Genetic and Molecular Analyses of Natural Variation Indicate *CBF2* as a Candidate Gene for Underlying a Freezing Tolerance Quantitative Trait Locus in Arabidopsis[1][w]

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Natural variation for freezing tolerance is a major component of adaptation and geographic distribution of plant species. However, little is known about the genes and molecular mechanisms that determine its naturally occurring diversity. We have analyzed the intraspecific freezing tolerance variation existent between two geographically distant accessions of Arabidopsis (*Arabidopsis thaliana*), Cape Verde Islands (Cvi) and Landsberg *erecta* (L*er*). They differed in their freezing tolerance before and after cold acclimation, as well as in the cold acclimation response in relation to photoperiod conditions. Using a quantitative genetic approach, we found that freezing tolerance differences after cold acclimation were determined by seven quantitative trait loci (QTL), named *FREEZING TOLERANCE QTL 1* (*FTQ1*) to *FTQ7*. *FTQ4* was the QTL with the largest effect detected in two photoperiod conditions, while five other *FTQ* loci behaved as photoperiod dependent. *FTQ4* colocated with the tandem repeated genes *C-REPEAT BINDING FACTOR 1* (*CBF1*), *CBF2*, and *CBF3*, which encode transcriptional activators involved in the cold acclimation response. The low freezing tolerance of *FTQ4*-Cvi alleles was associated with a deletion of the promoter region of Cvi *CBF2*, and with low RNA expression of *CBF2* and of several *CBF* target genes. Genetic complementation of *FTQ4*-Cvi plants with a *CBF2*-L*er* transgene suggests that such *CBF2* allelic variation is the cause of *CBF2* misexpression and the molecular basis of *FTQ4*.

Plants are continuously exposed to a wide range of adverse environmental conditions that fluctuate along their life cycles. To survive, they have developed different tolerance strategies that protect them against abiotic stresses. Among such stress conditions, freezing temperature is one of the most relevant factors limiting the distribution of plant species (Weiser, 1970; Levitt, 1980). Due to its basic and applied interest, freezing tolerance has been studied in all sorts of plants, from annual to woody species. Freezing tolerance is acquired as a result of a complex stress response involving multiple physiological, biochemical, and molecular changes (Weiser, 1970; Levitt, 1980). This response is triggered or increased by environmental factors that anticipate the freezing temperature, thus regulating the proper timing of freezing tolerance acquisition. Most plant species from temperate regions increase freezing tolerance in response to low nonfreezing temperatures, a process called cold acclimation. Additionally, short-day (SD) photoperiods enhance the tolerance of many species from temperate regions (Fowler et al., 2001; Welling et al., 2002; Karlson et al., 2003).

Our understanding of the molecular mechanisms of cold acclimation has dramatically improved in the past years, mainly due to the study of the model plant Arabidopsis (*Arabidopsis thaliana*; Thomashow, 1999; Salinas, 2002). It has been found that most physiological changes during cold acclimation are associated with changes in gene expression (Thomashow, 1999; Maruyama et al., 2004). Numerous cold-induced genes have been identified that encode proteins involved either in the molecular mechanisms of tolerance or in signal transduction and regulation (Maruyama et al., 2004). Furthermore, a small gene family of AP2-domain transcriptional regulators, known as C-repeat

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(CRT)-binding factor/dehydration-responsive element (DRE)-binding factor (CBF/DREB1; Stockinger et al., 1997; Liu et al., 1998), was identified by its binding capacity to the CRT or DRE regulatory sequences (Thomashow, 1999). Expression of the *CBF/DREB1* genes (*CBF1/DREB1B*, *CBF3/DREB1A*, and *CBF2/DREB1C*; hereafter referred to by their *CBF* names; Gilmour et al., 1998) is transiently induced by low temperature and precedes the expression of the CBF target genes whose promoters contain the CRT/DRE element (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999; Maruyama et al. 2004). Thus, a transcriptional cascade leads to the expression of the CRT/DRE class of genes, which act in concert to increase freezing tolerance. Overexpression of *CBF1*, *CBF2*, and *CBF3* increases freezing tolerance in acclimated and nonacclimated plants, suggesting that they are sufficient to trigger the cold acclimation response (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Gilmour et al., 2004). On the other hand, an insertion mutant of the *CBF2* gene shows higher freezing tolerance and overexpression of *CBF1* and *CBF3* (Novillo et al., 2004), indicating that *CBF2* negatively regulates *CBF1* and *CBF3* expression.

Naturally occurring variation for freezing tolerance has been found not only among species occupying different environments but also within species with broad geographic distribution (Sackville Hamilton et al., 2002; Andaya and Mackill, 2003; Karlson et al., 2003; Vágújfalvi et al., 2003; Tsarouhas et al., 2004). Intraspecific differences have often been associated with environmental parameters from the locations where the populations evolved, such as winter temperature and photoperiod (Sackville Hamilton et al., 2002; Karlson et al., 2003). Thus, intraspecific genetic variation is, presumably, a fundamental component determining adaptation to different environments. However, little is known about the molecular basis of the natural variation for freezing tolerance. Essentially, a number of studies have just initiated the genetic dissection of these differences in crop and forestry species, showing that it is a complex quantitative trait controlled by multiple quantitative trait loci (QTLs; Andaya and Mackill, 2003; Vágújfalvi et al., 2003; Francia et al., 2004; Tsarouhas et al., 2004).

