CBF-dependent and CBF-independent regulatory pathways contribute to the differences in freezing tolerance and cold-regulated gene expression of two Arabidopsis ecotypes locally adapted to sites in Sweden and Italy

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Abstract

Arabidopsis thaliana (Arabidopsis) increases in freezing tolerance in response to low non-freezing temperatures, a phenomenon known as cold acclimation. The CBF regulatory pathway, which contributes to cold acclimation, includes three genes—CBF1, CBF2 and CBF3—encoding closely-related transcription factors that regulate the expression of more than 100 genes—the CBF regulon—that impart freezing tolerance. Here we compare the CBF pathways of two Arabidopsis ecotypes collected from sites in Sweden (SW) and Italy (IT). Previous studies showed that the SW ecotype was more freezing tolerant than the IT ecotype and that the IT ecotype had a nonfunctional CBF2 gene. Here we present results establishing that the difference in CBF2 alleles contributes to the difference in freezing tolerance between the two ecotypes. However, other differences in the CBF pathway as well as CBF-independent pathways contribute the large majority of the difference in freezing tolerance between the two ecotypes. The results also provided evidence that most cold-induced CBF regulon genes in both the SW and IT ecotypes are coregulated by CBF-independent pathways. Additional analysis comparing our results with those published by others examining the Col-0 accession resulted in the identification of 44 CBF regulon genes that were conserved among the three accessions suggesting that they likely have important functions in life at low temperature. The comparison further supported the conclusion that the CBF pathway can account for a large portion of the increase in freezing tolerance that occurs with cold acclimation in a given accession, but that CBF-independent pathways can also make a major contribution.
Introduction

The CBF pathway has a prominent role in cold acclimation, the process whereby plants increase in freezing tolerance in response to low temperatures [1–3]. In Arabidopsis thaliana (hereafter referred to as Arabidopsis), the pathway includes action of three CBF genes which are physically linked in tandem array on chromosome 4 and encode closely related AP2/ERF family transcription factors that bind to the CRT/DRE DNA regulatory element present in the promoters of CBF-regulated genes [4–6]. Within minutes of exposing Arabidopsis to low non-freezing temperatures, CBF1, CBF2 and CBF3 [5, 7, 8]—also known as DREB1b, DREB1c and DREB1a, respectively [9]—are induced followed by induction of CBF-targeted cold-regulated (COR) genes, known as the CBF regulon [2, 10]. Expression of the CBF regulon leads to an increase in freezing tolerance as indicated by the findings that overexpression of CBF1, CBF2, or CBF3 in transgenic plants results in an increase in freezing tolerance without exposing plants to low temperature [9, 11, 12] and that down-regulation of the CBF pathway in plants exposed to low temperature results in a decrease in freezing tolerance [13, 14].

The natural range of Arabidopsis extends from North Africa to northern Europe and east to Central Asia encompassing geographical locations that differ greatly in winter freezing temperatures [15, 16]. Accordingly, natural variation in freezing tolerance has been observed among Arabidopsis accessions with those collected from cooler northern latitudes generally more freezing tolerant than those collected from warmer southern latitudes [17, 18]. Given the role of the CBF pathway in freezing tolerance, a question that arises is whether natural variation in the pathway has contributed to local adaptation in Arabidopsis. Recent studies provide strong evidence that it has. Ägren and Schemske (2012) [19] conducted reciprocal transplant experiments using Arabidopsis populations collected from sites in Sweden (SW) and Italy (IT) and found that the SW genotype was more fit (measured as survival and total fruit production) than the IT genotype when both genotypes were grown at the Sweden site, and that the reverse was true when both genotypes were grown at the Italy site. Moreover, the relative survival of the IT ecotype at the Sweden site was positively correlated with the minimum soil freezing temperature [19] implicating freezing tolerance as an adaptive trait. Indeed, direct testing showed that the SW ecotype was more freezing tolerant than the IT ecotype and a QTL for both fitness and freezing tolerance was mapped to a site on chromosome 4 that overlapped the CBF locus (the QTL was mapped using a RIL population developed from a cross between the IT and SW ecotypes) [20]. These results suggested that differences in the CBF locus of the IT and SW ecotypes contribute to the differences in freezing tolerance and the local adaptation observed in the IT and SW ecotypes. Consistent with this suggestion was the finding that the IT ecotype had a nonfunctional cbf2 gene and that transformation of the SW CBF2 gene into the IT plants resulted in an increase in freezing tolerance [21].

Here we used CRISPR/Cas9 technology to determine the extent to which the differences in freezing tolerance and cold-regulated gene expression observed between the IT and SW ecotypes are due to differences in their CBF pathways. Our results indicate that the non-functional IT CBF2 allele contributes to the difference in freezing tolerance between the SW and IT ecotypes, but that other differences in CBF-dependent and CBF-independent function account for the majority of the difference in freezing tolerance between the two ecotypes. Our results also provide evidence that most cold-induced CBF regulon genes in both the SW and IT ecotypes are coregulated by CBF-independent pathways. Finally, we compared our results with those recently published by Zhao et al. (2016) [22] and Jia et al. (2016) [23] who used CRISPR/Cas9 technology to test the role of the CBF pathway in the Col-0 and Col-3 accessions; Col-0 and Col-3 are reported to be derived from the Columbia accession [24, 25]. The comparison identified 44 CBF regulon genes that are conserved among the three accessions, but also...
revealed that there is considerable diversity in the genes that comprise the IT, Col-0 and SW CBF regulons.

Materials and methods

Plant material and growth conditions

The Arabidopsis thaliana IT and SW accessions used in this study have been described elsewhere [21]. 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 or 16-h photoperiod with a light intensity of 100–120 μmol m⁻² sec⁻¹ as described [26]. For RNAseq experiments, the plants were grown on soil in pots for 23 days and then exposed to low temperature (4°C) for 24 h or 2 weeks at about 35 μmol m⁻² sec⁻¹ under a 12-h photoperiod. For evaluation of the vernalization response, the plants were grown on soil in pots at 22°C under a 16-h photoperiod for 12 days and exposed to 4°C under a 16-h photoperiod for 0, 2, 4, 6 and 8 weeks, after which time the plants were moved back to 22°C for flowering.

