4 at a position that overlapped the *CBF* locus, a finding that was consistent with freezing tolerance contributing to local adaptation. Oakley *et al.* (2014) tested this hypothesis by conducting growth chamber experiments to determine the relative freezing tolerance of the IT and SW parental lines and the RILs developed by Ågren *et al.* (2013). The results indicated that the SW ecotype was indeed more tolerant of freezing than the IT ecotype. Moreover, they identified seven QTL that conditioned freezing tolerance, one of which (accounting for about 18% of the variance) co-localized with the fitness QTL on chromosome 4 that overlapped the *CBF* locus.

The studies with the SW and IT accessions indicate that they are locally adapted (Ågren and Schemske, 2012; Ågren *et al.*, 2013), that the SW ecotype is more tolerant of freezing than the IT ecotype (Oakley *et al.*, 2014) and that QTL for both fitness and freezing tolerance map to a site on chromosome 4 that overlaps the *CBF* locus (Oakley *et al.*, 2014). These results raise the possibility that differences in the IT and SW *CBF* loci contribute to the differences in freezing tolerance observed between the IT and SW ecotypes. Here we address this possibility by comparing the low-temperature transcriptomes of the SW and IT ecotypes; mapping the expression QTL (eQTL) that affect expression of CBF regulon genes in the two ecotypes; determining the DNA sequences of the SW and IT *CBF* genes; comparing the predicted amino acid sequences of the CBF proteins; and determining the effects that expression of the SW CBF2 protein in the IT ecotype has on expression of CBF regulon genes and freezing tolerance. Taken together, the results support the model that differences in the SW and IT *CBF* loci contribute to their differences in freezing tolerance and local adaptation.

RESULTS

The SW and IT plants have similar sets of COR genes

The increase in freezing tolerance that occurs in plants in response to low temperature involves the action of COR

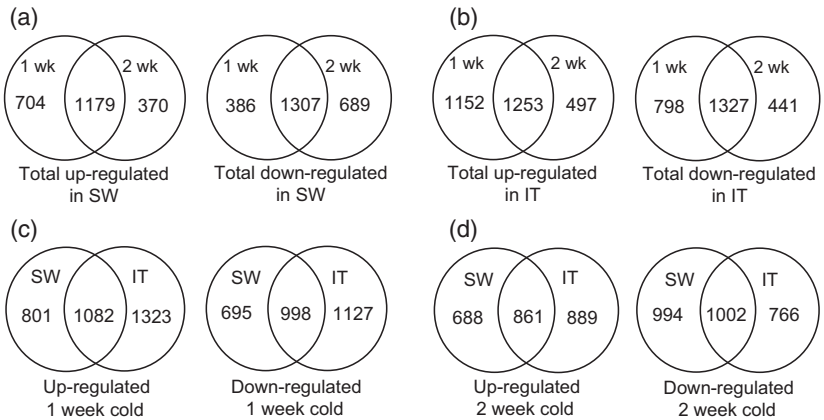

Figure 1. Comparison of cold-regulated genes in Swedish (SW) and Italian (IT) plants.
Plants were grown at 22°C and rosette leaf tissue was either harvested directly or after plants had been transferred to 4°C for 1 or 2 weeks. Gene expression was measured by RNA sequencing (RNAseq). Genes with at least a two-fold change (false discovery rate = 0.01) were considered differentially expressed.
(a, b) Venn diagrams comparing the total number of up- or downregulated genes in SW (a) or IT (b) plants exposed to low temperature for 1 or 2 weeks.
(c, d) Venn diagrams comparing the total number of up- or downregulated genes in SW and IT plants exposed to low temperature for either 1 (c) or 2 (d) weeks.

genes (see Thomashow, 2010). Thus, we compared the COR gene sets of the SW and IT plants, as differences could potentially contribute to the differences in freezing tolerance exhibited by the two plant genotypes. Rosettes were harvested from non-acclimated plants and plants that had been exposed to low temperature for 1 or 2 weeks and the transcriptome compositions of the plants were determined by RNA sequencing (RNAseq) analysis. Freezing tolerance tests indicated that under our experimental conditions, which were very different from those used by Oakley *et al.* (2014) in their analysis of freezing tolerance, the SW plants were also more tolerant of freezing than the IT plants: freezing tolerance of both the IT and SW plants increased about equally in response to low temperature for 1 week, but after 2 weeks of cold acclimation, the freezing tolerance of the SW plants was greater than that of the IT plants (Figure S1 in Supporting Information).

The RNAseq analysis indicated that more than 4600 SW genes and more than 5400 IT genes were differentially expressed at least two-fold [$\log_2 = 1$, false discovery rate (FDR) = 0.01] in plants exposed to low temperature for either 1 or 2 weeks (Figure 1a,b, Table S1). In both ecotypes, most of the COR genes that were differentially expressed at 1 week were also differentially expressed at 2 weeks (Figure 1a,b). At the 1- and 2-week time points, 34–36% of the COR genes were up- or downregulated in both the SW and IT plants, indicating a substantial percentage of 'common' COR genes (Figure 1c,d; the 34–36% range indicates the low and high percentages for the four comparisons shown in Figure 1c,d). However, there also appeared to be considerable differences in the sets of COR genes. At 1 week of cold acclimation, 25–27% of the up- and downregulated COR genes were categorized as 'specific' to the SW plants and 40–41% specific to the IT plants. Similarly, at 2 weeks of cold acclimation, 28–36% of the up- and downregulated COR genes were specific to the SW plants and 28–36% were specific to the IT plants.