To further understand the molecular basis of the naturally occurring variation for freezing tolerance, we have exploited the intraspecific differences existing for this trait in the model plant Arabidopsis, an annual wild species with a wide geographic distribution (Cook et al., 2004; Klotke et al., 2004; Koornneef et al., 2004; Rohde et al., 2004). We found that the Cape Verde Islands (Cvi) and Landsberg *erecta* (L*er*) accessions differ in their freezing tolerance before and after cold acclimation and in their freezing tolerance response under different photoperiods. Using a QTL mapping approach, we have determined the genetic basis of this variation. Furthermore, we have pursued the molecular basis of the largest effect QTL, showing that allelic variation at the *CBF2* gene might underlie this locus.

RESULTS

L*er* and Cvi Differ in Freezing Tolerance

To identify Arabidopsis strains that differ in their natural freezing tolerance, we chose accessions L*er* and Cvi because they originally come from very different geographic areas, Northern Europe and the subtropical Cape Verde Islands, respectively. Both accessions were grown under a long-day (LD) photoperiod, frozen at different temperatures, and the lethal temperature for 50% survival (LT_{50}) estimated from fitted response curves (Fig. 1A). Cvi plants directly exposed to freezing temperatures showed a LT_{50} of −4.8°C, while L*er* had a value of −6.3°C. In addition, we evaluated their freezing tolerance after cold acclimation, with Cvi and L*er* plants displaying, respectively, LT_{50} values of −7°C and −9.2°C. Therefore, Cvi plants show a lower freezing tolerance than L*er*, before and after cold acclimation. The L*er*/Cvi freezing tolerance difference was maximal at a temperature of −8°C provided after cold acclimation, where 90% of L*er* plants survived and about 90% of Cvi plants died (Fig. 1B). To determine the overall dominance of the trait, we evaluated the freezing tolerance of F_1 (L*er* × Cvi)

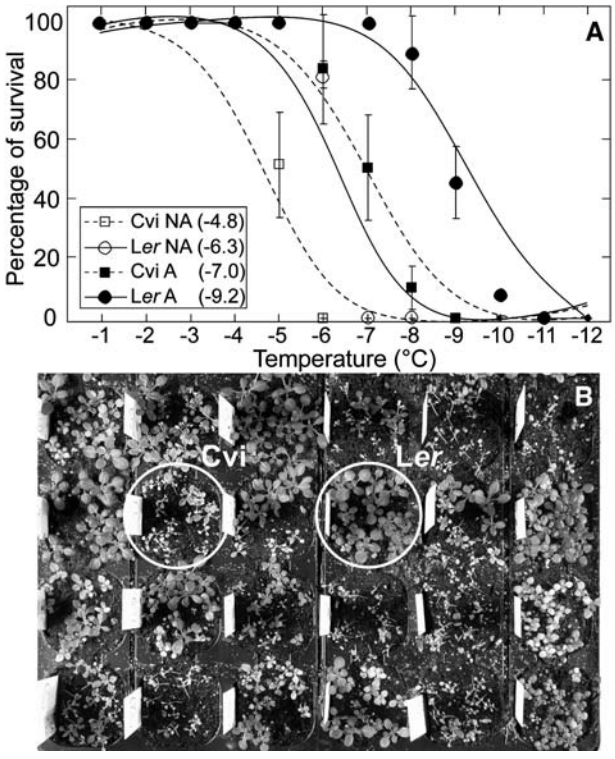

Figure 1. Freezing tolerance of L*er* and Cvi accessions. A, Percentage of surviving plants to different freezing temperatures with or without previous cold acclimation. Bars correspond to the mean ± SE of three replicates. Sigmoidal curves were fitted by a distance-weighted least-squares procedure, from which the LT_{50} values shown in the legend were calculated. A, Cold acclimated; NA, non-cold acclimated. B, Cold-acclimated plants of L*er* and Cvi (surrounded by circles) and various RIL and transgenic lines 2 weeks after freezing at −8°C.

plants in these conditions. Hybrid plants behaved similar to L*er*, indicating that the increased L*er* freezing tolerance was dominant. Furthermore, we analyzed the tolerance to −8°C after cold acclimation, when both accessions were grown under a SD photoperiod. In this assay, 97% of L*er* plants survived, with SD leading to a small increase in freezing tolerance compared to LD. However, Cvi plants behaved similarly under both conditions, suggesting that Cvi responds less than L*er* to a SD photoperiod (Supplemental Fig. 1).