Generation of cbf mutants using CRISPR/Cas9-mediated genome editing technology

To obtain the mutants with all three CBF proteins being nonfunctional, we utilized the multiplex genome engineering capability of the CRISPR/Cas9 system [27]. A plasmid for CRISPR was obtained from Dr. Jian-Kang Zhu. Briefly, three 19bp oligonucleotides designed to target the coding region of CBF1, CBF2 or CBF3 under control of the AtU3b, AtU6 or At7SL promoter, respectively, were cloned to a single binary vector (pCambia1300): CBF1, 5’-TCGCTGCATTAGCCCTCCG-3’; CBF2, 5’- TCGCCGCCATAGCTCTCCG-3’; CBF3, 5’- GCGGCGGCTGAAGCTGCGT-3’. The binary vector contained three single-guide RNA (sgRNA) modules, each of which was designed to target the coding region of each of the three CBFs. To prevent possible insertional effects by the T-DNA containing the CRISPR/Cas9 transgene, we removed the T-DNA from the CRISPR mutants by backcrossing the homozygous T3 lines of the mutants to the corresponding parents. Independent F2 lines from the backcrosses were used for the experiments to mitigate the formal possibility of off-site mutations affecting the phenotypes of interest.

The IT ecotype has a non-functional cbf2 allele due to a 13-bp deletion in the C-terminal region of the gene [21]. The frame-shift mutation results in a truncated protein that lacks the C-terminal activation domain (Fig 1; S1B and S1E Fig) and thus, overexpression of the protein does not induce expression of CBF regulon genes [21]. Using the CRISPR/Cas9 system, we made an IT line, it:cbf123, that had inactive alleles of all three CBF genes. The CRISPR cbf1 gene had an 18-bp deletion within the DNA binding domain (Fig 1; S1A and S1D Fig). Given that the deletion did not change the reading frame, the modified protein potentially had a functional activation domain and thus might be active. This was not the case as overexpression of the CRISPR cbf1 protein in Ws-2 did not induce expression of CBF regulon genes; in contrast, overexpression of the IT CBF1 gene did (S2 Fig). The CRISPR cbf2 gene, which was already inactive had an additional 3-bp deletion in the DNA binding domain (Fig 1; S1B and S1E Fig). The CRISPR cbf3 gene had a 1-bp mutation predicted to produce a truncated protein that did not have the activation domain (Fig 1; S1C and S1F Fig). Indeed, whereas overexpression of the IT CBF3 gene induced expression of CBF regulon genes, overexpression of the it:cbf123 CRISPR cbf3 gene did not (S2 Fig).

Using the CRISPR/Cas9 system, we also made an SW line with an inactive cbf2 gene, sw:cbf2, and a line that had inactive alleles of all three CBF genes, sw:cbf123 (Fig 1). The cbf2 gene
in sw:cbf2 had a 19-bp deletion in the DNA binding domain resulting in predicted protein that did not have an activation domain (Fig 1; S1B and S1E Fig). As would be expected, overexpression of this sw:cbf2 cbf2 gene in Ws-2 did not induce expression of CBF regulon genes, whereas overexpression of the SW CBF1 and CBF2 genes did (S3 Fig). In the sw:cbf123 plants, the CRISPR cbf1 gene had a 5-bp deletion in the DNA binding domain; the cbf2 gene had a 1-bp deletion in the DNA binding domain; and the cbf3 gene had a 1-bp deletion upstream of the activation domain (Fig 1; S1A–S1F Fig). None of these proteins had activation domains and thus were presumed to be inactive like the other CBF proteins without activation domains. Indeed, the CRISPR cbf3 allele in sw:cbf123 was predicted to form a truncated protein that ended at the same residue as the CRISPR cbf3 gene in the it:cbf123 plants, which, as indicated above, produced an inactive protein.

**Generation of transgenic plants overexpressing CBF alleles**

The coding regions of the cbf1 allele from it:cbf123, the cbf2 allele from sw:cbf2, and the cbf3 allele from it:cbf123, and corresponding WT alleles were amplified by PCR, cloned into the
Gateway vector pEarleyGate100 [28] under control of the CaMV 35S promoter, and transformed into Ws-2 plants using the floral dip procedure [29]. Homozygous T3 or higher plants were used for experiments. Primers used for PCR were: CBF1/cbf1, 5’-ACAGAGTACTCTGACTCAATGAACTC-3’ (forward) and 5’-GAATATTAGTAACTCCAAAGCGACACG-3’ (reverse); CBF2/cbf2, 5’-ATCTTCTACTTACTTACTCTACA3’ (forward) and 5’-GAATTTTAATAGCTCCATAAGGACACGTCATC-3’ (reverse); CBF3/cbf3, 5’-CAGAGTACTCTCAGCTC-3’ (forward) and 5’-GTTTTAATAACTCCATACGATACGTCGTCATC-3’ (reverse). The transcript levels for the various transgenes were determined by qRT-PCR (S2 and S3 Figs).

**Quantitative RT-PCR analysis**

Total RNA was extracted from plants using RNeasy Plant Mini kits (Qiagen, http://www.qiagen.com) as described [14]. Complementary DNA was synthesized from 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 [26, 30]. IPP2 (At3g02780) was used as a reference gene. The primers used for qRT-PCR are shown in S1 Table.

**Freezing tolerance tests**

Electrolyte leakage assays were performed as described [30, 31]. Plants were grown at 22˚C on soil under a 12-h photoperiod and then transferred to 4˚C under a 12-h photoperiod for 2 weeks as described above. EL\textsubscript{50} values were calculated from fitted third-order linear polynomial trends from the electrolyte leakage curves (S2 Table). Two-way analysis of variance was performed and significance of comparisons of responses among genotypes was determined by orthogonal contrast (S2 Table).

**Promoter motif analysis**

Motif enrichment was determined by counting the number of CRT/DRE DNA-binding motifs (rCCGAC) within the region 1 kb upstream of the translation start site for each gene based on TAIR version 10 annotation (Arabidopsis Information Resource; http://www.arabidopsis.org/). Motif enrichment in a group of genes was tested against a background distribution generated by 1000 random samplings from all genes in the Arabidopsis genome and presented as the Z-score and P value.

**RNAseq analysis**

Aerial tissue was collected from the IT, SW, CBF2-overexpressing, and cbf mutant plants (two experimental replicates) exposed to low temperature (4˚C) for 0 h, 24 h 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 sequencing. Sequencing was performed on an Illumina Genome Analyzer II (http://www.illumina.com). The RNAseq reads were mapped to the Arabidopsis thaliana reference genome (TAIR10) using Bowtie2 version 2.2.6 [32] and Tophat version 2.1.0 [33]. The resulting TopHat BAM files were used to estimate transcript abundance (FPKM) and identify differentially expressed genes using the Cuffdiff program within Cufflinks (version 2.2.1) [33]. Genes with a two-fold change (log\textsubscript{2} = 1) or more and an FDR = 0.01 were designated as differentially expressed. Hierarchical clustering analyses were performed using the hclust method of ‘amap’ in the R package (https://www.r-project.org/) and the resulting clusters were visualized with treeview (http://rana.lbl.gov/EisenSoftware.htm). The RNAseq data...
have been deposited in the Gene Expression Omnibus under accession number GSE106284. Gene ontology term enrichment was performed on the differentially expressed genes for biological processes with the entire nuclear gene set of 33278 (TAIR v10) as a background. P-values were calculated using the hypergeometric test.