The assignment of COR genes to the 'common' and 'specific' categories was based on the arbitrary cut-off of a two-fold change ($\log_2 = 1$, FDR = 0.01). Thus, it was possible that some of the specificity was due to the specific cut-off used. To gain additional insight into the similarities and differences between SW and IT COR gene sets and their regulation by low temperature, we examined heat maps prepared from the RNAseq data. The heat map prepared from the 'common' COR genes, as expected, indicated that these genes were induced or repressed, respectively, in response to low temperature in both the SW and IT plants at either the 1- or 2-week time point or both (Figure 2a). However, this was also true for a large percentage of the 'specific' COR genes (Figure 2b). Indeed, only about one-third of the 'specific' genes showed clear differences in regulation between the two ecotypes. These genes comprised eight groups labeled 1–8 (Figure 2b): group 1 genes were induced in the SW plants, but largely unaffected in the IT plants; group 2, 3 and 7 genes were largely unaffected in SW plants but the former two were induced and the latter unaffected in the IT plants; group 4, 6 and 8 genes were downregulated in SW plants but either induced or largely unaffected in the IT plants; and group 5 genes were induced in SW plants but repressed in IT plants. Thus, of the total number of 'common' and 'specific' COR genes, about 80% were COR genes in both the SW and IT plants, with 75% correspondingly induced or repressed in both ecotypes and 5% differentially cold regulated, but expressed in the opposite direction in the two ecotypes.

The CBF regulon genes are more highly induced in SW plants than in IT plants

Given the established role of the CBF pathway in freezing tolerance, we asked whether there were differences in the expression of CBF regulon genes in the SW and IT plants. A set of 133 cold-induced CBF regulon genes identified in Arabidopsis Ws-2 (Park *et al.*, 2015) was used for the comparison. A heat map prepared from the RNAseq data (Figure 3a, Table S2) showed that many CBF regulon genes were highly induced in both the SW and IT ecotypes

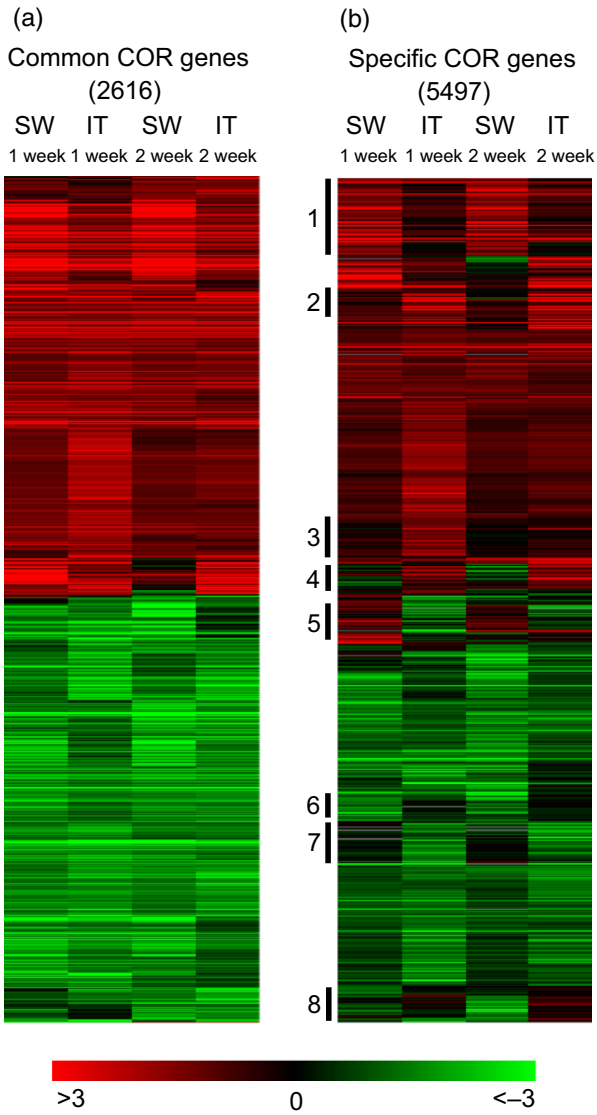

Figure 2. Heat maps comparing expression of 'common' and 'specific' Swedish (SW) and Italian (IT) CBF-targeted cold-regulated (COR) genes.
Hierarchical clustering of differentially expressed COR genes determined to be 'common' (a) or 'specific' (b) in the SW and IT ecotypes (see text for details of comparisons). Those groups of genes labeled 1–8 in (b) showed clear differences in cold regulation between the two ecotypes. The color scale represents $\log_2$-fold change.

(labeled 'A'). However, the heat maps also showed that a considerable number of the CBF regulon genes that were highly induced in the SW plants were induced to a much lower extent in the IT plants (labeled 'C') or barely at all (labeled 'B'). Indeed, graphs presenting the ratio of CBF regulon gene induction in the IT plants compared with their induction in the SW plants show that most of the CBF regulon genes were 'downregulated' in the IT plants (Figure 3b,c; green lines). However, the differences in cold induction varied greatly across the population of CBF regulon genes. Whereas cold induction of some genes was reduced by more than 90% in the IT plants, compared with the SW plants, others were little affected and some were actually induced to higher levels in the IT plants (Figure 3b,c; red lines).

The IT *CBF2* gene encodes a non-functional protein

One possible explanation for the lower level of cold induction of CBF regulon genes in the IT plants was that cold induction of the *CBF* genes might have been greatly reduced in the IT plants. The RNAseq data indicated that the transcript levels for *CBF1*, *CBF2* and *CBF3* were nearly the same in the IT and SW plants exposed to low temperature for 1 week, but that at 2 weeks the levels for *CBF1* and *CBF2* were about two-fold higher in the SW plants (Figure 4a). Thus, there were differences in levels of *CBF* gene expression between the IT and SW plants, but it seemed unlikely that these relatively small differences could fully account for the large differences in CBF regulon gene expression observed in the IT and SW plants.

Another explanation for the lower level of CBF regulon expression in the IT plants was that one or more of the IT CBF proteins might have been functionally impaired. To test this possibility, we determined the DNA sequences of the protein-coding regions of the IT and SW *CBF* genes and compared the predicted amino acid sequences of the CBF proteins. The results indicated that the DNA sequences of the *CBF1* and *CBF3* genes (Figures S2 and S4) and the predicted amino acid sequences of the CBF1 and CBF3 proteins (Figure S5) are nearly identical in the IT and SW plants. However, we found that the IT *CBF2* gene had a 13-bp deletion that resulted in a premature stop codon at amino acid 129 (Figures 4b, S3 and S5). The IT CBF2 protein retains the AP2/ERF DNA-binding domain, but lacks the C-terminal region, which encodes multiple transcriptional activation domains (Wang *et al.*, 2005). Thus, the IT CBF2 protein would not be expected to induce expression of CBF regulon genes or affect freezing tolerance.