L*er*/Cvi QTL Mapping for Freezing Tolerance

To map the loci causing the freezing tolerance differences observed between L*er* and Cvi after cold acclimation, we characterized a L*er*/Cvi recombinant inbred line (RIL) population (Alonso-Blanco et al., 1998b). The freezing tolerance of 132 RILs was estimated under a LD photoperiod at the −8°C discriminatory temperature provided after cold acclimation. In addition, the freezing tolerance of a subset of 53 RILs was also evaluated in a similar assay performed under a SD photoperiod (Supplemental Fig. 1). Significant genotype (RIL) by environment (photoperiod) interaction was detected ($P < 0.001$), in agreement with the low correlation observed between tolerances under both photoperiods ($r = 0.28$). These data were used to map QTLs under LD and SD photoperiods (Fig. 2; Supplemental Table I), identifying five and four QTLs, respectively, whose total additive effects account for 46% and 58% of the phenotypic variance. A genome-wide search for two-way interactions did not detect any significant interaction among those QTLs or among any other pair of genomic regions ($P > 0.05$). Conservatively, we found a minimum number of seven different loci on chromosomes 1, 4, and 5, which we named as *FREEZING TOLERANCE QTL 1* (*FTQ1*) to *FTQ7*. The relative effect of the *FTQ* loci varied from small (<5%) to very large (>15%). In all of them, the L*er* allele increased freezing tolerance compared to Cvi, in agreement with the parental values. Only two QTLs, *FTQ4* and *FTQ6*, were detected in both photoperiod conditions, suggesting that there is a limited common genetic component determining the variation in both photoperiods.

The *CBF* Cluster as a Candidate for *FTQ4*

Since *FTQ4* was the strongest effect QTL detected under both photoperiods, we pursued its molecular characterization by a candidate gene approach. Analysis of the chromosome 4 genomic sequence (Arabidopsis Genome Initiative, 2000) detected the cluster of *CBF1*, *CBF3*, and *CBF2* genes within the *FTQ4* support interval. Location of these genes in the middle of the *FTQ4* interval was confirmed by mapping of a *CBF2* PCR marker in a subset of 33 L*er*/Cvi RILs, including those with recombination events in the *FTQ4* confidence interval (Fig. 2). To explore whether the *CBF* genes might underlie *FTQ4*, we analyzed their cold-induced expression in both parental lines and in the commonly used Columbia (Col) laboratory strain, which shows an overall freezing tolerance similar to L*er* (Rohde et al., 2004; Fig. 3A). RNA expression analysis of *CBF* genes confirmed their cold induction in the three accessions, but there were considerable quantitative differences among accessions for the amount and timing of expression. Col plants displayed maximal expression of the three *CBF* genes after 2 h at 4°C and their expression was reduced following 4 h of cold treatment. In contrast, L*er* plants showed a higher expression of *CBF2* and *CBF3* than Col after 1 h of cold treatment, indicating a faster induction. In addition, abundant expression was still detected after 4 h at 4°C, suggesting a slower reduction of expression in L*er* than Col. The Cvi expression pattern of the *CBF* genes was distinct, with two main differences being detected with respect to L*er*. First, the expression of *CBF2* was greatly reduced in Cvi, showing a very limited maximal induction after 1 h at 4°C. Second, Cvi plants exhibit a slightly higher expression of *CBF1* and *CBF3* after 1 or 2 h of exposure to cold.

We also analyzed the expression of cold-induced genes that are activated by the CBF transcription regulators (Fig. 3B). The CBF target genes *LTI78*, *COR47*, and *KIN1* exhibited much lower cold-induced expression in Cvi than in L*er*, suggesting that gene expression regulated by the *CBF* genes was reduced in Cvi compared to L*er*. Therefore, the *FTQ4* freezing tolerance effect correlated with the expression of the *CBF2* and *CBF* target genes, supporting a causal relationship between *FTQ4* and the *CBF* cluster.

To find DNA polymorphisms in the *CBF* genes that could underlie *FTQ4*, we sequenced the Cvi genomic region containing the *CBF* cluster and compared it to available L*er* and Col sequences (Fig. 4). A total of 83 polymorphisms were found among the three accessions in the 8.7-kb sequence. L*er* and Cvi differed in 62 polymorphisms, while L*er* and Col differed only in 33. Two relevant aspects were found when analyzing the 62 L*er*-Cvi polymorphisms. First, Cvi carried a deletion of 1,630 bp in the promoter region of the *CBF2* gene, located 160 bp upstream from the predicted transcription start. This polymorphism reduces the size of the *CBF3*-*CBF2* intergenic region to 378 bp and determines a minimum *CBF2* promoter. Second, the remaining 61 polymorphisms were not evenly distributed among the three *CBF* genes, *CBF3* showing much more DNA allelic variation than *CBF1* and *CBF2*. This was especially evident when comparing the promoter regions, since the first kilobase located 5′ from the *CBF3* start codon contains 23 polymorphisms, while the equivalent regions of the *CBF1* and *CBF2* genes carry only seven and three polymorphisms, respectively (Fig. 4). In addition, the *CBF3* coding sequence contains more synonymous and replacement polymorphisms than the coding sequences of *CBF1* and *CBF2*. L*er* and Cvi differ in three-, two-, and three-amino acid substitutions for CBF1, CBF2, and CBF3, respectively. The Pro-49 to Thr-49 CBF2 change affects the first amino acid of the AP2 domain, whereas the remaining