Results

Differences in CBF2 alleles contribute to the difference in freezing tolerance between the SW and IT ecotypes

Oakley et al. (2014) [20] showed that the SW ecotype is more freezing tolerant than the IT ecotype and identified a QTL for freezing tolerance that overlapped the CBF locus. Gehan et al. (2015) [21] then showed that the IT CBF2 gene was nonfunctional and proposed that this accounted for the freezing tolerance QTL that overlapped the CBF locus. To test this possibility, we used CRISPR/Cas9 technology to inactivate the SW CBF2 gene, creating sw:cbf2 (Fig 1; S1B and S1E Fig; see Materials and Methods) and determined the effects that this had on freezing tolerance (Fig 2; S2 Table). The results indicated that there was little, if any, difference in freezing tolerance between non-acclimated SW and sw:cbf2 plants (Fig 2A). There was, however, a difference in freezing tolerance between cold-acclimated SW and sw:cbf2 plants: whereas the EL50 (the temperature at which freezing damage results in leakage of 50% of the total cellular electrolytes) of cold-acclimated SW plants was -12.4˚C, the EL50 of and sw:cbf2 plants was -11.6˚C (Fig 2B; S2 Table). Thus, loss of SW CBF2 function resulted in a 0.8˚C decrease in freezing tolerance indicating that differences in SW and IT CBF2 alleles did indeed contribute to the difference in freezing tolerance between the two ecotypes.

Both CBF-dependent and CBF-independent pathways contribute to the freezing tolerance of the SW and IT ecotypes

To determine the extent to which the freezing tolerance of SW and IT plants was dependent on the CBF pathway, we used CRISPR/Cas9 technology to completely inactivate the CBF locus of each ecotype—resulting in sw:cbf123 and it:cbf123, respectively (Fig 1; S1A–S1G Fig)—and determined the effects that this had on freezing tolerance (Fig 2; S2 Table). In the case of the SW plants, about 40% of the cold-induced increase in freezing tolerance was CBF-dependent: whereas the freezing tolerance of SW plants increased 7.3˚C in response to cold acclimation (EL50 values decreased from -5.1 to -12.4˚C), the freezing tolerance of sw:cbf123 plants increased 4.4˚C (EL50 values decreased from -4.5 to -8.9˚C) (Fig 2A and 2B). In contrast, only about 10% of the increase in freezing tolerance that occurred in IT plants in response to low temperature was CBF-dependent: whereas the freezing tolerance of IT plants increased 4.0˚C in response to cold acclimation (EL50 values decreased from -4.7 to -8.7˚C), the freezing tolerance of it:cbf123 plants increased 3.5˚C (EL50 values decreased from -3.8 to -7.3˚C) (Fig 2C and 2D). In absolute terms, the CBF-dependent pathways of the SW and IT plants contributed, respectively, a 2.9˚C and 0.5˚C increase in freezing tolerance in response to low temperature whereas the CBF-independent pathways contributed, respectively, a 4.4˚C and 3.5˚C increase in freezing tolerance. Thus, both the CBF-dependent and CBF-independent freezing tolerance pathways of the SW plants imparted greater levels of freezing tolerance than did the respective pathways in the IT plants.

Most cold-induced CBF regulon genes are coregulated by CBF-independent pathways in both the SW and IT ecotypes

Using the Ws-2 accession, Park et al. (2015) [14] identified 136 genes that were induced in plants exposed to low temperature for 24 h and in transgenic plants overexpressing CBF1,
CBF2 or CBF3 grown at warm temperature. They designated these genes members of the CBF regulon. Of these 136 genes, 117 (86%) and 89 (65%) were cold-induced (>2-fold, FDR = 0.01), respectively, in SW and IT plants exposed to low temperature for 24 h (Fig 3A; S3 Table). The transcript levels for most of these CBF regulon genes were reduced in the sw:cbf123 and it:cbf123 plants (Fig 3B and 3C; S3 Table), as would be expected for genes induced by the CBF transcription factors. However, a large proportion of these genes were still significantly up-regulated in the sw:cbf123 and it:cbf123 plants. Of the 117 CBF regulon genes that were induced in the SW plants, the induction of only 50 genes (43%) was reduced more than

Fig 2. Inactivation of the CBF pathway in SW and IT plants impairs freezing tolerance. Electrolyte leakage freeze tests were conducted on non-acclimated plants grown at 22°C (A, C) and cold-acclimated plants grown at 22°C and then exposed to 4°C for 2 weeks (B, D). sw:cbf2, SW with CBF2 mutated; sw:cbf123, SW with CBF1, CBF2, and CBF3 mutated; it:cbf123, IT with CBF1, CBF2, and CBF3 mutated (see Fig 1). Error bars indicate SE of three biological replicates. Third-order linear polynomial trends were fitted for the electrolyte leakage data for each genotype. EL_{50} values were calculated from the fitted models. Two-way analysis of variance was performed; significance of comparisons of responses among genotypes was determined by orthogonal contrast. P values and EL_{50} values are shown in S2 Table.

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50% in the sw:cbf123 plants (Fig 3D; S3 Table). Similarly, of the 89 CBF regulon genes that were induced in the IT plants, the induction of only 49 genes (55%) was reduced more than 50% in the it:cbf123 plants (Fig 3E; S3 Table). These results provide evidence that most of the SW and IT CBF regulon genes were co-regulated by one or more transcription factors in addition to the CBF transcription factors.

Similar results were obtained when the SW and IT plants were exposed to low temperature for two weeks, though fewer CBF regulon genes were induced at this time point: of the 136 CBF regulon genes, 76 (56%) and 40 (29%) were cold-induced, respectively, in SW and IT plants (Fig 4A; S4 Table). However, there was a striking difference regarding the expression of the CBF regulon genes in the sw:cbf123 and it:cbf123 plants. As observed with sw:cbf123 plants exposed to low temperature for 24 h, the transcript levels for most of the CBF regulon genes were reduced in the sw:cbf123 plants treated at low temperature for 2 weeks, although again, a large proportion remained significantly up-regulated: of the 76 CBF regulon genes that were
induced in the SW plants, the induction of only 39 genes (51%) was reduced more than 50% in the sw: cbf123 plants (Fig 4B and 4D; S4 Table). In contrast, of the 40 CBF regulon genes that were induced in the IT plants, the induction of only 8 genes (20%) was reduced more than 50% in the it: cbf123 plants (Fig 4C and 4E; S4 Table). Thus, in the IT genotype, it would appear that most of the CBF regulon genes that were induced after two weeks of cold treatment were up-regulated by transcription factors that were members of CBF-independent pathways.