To assess the functional activity of the IT CBF2 protein, we placed the IT CBF2 coding sequence under control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter, transformed the gene fusion into IT plants and determined the expression levels of CBF regulon genes and plant freezing tolerance. Three transgenic lines (60-1, 60-4 and 60-5) were obtained that expressed the IT *CBF2* transgene at levels between 400- and 1300-fold higher than the endogenous IT *CBF2* gene in plants grown at 22°C (Figure 5a) (the IT *CBF2* transgene levels were between 40- and 130-fold higher than that of the endogenous *CBF2* gene in plants exposed to low temperature for 2 weeks). However, despite this very high level of IT *CBF2* expression, freezing tolerance

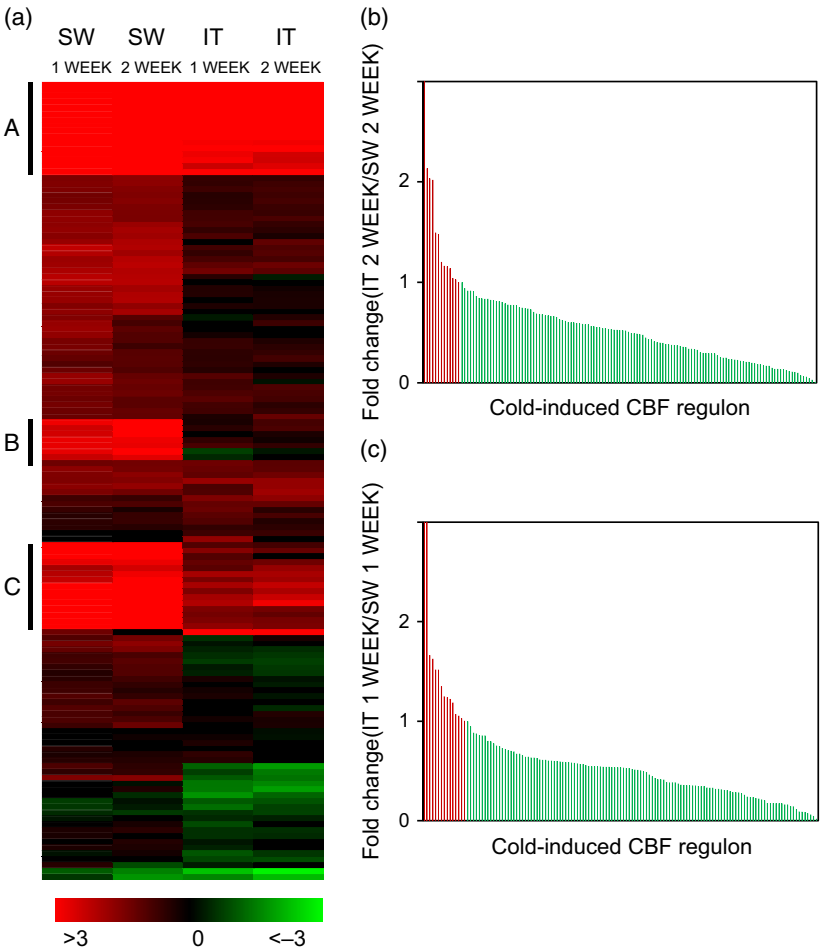

Figure 3. Most cold-induced C-repeat binding factor (CBF) regulon genes are more highly induced in Swedish (SW) plants than in Italian (IT) plants.
(a) Hierarchical clustering of CBF regulon transcript levels. Log_2-fold change in transcript levels for CBF regulon genes in SW and IT plants exposed to low temperature (4°C) for 1 or 2 weeks compared with warm-grown plants (22°C). The color scale represents log_2-fold change.
(b, c) Ratio of log_2-fold change values in transcript levels for cold-induced CBF regulon genes in IT plants vs. SW plants. Plants were exposed to low temperature (4°C) for 1 (b) or 2 weeks (c). Genes induced to a lesser or greater degree in IT plants compared with SW plants are indicated with green and red lines, respectively.

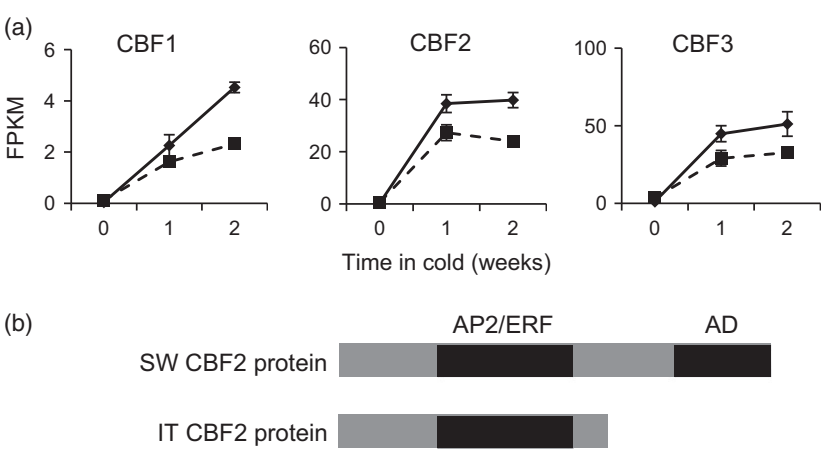

Figure 4. Comparison of *CBF* transcript levels and CBF2 proteins in Swedish (SW) and Italian (IT) plants.
(a) Transcript levels for *CBF1*, *CBF2* and *CBF3* were measured by RNA sequencing (RNAseq) (FPKM, fragments per kilobase of transcript per million mapped reads) (Table S1). Plants were grown at 22°C and rosette tissue was sampled either directly or after plants had been exposed to low temperature (4°C) for 1 or 2 weeks. Solid line, SW; dashed line, IT. The results presented are average values from three independent experiments ($n = 3$). Error bars indicate ±SEM.
(b) Schematic of the CBF2 proteins from the IT and SW ecotypes. AP2/ERF is the DNA-binding domain; AD is the transcriptional activation domain.