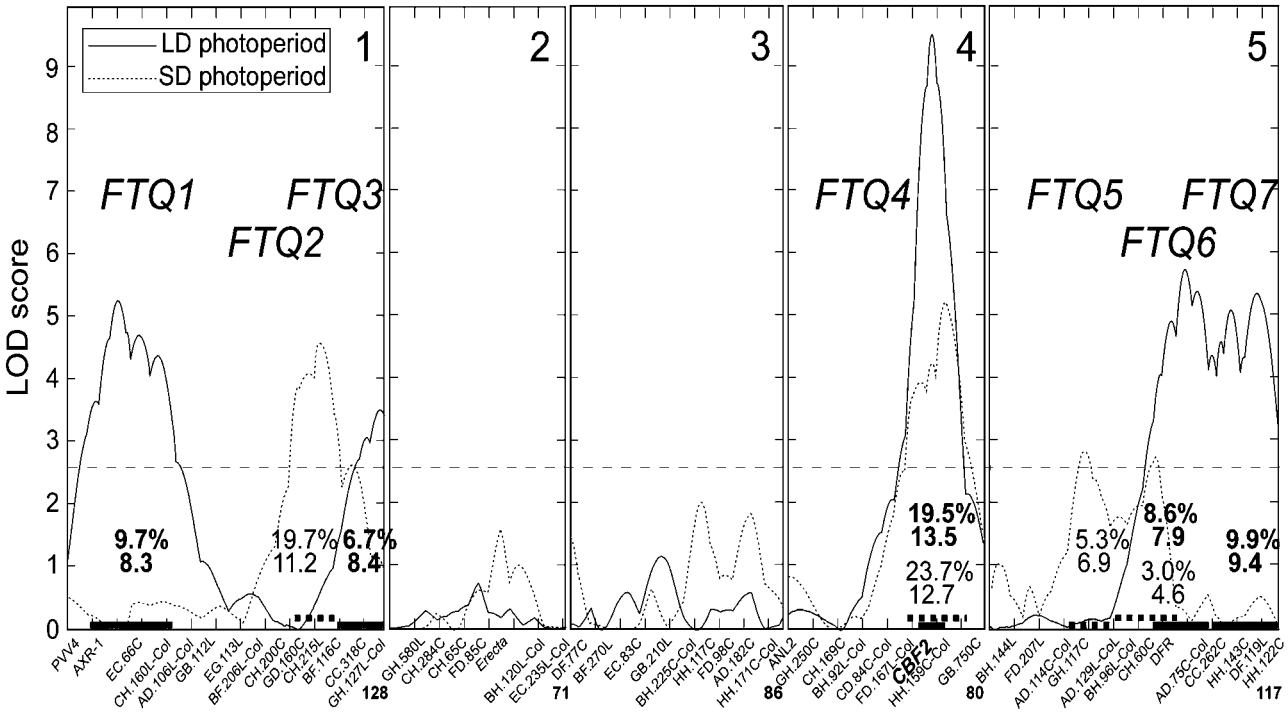

Figure 2. QTL likelihood maps for freezing tolerance in the L*er*-Cvi RIL population grown under two photoperiod conditions. Genetic maps of the Arabidopsis linkage groups are shown in the abscissa and LOD scores in the ordinate. Chromosome numbers are indicated in the top right corner of each image. The LOD threshold used for QTL detection is shown as a hatched horizontal gray line and the 2-LOD support intervals of the detected QTL are depicted as thick lines on the genetic maps. Continuous or hatched LOD profiles and 2-LOD intervals correspond to QTL mapping under LD or SD photoperiods, respectively. For each QTL, its name, the percentage of phenotypic variance explained, and its allele additive effect are shown. Allele effects are given in survival percentage units, positive values indicating that L*er* genotypes show higher freezing tolerance than Cvi.

replacements do not lead to meaningful changes in potential nuclear localization signals, phosphorylation sites, or protein acidic regions. Interestingly, the Cvi allele of *CBF3* is Cvi specific, since no *CBF3* polymorphism was found between L*er* and Col.

A L*er CBF2* Transgene Complements the *FTQ4*-Cvi Allele

To further evaluate the *CBF* genes as candidates for *FTQ4*, we tested whether expression of a *CBF2* gene from L*er* complements the low freezing tolerance of lines carrying *FTQ4*-Cvi alleles. To this end, we isolated a binary cosmid clone carrying a L*er* genomic insert that contains the complete coding sequence of *CBF2* with 275 bp of its 5′ sequence from the start codon, and six other predicted open reading frames located 3′ downstream to *CBF2* (see ''Materials and Methods''). This genomic clone was used to generate transgenic plants in three different homozygous *FTQ4*-Cvi genetic backgrounds, Cvi, RIL42, and RIL44, as well as in the L*er* parental background. These RILs were selected as carrying Cvi alleles at *FTQ4* and L*er* alleles at *FTQ6* and *FTQ7*, which were the next largest effect QTLs detected under a LD photoperiod. RIL42 also carries L*er* alleles at *FTQ3*.