Park et al. (2015) [14] identified 27 genes in Arabidopsis Col-0 that encoded transcription factors and were rapidly induced in response to low temperature in parallel with CBF1, CBF2 and CBF3. Further, they established that five of these “first-wave” genes—HSFC1, ZAT12, ZAT10, ZF, and CZF—encoded transcription factors that were able to induce one or more of 35 CBF regulon genes. Our results indicated that a majority of the 27 first-wave transcription factor genes of the Col-0 accession—including HSFC1, ZAT12, ZAT10, ZF, CZF—were also rapidly cold-induced in IT and SW plants indicating that they might also have a role in co-regulating CBF regulon genes in the SW and IT ecotypes (Fig 5). Moreover, cold-induction of
HSFC1, ZAT12, ZF, and CZF appeared to be independent of the CBF pathway as they were induced in the sw:cbf123 and it:cbf123 plants (Fig 5). Thus, induction of CBF regulon genes in the SW and IT ecotypes might also involve co-regulation by a common set of first-wave transcription factors.
There is considerable diversity in the genes that comprise the CBF regulons of the SW, IT and Col-0 genotypes

To further explore the issue of CBF regulon diversity, we identified the CBF regulons of the SW and IT ecotypes and compared them with each other and with the CBF regulon of Col-0 determined by Zhao et al (2016) [22]. In this analysis, we defined members of the SW, IT and Col-0 CBF regulons as genes that were cold-induced at 24 h and were reduced at least two-fold in their cold induction in their corresponding cbf123 triple mutants. The results indicated that there were considerable differences in the number of genes that comprise the CBF regulons of the three genotypes: there were 112, 321 and 421 genes for the IT, Col-0 and SW genotypes, respectively (Fig 6A; S5 and S6 Tables). Moreover, a comparison of the CBF regulon gene lists indicated that there were only 44 genes common to all three genotypes (Fig 6A; Table 1). As would be anticipated, the promoters for these 44 genes in the Col-0 genotype (for which a complete genome sequence is available) were highly enriched in the CBF DNA binding motif, rCCGAC (Table 2). In addition, the motif was highly enriched in the 68 Col-0 CBF regulon genes that were in common with the SW CBF regulon, the 29 Col-0 CBF regulon genes that were in common with the IT CBF regulon, and the 180 Col-0 CBF regulon genes that were “specific” to the Col-0 CBF regulon (Table 2). In contrast, the CBF DNA binding motif was not enriched in the promoters of the 296 CBF regulon genes that were specific to the SW ecotype and the 13 CBF regulon genes that were specific to the SW and IT ecotypes (Table 2), results that were consistent with these genes not being members of the Col-0 CBF regulon.

In regard to function, the 44 common CBF regulon genes were very highly enriched in the GO terms “response to cold,” “response to water deprivation” and “response to abscisic acid” (Table 3). These three GO terms were also the top very highly enriched terms in the complete CBF regulons of the Col-0 and IT genotypes (Table 3). The CBF regulon of the SW ecotype was also highly enriched in the GO terms “response to water deprivation” and “response to cold”, though not “response to abscisic acid”. Moreover, the SW CBF regulon was very highly enriched in the GO terms related to biotic stress responses including “regulation of plant-type hypersensitive response,” which was not highly enriched in the IT and Col-0 CBF regulons, and “response to chitin” and “defense response to fungus”, which were enriched in the IT and Col-0 CBF regulons, but to a much lesser degree. These results point to the CBF regulons of different genotypes having functional differences beyond the common function of freezing tolerance.

Finally, we found that there was a highly significant correlation between the number CBF regulon genes in the IT, Col-0 and SW genotypes and the minimum monthly low temperature average of the region from which the genotypes are reported to have been isolated: 2.7˚C for IT, -5.0˚C for Col-0 and -12.6˚C for SW [19, 34] (the Col-0 accession is derived from the La-1 accession which was isolated in Landsberg an der Warthe, now known as Gorzów Wielkopolski, a city in western Poland [24, 25]. Thus, as the minimum monthly low temperature average decreased, the number of CBF regulon genes increased (Fig 6B). A highly significant correlation was also observed between the total number of COR genes and the minimum monthly low temperature average of the region from which the accession was isolated (Fig 6C). It should be noted that our list of CBF regulon and total COR genes was based on using a statistical cut-off of FDR = 0.01 whereas Zhao et al. (2016) [22] used an FDR = 0.05 in their analysis. Using an FDR = 0.05 for analyzing the SW and IT data resulted in a small increase number of CBF regulon genes: SW increased from 421 to 471 and IT increased from 112 to 126 (S4A Fig). However, there was still a highly significant correlation between the number of CBF regulon genes or total COR genes and the minimum monthly low temperature average of the regions from which the accessions were collected (S4B and S4C Fig).
The CBF pathways of the IT and SW ecotypes retard plant growth at low temperature

It is long established that overexpression of the CBF pathway retards plant growth [9, 31, 35]. Consistent with this finding, the fresh weights of IT and SW plants were less than those of it:

**Fig 6.** There is considerable diversity in the genes that comprise the CBF regulons of the SW, IT and Col-0 genotypes. (A) Venn diagram showing the number of common and specific CBF regulon genes. Genes were assigned to the CBF regulon if they were induced at least two-fold in response to low temperature (4˚C for 24 h) and down-regulated in the *cbf123* triple mutant by at least two-fold (log₂ = 1; FDR = 0.01 for IT and SW and FDR = 0.05 for Col-0, see text). (B, C) The minimum monthly low temperature average for the genotypes were plotted against the number of CBF regulon genes (B) or total number of COR genes (C). A regression line for the data and R² value are shown. Red indicates it: *cbf123*; black, col:*cbf123*; blue, sw:*cbf123*.