(Figure 5b) and the expression of three commonly studied cold-regulated genes – *COR47*, *COR78* and *COR314* – that are induced by the CBF1, CBF2 and CBF3 proteins from Arabidopsis Col-0 (Park *et al.*, 2015) (Figure 5a), were unaffected. These results provide direct evidence that the IT *CBF2* gene encodes a non-functional CBF2 protein.

A major eQTL conditioning the lower-level induction of CBF regulon genes in the IT ecotype maps to the *CBF* locus

To test the hypothesis that the non-functional IT CBF2 protein accounted for impaired cold induction of CBF regulon genes in the IT plants, we conducted an eQTL mapping

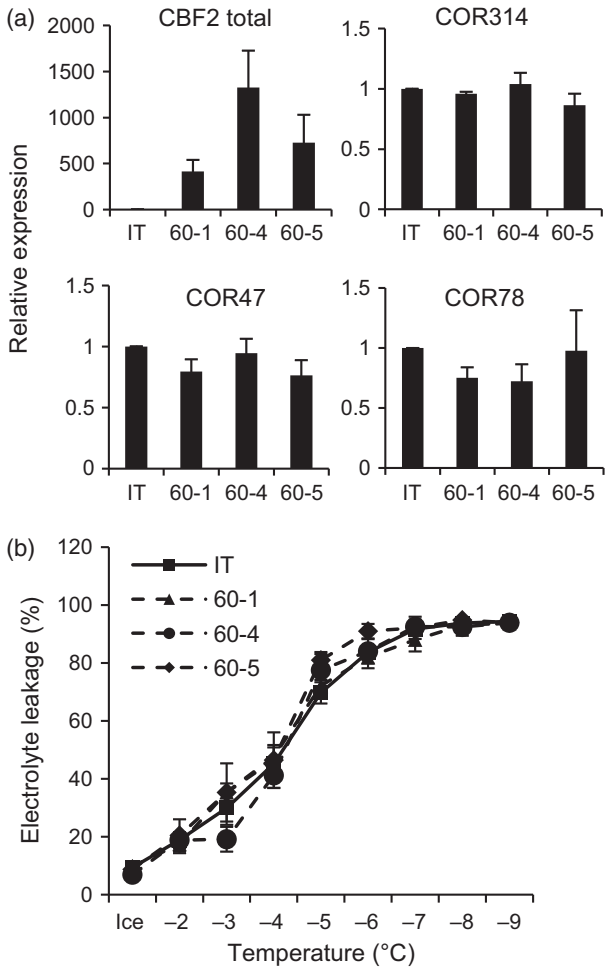

Figure 5. Overexpression of the Italian (IT) CBF2 protein-coding sequence does not induce expression of C-repeat binding factor (CBF) regulon genes or increase freezing tolerance.
Parent IT plants (IT) and transgenic IT plants constitutively overexpressing the IT CBF2 coding sequence placed under control of the strong constitutive CaMV 35S promoter (lines 60-1, 60-4, 60-5) were grown at 22°C and tested for gene expression and freezing tolerance.
(a) RNA was extracted from leaf tissue and the relative levels of the indicated transcripts were determined by quantitative real-time PCR. 'CBF2 total' includes transcripts for the endogenous *CBF2* gene and the *CBF2* transgene. Values for the IT parent plants were set to one.
(b) Freezing tolerance of the indicated plants was measured by the electrolyte leakage assay.
The results in (a) and (b) are from three independent experiments (n = 3). Error bars indicate ±SEM.

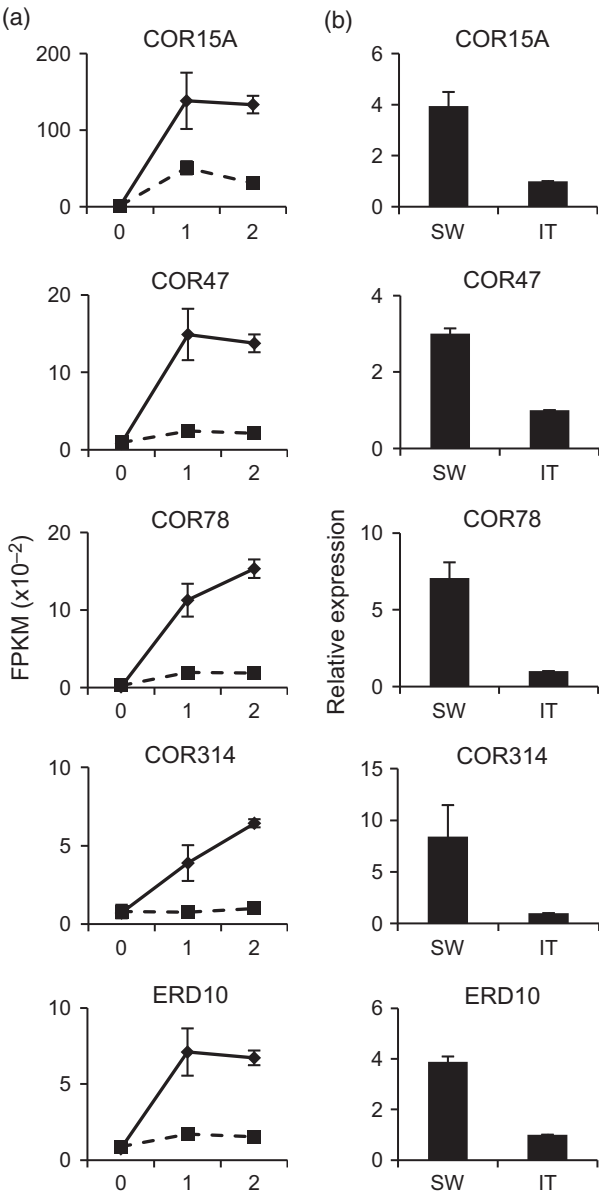

Figure 6. Cold induction of *COR15a*, *COR47*, *COR78*, *COR314* and *ERD10* is greater in Swedish (SW) plants than in Italian (IT) plants.
(a) Transcript levels (FPKM, fragments per kilobase of transcript per million mapped reads) of the indicated C-repeat binding factor (CBF) regulon genes in plants exposed to low temperature for 0, 1 and 2 weeks. Transcript levels were determined by RNA sequencing (RNAseq) (Table S2). Solid lines, SW; dashed lines, IT.
(b) Relative transcript levels for the indicated CBF regulon genes in plants exposed to low temperature for 2 weeks compared with plants grown at 22°C. Transcript levels were determined by quantitative real-time PCR.
The results presented in (a) and (b) are average values from three independent experiments (n = 3). Error bars indicate ±SEM.