To be able to test phenotypic complementation with this clone, we first determined the pattern of expression of the *CBF2*-L*er* transgene. Several transgenic lines were analyzed by RNA hybridization of a *CBF2* probe, before and after 2 h of cold treatment at 4°C (Fig. 5A). Unexpectedly, all tested transgenic lines showed considerably higher *CBF2* expression than the untransformed controls before the cold treatment, which indicated that the *CBF2*-L*er* transgene was overexpressed. However, a similar, strong cold-induced increment of *CBF2* expression was also observed in these lines, indicating that the *CBF2*-L*er* transgene responded to cold despite its small promoter region. The

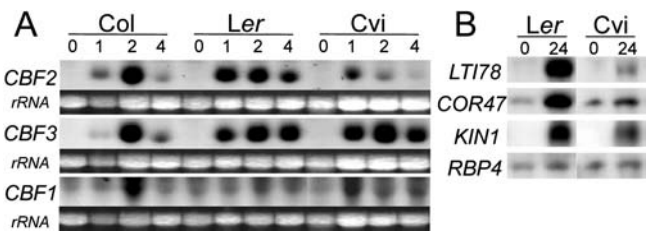

Figure 3. Expression of cold-induced genes in L*er*, Cvi, and Col. A, RNA-blot hybridizations of *CBF1*, *CBF2*, and *CBF3* in plants exposed to 4°C for 0, 1, 2, or 4 h. Ethidium bromide staining of the ribosomal RNA (*rRNA*) is shown as a loading control. B, RNA-blot hybridizations of CBF target genes in L*er* and Cvi control plants (0 h) and plants exposed to 4°C for 24 h. Hybridization of a *RBP4* probe is shown as a loading control.

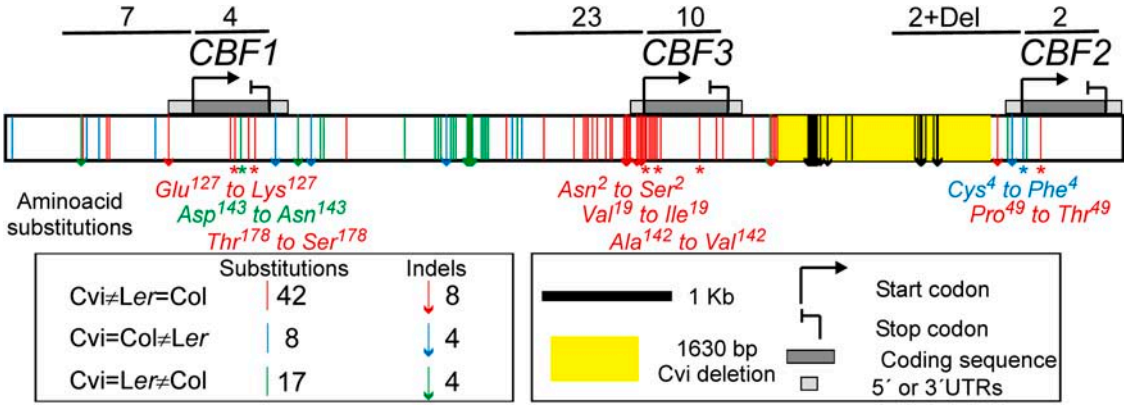

Figure 4. DNA sequence comparison of the genomic regions containing *CBF1*, *CBF2*, and *CBF3* genes from L*er*, Cvi, and Col. Genomic organization of the *CBF* cluster is shown in the top part. Single-nucleotide substitutions are depicted as vertical bars, different colors denoting allele compositions (indicated in the bottom left box). Indels are depicted as vertical arrows with similar color codes. Thirteen indels affect single nucleotides, one involves two nucleotides, and another five nucleotides. A large L*er*-Cvi deletion (Del) is represented as a yellow color box. Asterisks (*) indicate amino acid changes. A summary of the number of polymorphisms is presented in the bottom left box. L*er*-Col polymorphisms in the 1.6-kb genomic deletion of Cvi are not considered and are depicted in black. Numbers in the top part correspond to the L*er*-Cvi polymorphisms in the 0.6-kb coding regions of the *CBF* genes and in the first kilobase 5′ upstream from the start codons. GenBank accession numbers of Cvi, L*er*, and Col sequences are AY667247, AF076155, and AL022197, respectively.

molecular cause of the higher basal expression and increased cold response of this *CBF2*-L*er* transgene remains unknown, but since this behavior is independent of the genetic background, it seems mostly determined by the construct and not by the transgene positional effects. This higher basal expression of the *CBF2*-L*er* transgene could result from the lack of upstream promoter elements (Zarka et al., 2003) that could negatively regulate *CBF2*. In addition, due to the short promoter region, the expression of the *CBF2*-L*er* transgene might be enhanced by the cauliflower mosaic virus 35S promoter that activates the *nptII* reporter gene located 5′ to *CBF2* or by other sequences present in the cosmid vector.