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Table 1. CBF regulon genes in common between the SW, IT, and Col-0 genotypes.

| NAME | Description | %Reduction sw:cbf123/IT | %Reduction sw:cbf123/Col | %Reduction it:cbf123/IT |
|------|-------------|-------------------------|-------------------------|------------------------|
| AT2G43620 | Chitinase family protein | 92.7 | 83.9 | 92.9 |
| AT2G16990 | Major facilitator superfamily protein | 92 | 62.1 | 80.1 |
| AT3G22142 | Bifunctional inhibitor/lipid-transfer protein | 91.8 | 73.7 | 66.0 |
| AT4G30830 | Protein of unknown function DUF593 | 91.8 | 90.5 | 96.6 |
| AT1G9930 | GolS3 | 90.6 | 97.1 | 98.2 |
| AT3G50970 | LT30|dehydrin family protein | 88.5 | 94.9 | 91.9 |
| AT1G62570 | FMO G5-OX4 | 88.4 | 87.1 | 89.6 |
| AT5G2300 | RD29B|LTI65|CAP160 protein | 85.2 | 86.1 | 88.3 |
| AT4G3070 | Pyruvate decarboxylase family protein | 84.8 | 83.9 | 61.7 |
| AT4G23450 | RING/U-box superfamily protein | 83.7 | 60.9 | 61.9 |
| AT5G0720 | HVA22E|ATHVA22E|HVA22 homologue E | 81.8 | 91.5 | 75.8 |
| AT5G37500 | GORK|gated outwardly-rectifying K+ channel | 81.6 | 71.4 | 68.3 |
| AT3G55940 | Phospholipase C family protein | 80.7 | 69.5 | 92.6 |
| AT3G05660 | AtRLP33 | 79.9 | 70 | 95.0 |
| AT4G12470 | AZII|azelaic acid induced 1 | 77.5 | 65.4 | 70.3 |
| AT1G08890 | Major facilitator superfamily protein | 74.6 | 82 | 87.5 |
| AT1G51090 | Heavy metal transport/detoxification | 74.3 | 60.6 | 89.2 |
| AT5G15960 | KIN1 | 74.2 | 95.1 | 91.7 |
| AT3G17460 | unknown protein | 72.8 | 76.3 | 84.5 |
| AT1G46768 | RAP2.1 | 71.5 | 75.5 | 88.1 |
| AT3G17130 | Plant invertase/pectin methyltransferase inhibitor | 71.5 | 79.6 | 86.9 |
| AT3G53990 | Adenine nucleotide alpha hydrolases-like | 70 | 53.3 | 70.0 |
| AT1G47710 | Serine protease inhibitor | 69.2 | 52.1 | 80.0 |
| AT3G62740 | BGLU7|beta glucosidase 7 | 67.3 | 93.1 | 67.7 |
| AT1G08920 | ESL1, a transporter for monosaccharides | 66.9 | 56.4 | 59.5 |
| AT1G29395 | COR413-TM1 | 65.8 | 91.6 | 87.7 |
| AT5G47650 | ATNUDX2|nudix hydrolase homolog 2 | 62.9 | 55.9 | 66.4 |
| AT4G15910 | DII1|ATDII1|drought-induced 21 | 62.8 | 85 | 54.2 |
| AT4G21570 | Protein of unknown function (DUF300) | 62.7 | 61.9 | 81.9 |
| AT5G58700 | PLC4|phosphatidylinositol-phospholipase C4 | 60.5 | 66.4 | 78.1 |
| AT2G15970 | ATCOR413-PM1 | 58.9 | 73.6 | 84.1 |
| AT1G20450 | LTI45|ERD10 | 57.6 | 62.8 | 68.2 |
| AT1G64980 | Major facilitator superfamily protein | 57.5 | 52.5 | 83.9 |
| AT4G12000 | SNARE associated Golgi protein family | 56.4 | 59.2 | 79.9 |
| AT5G49900 | Beta-glucosidase GBA2 type family protein | 56.1 | 55.6 | 69.8 |
| AT4G34650 | SQS2|squale synthase 2 | 55.2 | 71.9 | 82.4 |
| AT4G24960 | HVA22 homologue D | 55 | 61.4 | 82.9 |
| AT1G53590 | NTMCT6.1|Calcium-dependent lipid-binding | 54.9 | 54.5 | 56.7 |
| AT5G10410 | ENTH/ANTH/VHS superfamily protein | 54.4 | 57.7 | 84.4 |
| AT2G42540 | cold-regulated 15a | 53.7 | 92.2 | 95.9 |
| AT5G57110 | ACA8|autoinhibited Ca2+ -ATPase isoform 8 | 53.4 | 54.6 | 72.9 |
| AT5G12140 | ATCYS1|CYS1|cystatin-1 | 52.7 | 62.6 | 70.0 |
| AT3G09540 | Pectin lyase-like superfamily protein | 50.9 | 51.8 | 57.6 |
| AT2G17280 | Phosphoglycerate mutase family protein | 50 | 50.8 | 70.0 |

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cbf123 and sw:cbf123 plants, respectively, when plants were grown at low temperature for three months (Fig 7).

Expression of the CBF pathway reduces the time to flowering of IT plants

During routine growth of the IT and it:cbf123 plants at warm temperature, it appeared that the it:cbf123 plants were generally delayed in flowering. Indeed, quantitative analysis indicated that this was the case: when IT and it:cbf123 plants were grown at warm temperature, the total number of leaves per generation were 70 and 86, respectively (Fig 8A) and the days to bolting were 40 and 51, respectively (Fig 8B). These results indicated that inactivation of the CBF pathway at warm temperature delayed flowering. Consistent with this observation was our finding that the transcript levels for the flowering repressor genes MAF5, MAF4 and FLC were higher in it:cbf123 plants than they were in IT plants (Fig 9A and 9B). An analogous comparison of the SW and sw:cbf123 plants was not possible as the SW ecotype requires vernalization to flower and thus there could not be a further delay in flowering of plants grown at warm temperature.

The CBF pathway does not affect the vernalization response of IT or SW plants

Given the effect of the CBF pathway on time to flowering of IT plants, we asked whether the pathway also affected the vernalization response. We found that it did not: the total number of leaves and days to bolting decreased in response to length of vernalization in both the IT and it:cbf123 plants (Fig 8A and 8B). Similar results were obtained with the SW and sw:cbf123 plants (Fig 8C and 8D).

Discussion

One goal of this study was to better understand the genetic basis for the difference in freezing tolerance between the SW and IT ecotypes. Previously, Oakley et al. (2014) [20] used a RIL population developed from a cross between the two ecotypes to map freezing tolerance QTL. Seven loci were identified, one of which overlapped the CBF locus with the SW CBF locus conditioning greater freezing tolerance than the IT CBF locus. Gehan et al. (2015) [21] found that the CBF2 gene of the IT genotype was nonfunctional and that transformation of the SW CBF2 gene into the IT plants increased plant freezing tolerance, thus providing indirect evidence that the SW CBF2 gene contributed to the difference in freezing tolerance between the two ecotypes. Here, we directly tested this hypothesis using CRISPR/Cas9 technology to inactivate the SW CBF2 gene. The results showed that cold-acclimated SW plants were 0.8°C more freezing tolerant than were cold-acclimated SW plants that had a nonfunctional cbf2 allele (Fig 2).
Table 3. Enrichment of GO biological processes for CBF regulon genes.