experiment using the population of RILs that Ågren *et al.* (2013) developed from a cross between the parent IT and SW genotypes. We exposed 530 RILs to low temperature for 2 weeks, isolated RNA from each line and used quantitative real-time PCR (qRT-PCR) to determine the relative transcript levels for five CBF regulon genes – *COR15A*, *COR47*, *COR78*, *COR314* and *ERD10* – that were induced to a greater degree in the SW parent plants than the IT parent plants (Figure 6, Table S3). These results were then used to map eQTL that affected expression of the five CBF regulon genes. For each CBF regulon gene tested, there was a major eQTL that mapped to the bottom end of chromosome 4 at a location that overlapped the *CBF* locus (Fig-

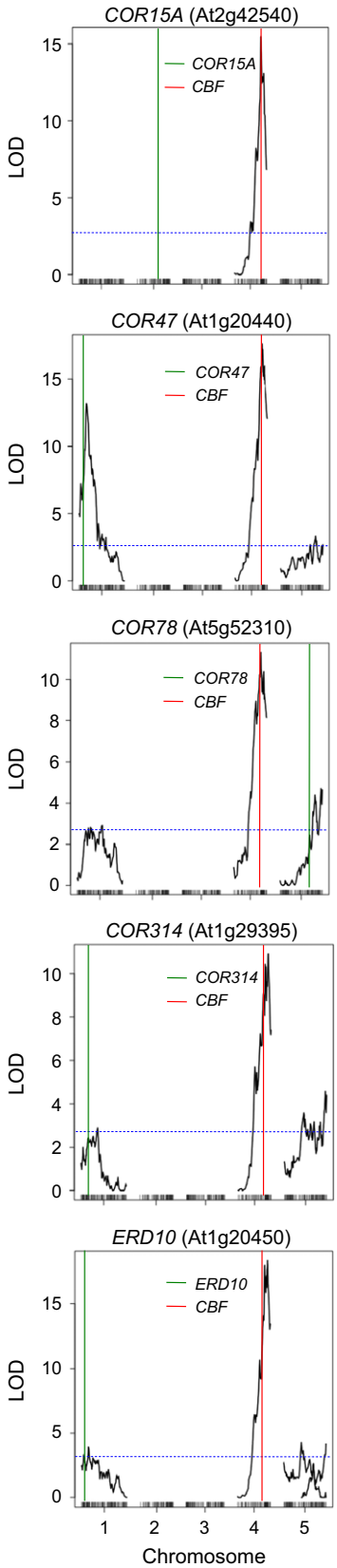

Figure 7. A major expression quantitative trait locus (eQTL) affecting cold induction of C-repeat binding factor (CBF) regulon genes maps to a region overlapping the *CBF* locus.
A total of 530 recombinant inbred lines developed from a cross between the parent Italian (IT) and Swedish (SW) genotypes (Ågren *et al.*, 2013) was used to map eQTL affecting cold induction of the indicated CBF regulon genes. Plants were grown at 22°C and then transferred to 4°C for 2 weeks. Rosette tissue was then harvested, total RNA isolated and the relative transcript levels of the CBF regulon genes determined by quantitative real-time PCR. The dashed blue horizontal lines indicate the 95% Bayesian credible interval. The vertical red and green lines indicate the physical locations of the *CBF* locus and CBF regulon genes, respectively. LOD, logarithm of odds.

ure 7, Table S4). In each case, the locus from the SW plants conditioned higher levels of expression of CBF regulon genes. These results were consistent with the truncated IT CBF2 protein contributing to the impaired induction of CBF regulon genes in the IT plants.

A second major eQTL was identified for *COR47* on chromosome 1, again with the SW locus conditioning the higher level of gene expression (Figure 7, Table S4). This eQTL overlapped the physical location of *COR47*, and thus might be a *cis*-acting eQTL. Hints of minor eQTL mapping to chromosomes 1 and 5 were also detected for *COR78*, *COR314* and *ERD10*. This common pattern suggests that these may be additional *trans*-QTL affecting the expression of these three CBF regulon genes. However, the eQTL detected for *COR314* and *ERD10* on chromosome 1 might be *cis*-eQTL as these two genes are also located on chromosome 1. Similarly, the eQTL on chromosome 5 for *COR78* might be a *cis*-eQTL, as *COR78* is located on chromosome 5 near the peak of the eQTL. Additional experiments will be required to decide between these different regulatory models.