The freezing tolerance of three to four independent transgenic lines homozygous for the transgenes was tested in a similar assay to that performed for QTL mapping under LD photoperiod conditions (Fig. 5B). All *FTQ4*-Cvi transgenic lines showed a very significant increase of freezing tolerance, on average exhibiting 49% higher survival to the −8°C freezing temperature than the untransformed controls. The observed increase was rather similar in the three *FTQ4*-Cvi genetic backgrounds. In contrast, a much smaller and not significant increase was obtained in the transgenic lines in the L*er* genetic background. Thus, the observed freezing tolerance effect, which is presumably produced by the *CBF2*-L*er* transgene, partly depends

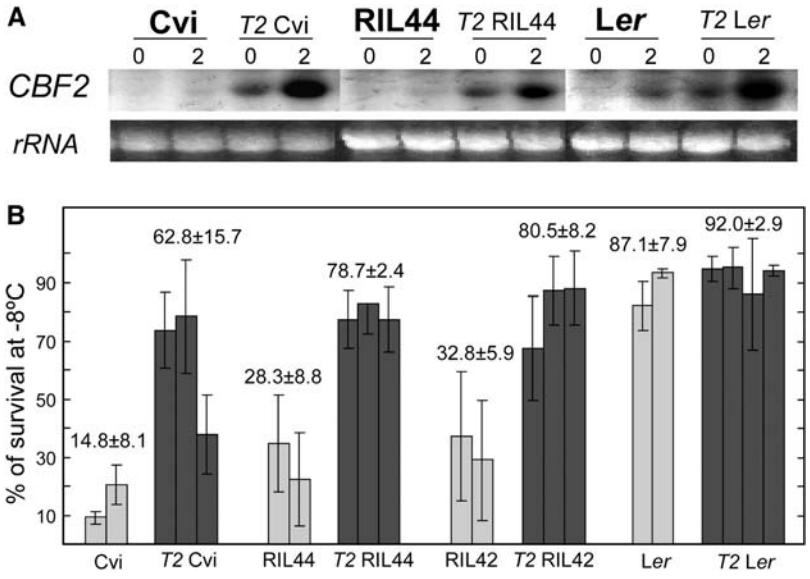

Figure 5. Expression of a *CBF2* transgene and freezing tolerance of transgenic lines. A, Expression of *CBF2* in Cvi, RIL44, and L*er* untransformed plants and in transgenic lines containing the L*er* *CBF2* transgene. Expression was analyzed in control plants not exposed to cold (0) and in plants of the same line exposed to 4°C during 2 h (2). The ribosomal RNA (*rRNA*) stained with ethidium bromide is shown as a loading control. B, Freezing tolerance after cold acclimation under a LD photoperiod of transgenic lines overexpressing a L*er* *CBF2* gene. Dark gray color bars depict transgenic lines, and light gray bars represent untransformed controls. Bars correspond to mean ± SE of each transgenic line derived from four replicates. Numbers in the top part of the images show the mean ± SE of the various transgenic lines analyzed for each background. *T2* Cvi, *T2* RIL42, *T2* RIL44, and *T2* L*er* denote different independent T2 transgenic homozygous lines in the corresponding genetic backgrounds.

on the genetic background. However, L*er* alleles at other QTLs than *FTQ4* seem unnecessary for this *CBF2*-mediated tolerance. We concluded that the highly expressed *CBF2*-L*er* transgene complemented the low freezing tolerance of *FTQ4*-Cvi alleles. These results suggest that Cvi is not defective in any trans-regulator necessary for the cold induction of *CBF2* expression or in any essential downstream element mediating *CBF2* freezing tolerance.

DISCUSSION

We have performed a genetic and molecular analysis of natural variation for freezing tolerance in two Arabidopsis accessions, L*er* and Cvi, originating from very different geographic areas. Both accessions differ considerably in their basal freezing tolerance and in their acquired freezing tolerance after cold acclimation. In addition, L*er* and Cvi differ in the cold acclimation response in relation to photoperiod conditions. Using a QTL mapping approach, we have shown that the variation in freezing tolerance after cold acclimation is a multigenic trait determined by at least seven *FTQ* loci. Comparison of the QTLs identified in plants grown under LD and SD photoperiods reveals that photoperiod is an important environmental factor affecting freezing tolerance in Arabidopsis. Different photoperiods seem to affect the phenotypic expression of different QTLs, since three *FTQ* loci were detected only under LD while two others were only found under SD. Testing the adaptive relevance of these differential freezing tolerances and photoperiod responses awaits a detailed study of the genetic variation present in large collections of Arabidopsis wild accessions.

The L*er*/Cvi RIL population has been previously studied for flowering time in relation to the same environmental factors, with L*er* and Cvi differing in the flowering response to photoperiod and cold treatments (called vernalization; Alonso-Blanco et al., 1998a). Allelic variation at the blue-light photoreceptor gene *CRY2* accounted for the different flowering photoperiod responses of these accessions (El-Assal et al., 2001). However, although *CRY2* is genetically linked to the photoperiod-dependent locus *FTQ1*, its map position suggests that *CRY2* does not affect freezing tolerance. In addition, we cannot ignore that PhyA could mediate the photoperiodic regulation of freezing tolerance in Arabidopsis, as shown for hybrid aspen (Welling et al., 2002). Supporting this hypothesis, *PhyA* is colocating with *FTQ1*, and this photoreceptor has been shown to regulate other Arabidopsis photoperiod responses (Yanovsky and Kay, 2002). Furthermore, *FTQ1* colocates with the *ESPRESSO* locus, which affects the circadian system and segregates in the L*er*-Cvi RIL population (Swarup et al., 1999). Thus, *FTQ1* might also be involved in the circadian regulation of *CBF* expression and/or freezing tolerance (Fowler et al., 2005). Alternatively, *FTQ1* colocates with the dehydrin genes *COR47* and *ERD10* as well (Maruyama et al., 2004). Differential dehydrin expression has been correlated with natural variation for freezing tolerance in Arabidopsis (this work) and other species (Karlson et al., 2003), and a dehydrin gene has been pointed to as the genetic cause of chilling tolerance variation in cowpea (Ismail et al., 1999). However, although these Arabidopsis genes are CBF targets (Maruyama et al., 2004), further analyses will reveal the effect of their allelic variation on freezing tolerance.