### CBF regulon genes common to the SW, IT and Col-0 genotypes (44)

| GO ID     | GO Term            | Target (%) | Genome (%) | P value |
|-----------|--------------------|------------|------------|---------|
| GO:0009409 | response to cold   | 36.4       | 1.2        | 5.5E-21 |
| GO:0009414 | response to water deprivation | 34.1       | 1          | 1.8E-20 |
| GO:0009737 | response to abscisic acid | 34.1       | 1.3        | 1.4E-18 |
| GO:0009631 | cold acclimation   | 13.6       | 0.1        | 2.2E-12 |
| GO:0009269 | response to desiccation | 11.4       | 0.1        | 9.7E-10 |

### CBF regulon genes of the Col-0 genotype (321)

| GO ID     | GO Term                          | Target (%) | Genome (%) | P-value |
|-----------|----------------------------------|------------|------------|---------|
| GO:0009409 | response to cold                 | 13.1       | 1.2        | 9.9E-32 |
| GO:0009737 | response to abscisic acid        | 13.1       | 1.3        | 5.5E-30 |
| GO:0009414 | response to water deprivation   | 10.9       | 1          | 1.2E-26 |
| GO:0009269 | response to desiccation         | 3.1        | 0.1        | 1.2E-12 |
| GO:0009651 | response to salt stress         | 9          | 1.9        | 1.7E-12 |
| GO:0042538 | hyperosmotic salinity response  | 4.7        | 0.5        | 2.1E-11 |
| GO:0010286 | heat acclimation                | 3.4        | 0.2        | 1.5E-10 |
| GO:0006970 | response to osmotic stress      | 3.7        | 0.4        | 6.3E-09 |
| GO:0006979 | response to oxidative stress    | 4          | 0.5        | 1.2E-08 |
| GO:0009631 | cold acclimation                | 2.2        | 0.1        | 1.6E-08 |
| GO:0030832 | defense response to fungus      | 5          | 0.9        | 3.5E-08 |
| GO:0010200 | response to chitin              | 5.3        | 1.3        | 3.5E-07 |
| GO:0009753 | response to jasmonic acid       | 4          | 0.8        | 9.2E-07 |
| GO:0009751 | response to salicylic acid      | 2.8        | 0.4        | 5.6E-06 |
| GO:0010310 | regulation of hydrogen peroxide metabolic process | 3.1 | 0.5 | 7.3E-06 |
| GO:0009611 | response to wounding            | 4          | 1          | 9.7E-06 |

### CBF regulon genes of the IT genotype (112)

| GO ID     | GO Term                                | Target (%) | Genome (%) | P-value |
|-----------|----------------------------------------|------------|------------|---------|
| GO:0009409 | response to cold                       | 25         | 1.2        | 1.1E-30 |
| GO:0009414 | response to water deprivation         | 20.5       | 1          | 4.5E-25 |
| GO:0009737 | response to abscisic acid             | 22.3       | 1.3        | 4.9E-25 |
| GO:0009631 | cold acclimation                       | 6.3        | 0.1        | 7.0E-12 |
| GO:0009269 | response to desiccation               | 6.3        | 0.1        | 2.0E-11 |
| GO:0009651 | response to salt stress               | 14.3       | 1.9        | 8.5E-11 |
| GO:0006970 | response to osmotic stress            | 8          | 0.4        | 4.2E-10 |
| GO:0042538 | hyperosmotic salinity response        | 7.1        | 0.5        | 3.0E-08 |
| GO:0050832 | defense response to fungus            | 8          | 0.9        | 4.6E-07 |
| GO:0009415 | response to water                     | 2.7        | 0          | 2.2E-06 |

### CBF regulon genes of the SW genotype (421)

| GO ID     | GO Term                                 | Target (%) | Genome (%) | P-value |
|-----------|-----------------------------------------|------------|------------|---------|
| GO:0010200 | response to chitin                     | 14         | 1.3        | 5.6E-44 |
| GO:0009414 | response to water deprivation          | 9.7        | 1          | 3.6E-28 |
| GO:0006612 | protein targeting to membrane          | 9.3        | 1.1        | 1.0E-24 |
| GO:0010363 | regulation of plant-type hypersensitive response | 9.3 | 1.1 | 1.3E-24 |
| GO:0050832 | defense response to fungus             | 8.6        | 0.9        | 6.8E-24 |
| GO:0009867 | jasmonic acid mediated signaling pathway | 7.8        | 0.8        | 1.3E-22 |
| GO:0009611 | response to wounding                   | 8.3        | 1          | 2.4E-22 |
| GO:0009723 | response to ethylene                   | 7.4        | 0.8        | 1.4E-21 |
| GO:0009738 | abscisic acid-activated signaling pathway | 6.9        | 0.6        | 1.9E-21 |

(Continued)
Thus, the SW CBF2 gene does indeed contribute to the difference in freezing tolerance between the SW and IT ecotypes.

Although the nonfunctional IT cbf2 allele contributes to the difference in freezing tolerance between the IT and SW ecotypes, the amount that it contributes would appear to be relatively small; i.e., the increase in freezing tolerance that occurred with cold acclimation of the SW plants was reduced by only about 10% in the sw:cbf2 plants. Thus, the large majority of the difference in freezing tolerance between the ecotypes would appear to due to differences beyond differences in CBF2 alleles. In fact, additional differences in the CBF pathways of the IT and SW ecotypes appear to contribute to their difference in freezing tolerance. This suggestion

Table 3. (Continued)

| GO ID          | GO Term                               | FDR  | q-value | p-value  |
|----------------|---------------------------------------|------|---------|----------|
| GO:0006865     | amino acid transport                  | 5.9  | 0.4     | 2.1E-21  |
| GO:0009409     | response to cold                      | 8.6  | 1.2     | 4.5E-20  |
| GO:0009697     | salicylic acid biosynthetic process   | 5.9  | 0.6     | 2.0E-17  |
| GO:0035556     | intracellular signal transduction     | 4.5  | 0.4     | 1.2E-14  |
| GO:0030968     | endoplasmic reticulum unfolded protein response | 5   | 0.6     | 2.5E-14  |
| GO:0010310     | regulation of hydrogen peroxide metabolic process | 4.5 | 0.5     | 2.2E-12  |
| GO:0010286     | heat acclimation                      | 2.9  | 0.2     | 3.1E-10  |
| GO:0009631     | cold acclimation                      | 1.4  | 0.1     | 3.1E-06  |
| GO:0006979     | response to oxidative stress          | 2.9  | 0.5     | 3.2E-06  |
| GO:0015824     | proline transport                     | 1.9  | 0.2     | 4.2E-06  |