Transformation of IT plants with the SW *CBF2* gene increases expression of CBF regulon genes and freezing tolerance

The results presented above indicated that the IT CBF2 protein was non-functional and that this was likely to account, at least in part, for the lower level of CBF regulon expression and freezing tolerance observed in the IT plants compared with the SW plants. To test this hypothesis, we transformed IT plants with either the IT *CBF2* gene or the SW *CBF2* gene (regulated by their endogenous promoters) and determined the effects that this had on cold induction of CBF regulon genes and freezing tolerance. The results indicated that transformation of the IT plants with the IT *CBF2* gene had no effect on cold induction of CBF-targeted COR genes (Figure 8a) or freezing tolerance (Figure 8b). In contrast, the IT plants transformed with the SW *CBF2* gene expressed the CBF-targeted COR genes at higher levels (Figure 8a) and were more tolerant of freezing (Figure 8b) than the IT parent plants. These results support the

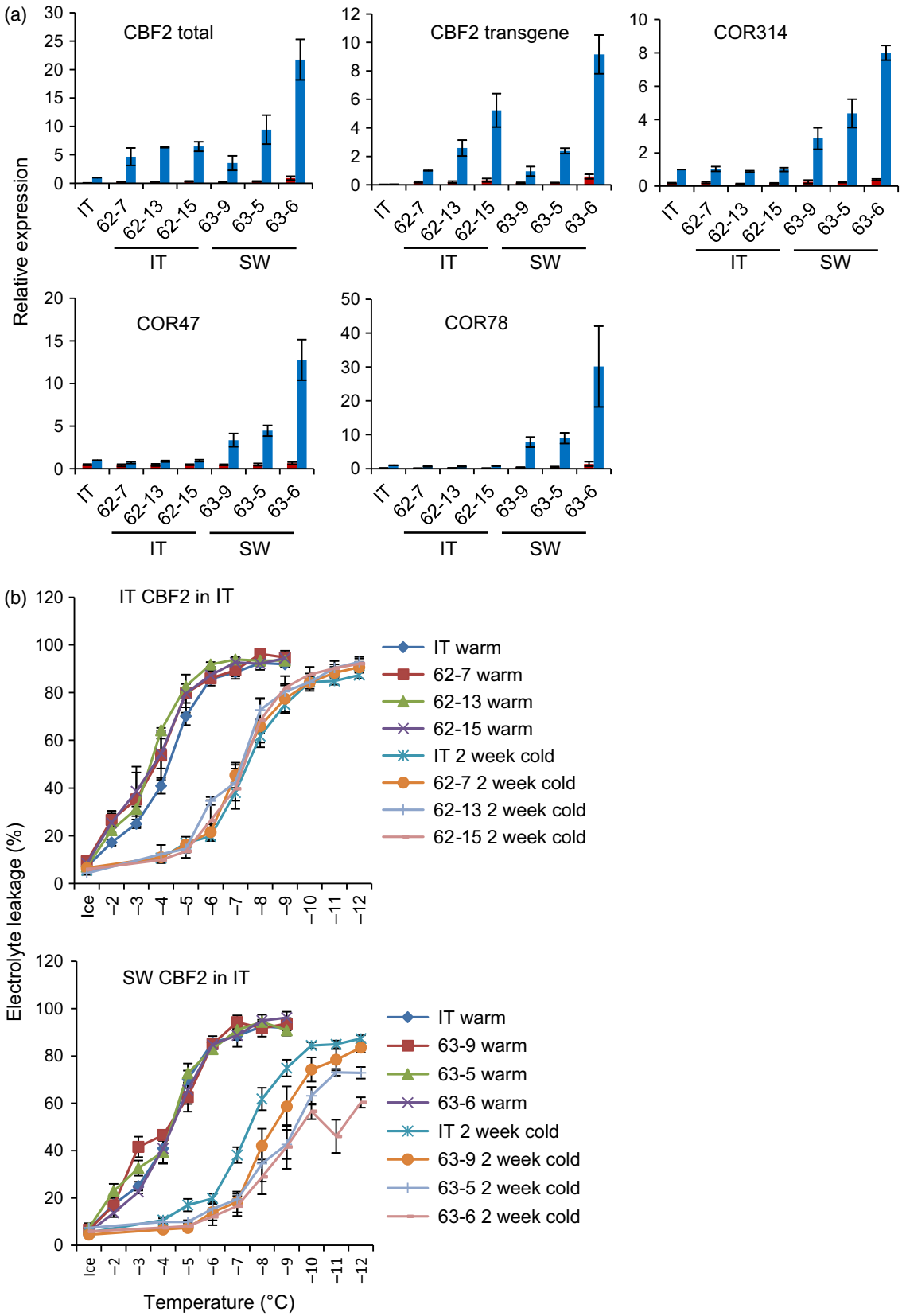

Figure 8. Transformation of Italian (IT) plants with the Swedish (SW) *CBF2* gene increases cold-induced expression of C-repeat binding factor (CBF) regulon genes and plant freezing tolerance.
Italian plants transformed with the IT *CBF2* gene (lines 62-7, 62-13, 62-15) or the SW *CBF2* gene (lines 63-9, 63-5, 63-6) were grown at 22°C and then transferred to 4°C for 2 weeks.
(a) RNA was extracted from leaf tissue and the relative levels of the indicated transcripts were determined by quantitative real-time PCR. Red bars, plants grown at 22°C; blue bars, plants treated at 4°C for 2 weeks.
(b) Freezing tolerance was measured by the electrolyte leakage assay.
The results in presented in (a) and (b) are from three independent experiments. Error bars indicate ±SEM.

hypothesis that the non-functional IT *CBF2* gene contributes to the lower level of CBF regulon expression and freezing tolerance observed in the IT plants compared with the SW plants.

DISCUSSION

Ågren and Schemske (2012) collected Arabidopsis accessions from sites in Italy and Sweden, established that these natural populations were locally adapted and presented evidence implicating freezing tolerance as an adaptive trait. In follow-up studies (Ågren *et al.*, 2013; Oakley *et al.*, 2014), the Ågren and Schemske laboratories identified QTL associated with fitness and freezing tolerance in a RIL population developed from the Arabidopsis IT and SW genotypes. Of particular interest was that QTL for both fitness and freezing tolerance mapped to a region on chromosome 4 that overlapped the *CBF* locus. In field experiments conducted over many years it was found that the IT *CBF*-containing region conditioned greater fitness in plants grown at the Italian site and that the SW *CBF*-containing region produced greater fitness in plants grown at the Swedish site (Ågren *et al.*, 2013). In growth chamber experiments, it was found that the SW ecotype was more tolerant of freezing than the IT ecotype and that the SW *CBF*-containing region conditioned greater freezing tolerance than did the IT *CBF* region (Oakley *et al.*, 2014). Given the role of the CBF pathway in freezing tolerance, the possibility raised was that one or more of the *CBF* genes accounted for the difference in freezing tolerance between the IT and SW genotypes and thereby contributed to local adaptation. Here we present strong evidence in support of this possibility, demonstrating that differences in the IT and SW *CBF2* genes contribute to the difference in freezing tolerance observed between the IT and SW ecotypes.