Two out of three QTLs that affect the vernalization response of the L*er*-Cvi population, *FLG* and *FLH*, overlapped with *FTQ5* and *FTQ7*, suggesting that they might affect cold perception and transduction. A similar role has been speculated for the Triticeae *VRN* loci (Vágújfalvi et al., 2003; Francia et al., 2004) and indicated for the Arabidopsis *HOS1* and *FVE* genes (Lee et al., 2001; Kim et al., 2004).

Finally, *FTQ2* overlaps with a major QTL, explaining the low raffinose and stachyose seed content of Cvi compared to L*er* (Bentsink et al., 2000). These sugars function as osmoprotectants (Taji et al., 2002), and variation in raffinose content has been correlated with natural variation for freezing tolerance between Col and C24 accessions (Klotke et al., 2004). Thus, we hypothesize that *FTQ2* might be one of the genes encoding a raffinose synthase or a galactinol synthase that were previously mapped within the oligosaccharide QTL interval (Bentsink et al., 2000).

We have used a candidate gene approach to identify the molecular basis of the largest effect QTL, *FTQ4*. This locus is closely linked to a complex locus containing a tandem repeat of three highly similar *CBF* genes. Presumably, this cluster originated by two consecutive duplication events of an ancestral gene and subsequent divergence by single-nucleotide mutations (Medina et al., 1999). The *CBF* genes seem to play a central role in cold acclimation (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999), and the observed allelic variation in all three genes might contribute to the *FTQ4* effect. However, our data strongly suggest that allelic variation at *CBF2* is underlying *FTQ4*. The low freezing tolerance of Cvi and of the *FTQ4*-Cvi allele correlated with low cold-induced expression of *CBF2* and of several CBF target genes, in agreement with a comparison of Cvi and Wassilewskija-2 accessions (Cook et al., 2004). This suggested two main alternative genetic scenarios for *FTQ4*: Either the allelic variation at *FTQ4* affected a trans-regulator of *CBF2* expression located within the *FTQ4* support interval, or *FTQ4* actually corresponded to the structural *CBF2* gene or its cis-regulatory sequences. Analysis of Cvi and *FTQ4*-Cvi transgenic lines carrying a highly expressed *CBF2*-L*er* transgene supports that Cvi is not defective in any trans-regulator involved in the cold induction of *CBF2*, since these lines show strong cold-induced expression of *CBF2*. In addition, genetic complementation of the low freezing tolerance of *FTQ4*-Cvi genotypes with this transgene indicates that Cvi is not defective in any essential downstream regulatory element participating in the

CBF2-mediated freezing tolerance. Taken together, our results suggest that the 1.6-kb Cvi deletion is likely to be the functional polymorphism that affects *CBF2* expression, an activation regulatory element being probably absent in Cvi. Thus, Cvi probably carries a *CBF2* misfunctional allele that strongly reduces its cold-induced expression, in agreement with the overall dominance of the higher L*er* freezing tolerance. It has been previously shown that the *CBF2* promoter contains two regulatory elements called induction of *CBF* expression region 1 (ICEr1) and ICEr2 (Zarka et al., 2003). Cvi lacks the ICEr1 sequence located at position −190 bp in *CBF2* promoter and probably other regulatory elements, which might be the cause of the low cold induction. Nevertheless, we cannot ignore that another closely linked gene might underlie *FTQ4*. Furthermore, it is also possible that the existing allelic variation at *CBF1* and, especially, at *CBF3*, could contribute to the effects of *FTQ4*, as suggested by the large amount of *CBF3* molecular polymorphisms found. This potential *FTQ4* complexity is somehow supported by current molecular models on the function of the *CBF* cluster in cold acclimation, since the three *CBF* genes do not operate independently (Novillo et al., 2004).

Recently, an insect resistance QTL has been shown to involve a tandem repeat of two methylthioalkylmalate synthase genes (Kroymann et al., 2003). The analysis of genetic variation for the methylthioalkylmalate and *CBF* genes point to the complexity of the natural variation provided by clusters of gene families, 17% of the Arabidopsis genes belonging to this class (Arabidopsis Genome Initiative, 2000). First, several genes might contribute to a single QTL and significant genetic interactions among the various clustered genes might also be involved. Second, as elegantly shown by Kroymann et al. (2003), gene conversion between the various tandem repeated genes provides an additional mechanism to generate genetic variation. Detailed analysis of the molecular variation present in the *CBF* genes in other Arabidopsis wild accessions will help in understanding the relevance of these genes in intraspecific freezing tolerance variation and the evolutionary dynamics of this complex locus. The *CBF*-mediated pathway has also been found in Triticeae and Brassica species, indicating a conserved *CBF* function in freezing tolerance among species (Jaglo et al., 2001). Such *CBF*-like genes have been proposed as candidates for freezing tolerance QTLs in barley and wheat (Vágújfalvi et al., 2003; Francia et al., 2004). Further molecular analyses of *CBF* genes in these and other species will reveal whether they have a conserved role across species as major genetic determinants of intraspecific variation for freezing tolerance.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) accessions Col, L*er*, and Cvi and 132 RILs derived from crosses between L*er* and Cvi were analyzed (Alonso-Blanco et al., 1998b).