Thus, the SW CBF2 gene does indeed contribute to the difference in freezing tolerance between the SW and IT ecotypes. Although the nonfunctional IT cbf2 allele contributes to the difference in freezing tolerance between the IT and SW ecotypes, the amount that it contributes would appear to be relatively small; i.e., the increase in freezing tolerance that occurred with cold acclimation of the SW plants was reduced by only about 10% in the sw:cbf2 plants. Thus, the large majority of the difference in freezing tolerance between the ecotypes would appear to due to differences beyond differences in CBF2 alleles. In fact, additional differences in the CBF pathways of the IT and SW ecotypes appear to contribute to their difference in freezing tolerance. This suggestion.
follows from our finding that the CBF pathway accounted for a 2.9˚C increase in freezing tolerance in the SW plants, but only a 0.5˚C increase in the IT plants, a considerably greater difference than the 0.8˚C decrease in freezing tolerance brought about by inactivating the SW CBF2 gene (Fig 2). One obvious possibility is that either the CBF1 or CBF3 gene (or both) of the IT ecotype is not as effective in promoting freezing tolerance as are the corresponding SW CBF genes. The results of Oakley et al. (2014) [20] provide some support for this hypothesis. In particular, their genetic analysis indicated that the freezing tolerance QTL overlapping the CBF2 locus accounted for about 36% of the variance in freezing tolerance between the two ecotypes, considerably more than the 10% difference in freezing tolerance that we observed in
converting the SW CBF2 allele to a nonfunctional cbf2 allele. It is possible that the differences in the freezing tolerance assays used in the Oakley et al. (2014) [20] study and ours accounts for this difference. Alternatively, it could be due to the single amino acid differences between

Fig 9. Expression of flowering repression genes MAF5, MAF4 and FLC were higher in it:cbf123 plants than in IT plants. Expression levels of MAF4, MAF5, and FLC were determined either by RNAseq (A) or by quantitative RT-PCR (B) in IT and it:cbf123 plants grown at 22°C (warm) or at 22°C followed by 2 weeks at 4°C (2wkV) under a 16-h photoperiod. Error bars indicate SE of two (A) or three (B) biological replicates. FPKM = Fragments Per Kilobase of transcript per Million mapped reads. Asterisks indicate a significant difference in expression level between IT and it:cbf123 plants (Student t-test, * indicates P<0.05, ** indicates P<0.01).

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the SW and IT CBF1 and CBF3 proteins [21] or potentially differences in the level of expression of the genes in general or in specific cell types. These possibilities remain to be tested.

While our results indicate that the IT CBF pathway does not impart the same level of freezing tolerance as does the SW CBF pathway, the results also indicate that there are differences in the level of freezing tolerance promoted by CBF-independent pathways. Whereas CBF-independent pathway genes of the SW plants imparted a 4.4°C increase in freezing tolerance in response to cold acclimation, the CBF-independent pathway genes of the IT plants only imparted a 3.5°C increase in freezing tolerance. In this regard, it is of interest that a number of “first-wave” genes that encode transcription factors are expressed at higher levels in SW plants than they are in IT plants (Fig 5) including two—ZAT12 and HSFC1—that have been shown to impart a small degree of freezing tolerance when overexpressed in the Col-0 accession [14, 36]. Also, eight of the first-wave transcription factors map to the freezing tolerance QTL identified by Oakley et al. (2014) [20]—ZAT10 and ERF11, FrzT1:1; DoF1.10, FrzT1:2; WRKY33 and CZF1, FrzT2:2; CRF2, FrzT4:1; DEAR1 and ATMYB44, FrzT5:2—and thus potentially contribute to the difference in freezing tolerance between the SW and IT ecotypes.

A second goal of this study was to better understand the extent to which the CBF pathway contributes to freezing tolerance in Arabidopsis. Park et al. (2015) [14] addressed this question using a dominant negative version of the CBF2 protein to impair function of the CBF pathway in the Ws-2 accession and found that it accounted for about 50% of the increase in freezing tolerance associated with cold acclimation. Using CRISPR/Cas9 technology, Zhao et al. (2016) [22] compared the freezing tolerance of Col-0 and col-0:cbf123 plants and found that the CBF pathway accounted for about 60% of the increase in freezing tolerance that occurred with cold acclimation. Similarly, Jia et al. (2016) [23] compared the freezing tolerance of Col-3 and col-3:cbf123 plants using CRISPR/Cas9 technology—the Col-0 and Col-3 accessions are reported to be derived from the Columbia accession [24]—and obtained results that were consistent with those of Zhao et al. (2016) [22]. And finally, here we used CRISPR/Cas9 technology to create the sw:cbf123 triple mutant and found that the CBF pathway accounted for about 40% of the increase in freezing tolerance that occurred with cold acclimation. Thus, with certain Arabidopsis accessions, the CBF pathway can account for a major portion of the increase in freezing tolerance that occurs with cold acclimation. However, it is also clear that this is not always the case as we observed that the CBF pathway of the IT ecotype only contributed about 15% of the increase in freezing tolerance that occurred with cold acclimation.

While it is clear that the CBF pathway can contribute a large portion of the increase in freezing tolerance that occurs with cold acclimation, it is equally evident that CBF-independent pathways can also contribute significantly to freezing tolerance. At present, knowledge of such pathways is limited. As alluded to above, overexpression of ZAT12 and HSFA1, which encode transcription factors that are rapidly induced in parallel with the CBF genes, has been shown to cause an increase in Arabidopsis freezing tolerance [14, 36], though the effects are much less than those caused by overexpression of either CBF1, CBF2 or CBF3 [9, 11]. The CAMTA1, CAMTA2 and CAMTA3 transcription factors have also been shown to contribute significantly to freezing tolerance; about 50% of the increase in freezing tolerance that occurs in Arabidopsis Col-0 plants in response to low temperature [30, 37] involve the CAMTA proteins. However, the CAMTA proteins are positive regulators of CBF1, CBF2 and CBF3 and thus, it is unclear to what extent the effects that the CAMTA transcription factors have on freezing tolerance are independent of their regulation of the CBF pathway. In short, additional study will be required to better understand the nature of the CBF-independent pathways that contribute as much as half the increase in freezing tolerance that occurs with cold acclimation in the SW, IT, Col-0 and Ws-2 (and likely other) accessions.
A third goal of this study was to better understand the degree to which the composition of the CBF pathway is conserved among Arabidopsis accessions. What we found in comparing our results with those of Zhao et al. (2016) [22] is that the CBF regulons of the SW, IT and Col-0 genotypes have 44 genes in common (of course, this number would increase somewhat if the fold-increase and statistical cutoffs used were less strict). The conserved set of 44 genes was highly enriched for the GO terms “response to cold,” “response to water deprivation” and “response to abscisic acid” (Table 3) which reflects the fact that freezing tolerance must include tolerance to the severe dehydration stress associated with freezing [3, 38]. Indeed, among the 44 conserved CBF regulon genes was GolS3, which encodes a key enzyme in the biosynthesis of raffinose, a sugar with cryoprotective and osmoprotective activities [39–41], and multiple LEA (and LEA-like) proteins, including COR15A, LTI45, KIN1 and LTI65, which are thought to protect proteins and membranes against damage caused by severe dehydration [42, 43]. Presumably, the other conserved CBF regulon genes also have important roles in life at low temperature, though the nature of these roles remains to be determined.