Our results indicate that both the IT and SW ecotypes have three *CBF* genes that are induced in response to low temperature (Figure 4), but that the predicted IT CBF2 protein is severely truncated in comparison with the SW CBF2 protein (Figures 4b and S5): the IT CBF2 protein retains the AP2/ERF DNA-binding domain, but lacks the transcriptional activation domains present in the C-terminal region of the CBF proteins (Wang *et al.*, 2005). Thus, the IT CBF2 protein would be expected to be largely inactive. Indeed, constitutive overexpression of the IT CBF2 protein had no effect on the expression of CBF regulon genes or freezing tolerance in transgenic plants grown at warm temperatures (Figure 5). Moreover, cold induction of most CBF regulon genes was lower in the IT plants compared with SW plants (Figure 3) and major eQTL determining the degree to which five CBF regulon genes were induced in response to low temperature mapped to the *CBF* locus – the IT *CBF* locus imparting lower levels of cold induction than the SW *CBF* locus (Figure 7). Finally, IT plants transformed with the SW *CBF2* gene expressed CBF regulon genes at higher levels and were more tolerant of freezing than the parent IT plants (Figure 8). It should be noted that Oakley *et al.* (2014) also concluded that the IT *CBF2* gene encoded a non-functional protein, but the results cited in support of this assertion were 'unpublished data', and thus we are unable to compare their results with ours.

Taken together, our results along with those presented by the Ågren and Schemske labs (Ågren and Schemske, 2012; Ågren *et al.*, 2013; Oakley *et al.*, 2014), support the view that the CBF pathway has contributed to local adaptation in natural populations of Arabidopsis. Moreover, as plants growing at the Swedish site experience lower winter temperatures that persist for longer periods of time than do plants growing at the Italian site, the greater fitness brought about by the SW *CBF* locus in plants grown at the Swedish site is probably due to the greater freezing tolerance conditioned by this locus. However, the SW *CBF* locus has also been shown to be maladaptive in plants grown in the IT environment (Ågren and Schemske, 2012; Ågren *et al.*, 2013). Is there reason to think that downregulation of the CBF pathway might be advantageous in environments that have relatively mild winters? Support for this notion is provided by the studies of Alonso-Blanco *et al.* (2005) and Kang *et al.* (2013), who have also identified defective *CBF2* genes in Arabidopsis accessions collected from relatively warm environments.

Alonso-Blanco *et al.* (2005) found that an Arabidopsis accession collected from the Cape Verde Islands (Cvi) was less tolerant of freezing than the Arabidopsis Landsberg *erecta* (L*er*) genotype, which was derived from an accession (La-0) originally collected in Poland (Koornneef and Meinke, 2010). Using a RIL population generated from a cross between Cvi and L*er* parents, the investigators mapped seven QTL affecting freezing tolerance, one of which overlapped the *CBF* locus. Further analysis indicated that the expression levels of *CBF2* and three CBF regulon genes were lower in cold-treated Cvi plants than in cold-treated L*er* plants and that the promoter region of the Cvi

CBF2 gene had a 1.6-kb deletion, which likely accounted for the lower level of *CBF2* gene expression. Additional genetic complementation experiments supported the model that the Cvi *CBF2* gene accounted for the lower levels of CBF regulon gene expression and freezing tolerance observed in the Cvi plants.

Kang *et al.* (2013) studied a monophyletic group of four natural populations of Arabidopsis collected along the Yangtze River, China, from sites that differed in average mean winter temperatures. The investigators found that the freezing tolerances of the four populations were negatively correlated with the mean January temperature (the lower the January temperature the greater the freezing tolerance) and that induction of three CBF regulon genes, including *GOLS3*, positively correlated with the freezing tolerance of the four accessions. Using an F_2 population developed from the most and least freezing tolerant accessions, the investigators mapped two eQTL that affected the degree to which *GOLS3* was induced in response to low temperature, one of which mapped to the *CBF* locus. DNA sequencing indicated that the *CBF2* gene from the less freezing tolerant accession had a single base pair insertion within the protein-coding region that resulted in a premature stop codon. Remarkably, the resulting truncated CBF2 protein is nearly identical to the truncated CBF2 protein that we identified in the IT ecotype. Presumably the *cbf2* mutation identified by Kang *et al.* (2013) contributed to the lower freezing tolerance of the accession from the warmer environment, though this was not tested.

Unlike the situation with the SW and IT ecotypes, where QTL for local adaptation have been identified, the experiments required to demonstrate local adaptation have not been conducted with the Cvi and L*er* accessions or the Yangtze River accessions. However, the parallels in the results from these three studies are striking. In each case, Arabidopsis accessions originating from environments with milder winters had *CBF2* genes with mutations that impaired their function, resulting in lower levels of expression of CBF regulon genes. It seems likely that these null or hypomorphic *cbf2* alleles arose from functional C*BF2* genes and were fixed by natural selection in Arabidopsis populations where downregulation of the CBF pathway was advantageous. In this context, the results of Lee and Thomashow (2012) are of interest. These investigators found that the freezing tolerance of Arabidopsis plants is regulated by photoperiod: at warm temperature (22°C), plants grown under a long-day photoperiod (16 h) were found to be less tolerant of freezing than plants grown under a short-day photoperiod (8 h). This difference in freezing tolerance was found to be due to repression of *CBF* gene expression brought about by concerted action of PHYB, PIF4 and PIF7 under long-day conditions but not under short-day conditions. The authors suggested that repression of the CBF pathway during the active growing season, when photoperiods are generally long and temperatures warm, would promote growth by diminishing the allocation of energy and nutrient resources to unneeded frost protection. In a similar fashion, 'tuning' the expression level of the CBF pathway to the degree of freezing tolerance required in a given environment, i.e. not allocating carbon and energy sources to freezing tolerance significantly beyond what is needed for survival and competition, might also be advantageous and provide a driver for selection.