Growth Conditions and Freezing Tolerance Assays

Plants were grown in pots with soil and vermiculite at 3:1 proportion in 20°C growth chambers illuminated with cool-white fluorescent lamps. The LD photoperiod was 16 h light/8 h darkness, while the SD photoperiod was 10 h light/14 h darkness. Cold acclimation was supplied by growing 2-week-old plants at 4°C for 7 d under the appropriate photoperiod. Freezing treatments were provided in darkness by exposing 3-week-old plants to the corresponding freezing temperature during 6 h, after a temperature touch-down cycle consisting of 30 min at 4°C and a subsequent temperature lowering at a rate of 2°C/h. Thereafter, temperature was increased at a similar rate to 4°C, and plants were thawed for 5 h in darkness before being returned to the original growth conditions.

Freezing tolerance was recorded as the percentage of plants that survive to the corresponding freezing temperature. For that, 20 to 25 plants of each genotype were grown in an 80-mL pot and treated as described above. The number of surviving plants was visually scored 2 weeks after the freezing treatment. To evaluate the RIL population, two to four replicate pots per line were grown in a randomized incomplete block design, and the average tolerance per RIL was calculated. The various transgenic lines developed in this work were evaluated by growing four pots per genotype in a similar, randomized, incomplete block design.

QTL and Other Statistical Analyses

The mean RIL freezing tolerance was transformed by the angular transformation (=arcsin$\sqrt{}$). These data were used to map QTLs on a set of 99 markers covering the Arabidopsis genetic map (Alonso-Blanco et al., 1998a). MapQTL 4.0 software (Van Ooijen, 2000; Kyazma B.V.) was used to apply interval mapping and multiple QTL model mapping methods as described in its reference manual (http://www.mapqtl.nl). A logarithm of the odds (LOD) threshold value of 2.6 corresponding to a genome-wide significance $\alpha = 0.05$ was used for QTL detection. This was estimated with the permutation test implemented in MapQTL, using 10,000 permutations. Two LOD support intervals were established as ≈95% QTL confidence intervals (Van Ooijen, 1992). The additive allele effect, the percentage of variance explained by each QTL, and the total variance explained by all the QTLs were obtained from the multiple QTL model. The QTL additive allele effect corresponds to one-half the differences between the estimated means of the two RIL genotypic groups. Two-way interactions were searched using EPISTAT (Chase et al., 1997) with log-likelihood ratio thresholds corresponding to a significance of $P < 0.0005$. Fifty thousand trials were used in Monte Carlo simulations performed with EPISTAT to establish the statistical significance. Results shown are based on transformed data, but a similar outcome was obtained when using the original percentage data. Other statistical comparisons were performed with the statistical package SPSS 12.

Sequencing of the *CBF* Genomic Region and Isolation of a *CBF2* Cosmid Clone

A 7,066-bp genomic DNA fragment containing the *CBF1*, *CBF2*, and *CBF3* genes from Cvi was sequenced. This sequence has been deposited in GenBank (accession no. AY667247).

To isolate a L*er* clone containing the *CBF* genes, a 0.9-kb *CBF2* probe was used to screen a genomic library of L*er* DNA cloned in the binary cosmid vector pCLD 04541 (Soppe et al., 2000). Thus, cos10 was isolated, subjected to restriction analysis, and its insert borders sequenced. It was determined that cos10 carried a 20.4-kb insert corresponding to the *CBF2* coding sequence and 275 bp 5′ from the start codon (and 128 bp from the predicted transcription start) as well as six other Arabidopsis open reading frames located 3′ downstream to *CBF2* and annotated as *At4g25450*, *At4g25440*, *At4g25435*, *At4g25434*, *At4g2533*, and *At4g25430* (Arabidopsis Genome Initiative, 2000; http://www.arabidopsis.org).

Development of Transgenic Lines

Plant transformation was performed by the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected on medium supplemented with kanamycin. For each transformation assay, three or four independent transgenic plants carrying one single insertion were selected based on the antibiotic marker segregation. These were selfed to obtain the homozygous T2 lines and T3 plants analyzed.

Gene Expression Analysis

RNA expression was analyzed on 2-week-old plants by hybridization of blots containing 30 μg of total RNA. Specific probes for *CBF1*, *CBF2*, and *CBF3* were derived from the 3′ untranslated regions (Medina et al., 1999). Probes for *LTI178*, *COR47*, *KIN1*, and *RBP4* have been described elsewhere (Medina et al., 1999; Novillo et al., 2004). Plants for expression analyses were grown together under LD photoperiods, cold treated during the first hours of the light period, and harvested at the same time.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY667247.

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