In addition to identifying a core set of conserved CBF regulon genes, our results combined with those of Zhao et al. (2016) [22] indicate that CBF regulons can differ greatly in gene number: the CBF regulons of the IT, Col-0 and SW genotypes were composed of 112, 321 and 421 genes, respectively. These differences would seem to have biological significance as the number CBF regulon genes in the three genotypes highly correlated with the minimum monthly low temperature average of the region from which the genotypes were isolated (Fig 6B; S4B Fig): as the minimum monthly low temperature average decreased, the number of CBF regulon genes increased. This correlation suggests that those plants which experience severe cold temperatures might require specific gene functions that are not required by plants which inhabit environments with more moderate low temperatures. The same suggestion applies to genes outside the CBF regulon as the total number of COR genes also varied greatly between the IT, Col-0 and SW genotypes and there was a significant correlation of this number with the minimum monthly low temperature average of the environment (Fig 6C; S4C Fig). These results add to the previous work of Hannah et al. (2006) [44] who reported a correlation between the freezing tolerance of nine Arabidopsis accessions and the number of genes that were differentially expressed in response to low temperature.

Finally, our results suggest that CBF pathways may have evolved to include differences in gene composition that contribute to local adaptation beyond conditioning the core function of freezing tolerance. In particular, the SW CBF regulon was found to be very highly enriched for genes that comprise the GO categories “regulation of plant-type hypersensitive response” and “jasmonic acid mediated signaling pathway,” categories that are not highly enriched in either the IT or Col-0 genotypes (Table 3). We also found that inactivation of the CBF pathway delayed flowering in the IT ecotype, but not the SW ecotype, a difference that might contribute to IT fitness in its natural habitat. The ability to use gene editing technologies to inactivate the CBF1–CBF2–CBF3 locus will facilitate studies to better understand the evolution of CBF regulon functions that have contributed to local adaptation of Arabidopsis.

Supporting information

S1 Fig. Alignment of nucleotide and amino acid sequences for the CBF coding regions in SW, IT and CRISPR mutant plants. Nucleotide sequences for CBF1 (A), CBF2 (B) and CBF3 (C) and amino acid sequences for CBF1 (D), CBF2 (E) and CBF3 (F) in IT, it:cbf123, SW, sw:cbf2 and sw:cbf123 plants. Differences between IT and SW sequences are indicated in red; CRISPR-induced mutations are indicated in blue. In protein sequences, red and blue lines
indicate the AP2 DNA-Binding Domain and Activation Domain, respectively.

**S2 Fig. The cbf1 and cbf3 alleles from it:cbf123 plants encode non-functional proteins.** The cbf1 and cbf3 alleles and corresponding CBF1 and CBF3 WT coding sequences from the IT ecotype were overexpressed in Ws-2 plants to determine whether they could induce expression of CBF regulon genes. (A) Photographs show that overexpression of the cbf1 and cbf3 alleles did not retard plant growth consistent with the proteins being non-functional. (B) Overexpression of cbf1 and cbf3 did not induce expression of the CBF regulon genes Gols3, COR15a or COR47. Names in parentheses on the x-axis indicate the lines from which the overexpressed alleles are cloned. Error bars indicate SE for three biological replicates.

**S3 Fig. The cbf2 allele from sw:cbf2 plants encodes a non-functional protein.** The cbf2 allele and corresponding CBF2 coding sequence from the SW ecotype were overexpressed in Ws-2 plants to determine whether they could induce expression of CBF regulon genes. (A) Photographs show that overexpression of the cbf2 allele did not retard plant growth consistent with the protein being non-functional. (B) Overexpression of cbf2 did not induce expression of the CBF regulon genes Gols3, COR15a or COR47. Names in parentheses on the x-axis indicate the lines from which the overexpressed alleles are cloned. Error bars indicate SE for three biological replicates.

**S4 Fig. The CBF regulons of the IT, SW, and Col-0 genotypes are composed of conserved and specific genes.** (A) Venn diagram showing the number of common and specific CBF regulon genes. Genes were assigned to the CBF regulon if they were induced at least two-fold in response to low temperature (4°C for 24 h) and down-regulated in the cbf123 triple mutant by at least two-fold (log₂ = 1, FDR = 0.05). (B, C) The average minimum monthly temperatures for the genotypes were plotted against the number of CBF regulon genes (B) or total number of COR genes (C). A regression line for the data and R² value are shown. Red indicates it: cbf123; black, col:cbf123; blue, sw:cbf123.

**S1 Table. Primers used for quantitative RT-PCR.**

**S2 Table. EL₅₀ values and statistical analysis of temperature response curves.** EL₅₀ values were calculated from fitted third-order linear polynomial trends from the curves shown in Fig 2 and are shown in the diagonal boxes. Two-way analysis of variance was performed for the electrolyte leakage experiment; significance of comparisons of responses among genotypes was determined by orthogonal contrast. P values representing comparisons of the temperature response curves are indicated in the intersecting cells.

ns = not significantly different
Comparison of each genotype in both Fig 2A & 2B and in Fig 2C & 2D indicated the curves were very highly significantly different (P<0.0001) between warm and 2 wk cold.

**S3 Table. Transcript levels for the 136 Ws-2 CBF regulon genes [14] in SW, sw:cbf123, IT and it:cbf123 plants after a 24 h cold treatment.**
S4 Table. Transcript levels for the 136 Ws-2 CBF regulon genes [14] in SW, sw:cbf123, IT and it:cbf123 plants after a 2 week cold treatment.
(XLSX)

S5 Table. CBF regulon genes of SW, IT and Col-0 plants shown in the Venn diagram presented in Fig 6A.
(XLSX)

S6 Table. Expression of the 44 common CBF regulon genes in SW, sw:cbf123, IT, it:cbf123, Col-0, col-0:cbf123 plants exposed to low temperature for 24 h.
(XLSX)

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