It has been long established that the CBF pathway contributes to freezing tolerance in plants that acclimate to cold (Thomashow, 1999, 2010; Knight and Knight, 2012). Now, through the experiments conducted by Ågren, Schemske and colleagues (Ågren and Schemske, 2012; Ågren *et al.*, 2013; Oakley *et al.*, 2014) with the IT and SW ecotypes, and the molecular analysis presented here, there is compelling evidence that the CBF pathway has had a role in the adaptive evolution of Arabidopsis populations. Moreover, the results provide a new perspective on the adaptive value of freezing tolerance in contrasting thermal environments. In particular, the results of the field experiments with the RIL populations developed from the IT and SW ecotypes establish that the local genotypes at the *CBF* locus are strongly favored in the Italian and Swedish home environments, and more generally that freezing tolerance can contribute to a fitness tradeoff. The study of additional natural populations should provide further insight into the extent to which natural variation in the CBF pathway has contributed to local adaptation in Arabidopsis and the importance of freezing tolerance in the evolution of Arabidopsis ecotypes.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The Arabidopsis SW and IT accessions were collected from their native habitats in Sweden and Italy, respectively (Ågren and Schemske, 2012). The RILs used in this study were described previously (Ågren *et al.*, 2013). The IT (Castelnuovo-12) and SW (Rödåsen-47) ecotypes (deposited at the Arabidopsis Biological Resource Center) used in the experiments described here served as the parents used to make the RIL population.

Seeds were stratified for 3–5 days at 4°C and plants were grown on soil in pots at 22°C under a 12-h photoperiod with a light intensity of 100–120 μmol m^{-2} sec^{-1} as described (Dong *et al.*, 2011). Seeds were either sown directly onto soil and grown for 26 days (for the experiments shown in Figures 5 and 8) or were first grown for 7 days on sterile agar plates on Gamborg's B5 medium (Caisson Laboratories, http://www.caissonlabs.com/) with 1% sucrose before transfer to soil for an additional 11 days (all other experiments) and then exposed to low temperature (4°C) for 1 or 2 weeks at about 35 μmol m^{-2} sec^{-1} under a 12-h photoperiod.

Freezing tolerance tests

Electrolyte leakage assays were performed as described (Gilmour *et al.*, 2000; Doherty *et al.*, 2009). Plants were grown at 22°C on

soil under a 12-h photoperiod days and then transferred to 4°C (at ZT4, 4 h after dawn) under a 12-h photoperiod for 1 or 2 weeks as described above.

Cloning and transformation of *CBF2* genes

Constructs of the IT CBF2 coding sequence placed under control of the CaMV 35S promoter and the IT and SW CBF2 coding sequences controlled by their endogenous promoters were made as described in Methods S1.

Sequencing of IT and SW genes

Sequencing of the coding regions of *CBF1*, *CBF2* and *CBF3* was performed on DNA that had been amplified from genomic DNA by polymerase chain reaction (PCR) with Platinum Taq DNA Polymerase High Fidelity (Life Technologies; http://www.lifetechnologies.com/). The DNA fragment was either purified and sequenced directly (*CBF1*, *CBF3*) or cloned into PCR8/GW/TOPO (Life Technologies) and sequenced after amplification in *Escherichia coli* (all other genes). Sequencing was performed at the Research Technology Support Facility at Michigan State University.

Quantitative RT-PCR analysis

Total RNA was extracted from plants grown in soil using RNeasy Plant Mini kits (Qiagen, http://www.qiagen.com.) as described (Zarka *et al.*, 2003). Complementary DNA synthesis was performed on total RNA with random primers using the Reverse Transcription System (Promega, http://www.promega.com/). Complementary DNA was used as a template for qRT-PCR using fast SYBR Green master mix (Life Technologies) as described (Doherty *et al.*, 2009; Dong *et al.*, 2011). *IPP2* (*At3 g02780*) was used as a reference gene. The primers used for qRT-PCR are shown in Table S4.

RNAseq experiments

Rosette leaf tissue was collected from SW and IT plants (three experimental replicates) exposed to low temperature (4°C) for 0, 1 and 2 weeks. Total RNA was isolated for each experimental replicate using an RNeasy kit (Qiagen) and submitted to Michigan State University's Research Technology Support Facility (RTSF) for RNAseq library preparation and analysis as described in Methods S1. Genes with more than two-fold change and a *q*-value of <0.01 were designated as differentially expressed. Hierarchical clustering analyses were performed using CLUSTER (Eisen *et al.*, 1998) and the resulting clusters were visualized with TREEVIEW (http://rana.lbl.gov/EisenSoftware.htm). The SW and IT RNAseq data have been deposited in the Gene Expression Omnibus under accession number GSE67332.

Expression QTL mapping

We grew 530 RILs in soil for 18 days at 22°C under a 12-h photoperiod then cold-treated them at 4°C under a 12-h photoperiod for 2 weeks. Tissue was collected from the RILs and RNA was extracted as described above for the qRT-PCR experiments. The eQTL were determined as described in Methods S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Freezing tolerance of the Swedish and Italian plants.

Figure S2. Alignment of DNA sequences of the *CBF1* coding regions for Italian, Swedish and Col-0 plants.

Figure S3. Alignment of DNA sequences of the *CBF2* coding regions for Italian, Swedish and Col-0 plants.

Figure S4. Alignment of DNA sequences of the *CBF3* coding regions for Italian, Swedish and Col-0 plants.

Figure S5. Alignment of amino acid sequences for Italian, Swedish and Col-0 CBF1, CBF2 and CBF3.

Table S1. List of genes differentially expressed in Swedish and Italian plants after 1 and 2 weeks at 4°C.

Table S2. Expression levels of cold-induced CBF regulon genes in Italian and Swedish plants grown at low temperature.

Table S3. Expression quantitative trait locus mapping of COR genes with transformed data.

Table S4. Primer pairs used for quantitative real-time PCR.

Methods S1. Gene constructs, plant transformation, RNA sequencing analysis and expression quantitative trait locus mapping.

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