Published in final edited form as:

Plant Biotechnol J. 2012 January ; 10(1): 105–124. doi:10.1111/j.1467-7652.2011.00648.x.

The *Vitis vinifera* C-repeat binding protein 4 (*VvCBF4*) transcriptional factor enhances freezing tolerance in wine grape

Richard L. Tillett[1,*], **Matthew D. Wheatley**[1,*], **Elizabeth A.R. Tattersall**[1,2], **Karen A. Schlauch**[1], **Grant R. Cramer**[1], and **John C. Cushman**[1]

Richard L. Tillett: rltillett@unr.edu; Matthew D. Wheatley: wheatle8@gmail.com; Elizabeth A.R. Tattersall: eat@wnc.edu; Karen A. Schlauch: schlauch@unr.edu; Grant R. Cramer: cramer@unr.edu; John C. Cushman: jcushman@unr.edu

[1]Department of Biochemistry and Molecular Biology, University of Nevada, Mail Stop 330, Reno, NV 89557-0330, USA

Summary

Chilling and freezing can reduce significantly vine survival and fruit set in *Vitis vinifera* wine grape. To overcome such production losses, a recently identified grapevine C-repeat binding factor (CBF) gene, *VvCBF4*, was overexpressed in grape vine cv. “Freedom” and found to improve freezing survival and reduced freezing-induced electrolyte leakage by up to 2°C in non-cold-acclimated vines. In addition, overexpression of this transgene caused a reduced growth phenotype similar to that observed for CBF overexpression in *Arabidopsis* and other species. Both freezing tolerance and reduced growth phenotypes were manifested in a transgene dose-dependent manner. To understand the mechanistic basis of *VvCBF4* transgene action, one transgenic line (9–12) was genotyped using microarray-based mRNA expression profiling. Forty-seven and 12 genes were identified in unstressed transgenic shoots with either a greater than 1.5-fold increase or decrease in mRNA abundance, respectively. Comparison of mRNA changes with characterized CBF regulons in woody and herbaceous species revealed partial overlaps suggesting that CBF-mediated cold acclimation responses are widely conserved. Putative *VvCBF4*-regulon targets included genes with functions in cell wall structure, lipid metabolism, epicuticular wax formation, and stress-responses suggesting that the observed cold tolerance and dwarf phenotypes are the result of a complex network of diverse functional determinants.

Keywords

CBF transcription factor; freezing; cold tolerance; dwarf; wine grape; *Vitis vinifera*

Introduction

Vitis vinifera or wine grape was domesticated more than 7000 years ago and continues to the present day to produce one of the world’s most important fruit crops (Arroyo-Garcia et al., 2006; This et al., 2006). Cultivation of *V. vinifera* and other *Vitis spp*. encompasses ~8

Corresponding author: Professor John C. Cushman, University of Nevada, Reno, Mail Stop 330, Reno, Nevada 89557-0330; jcushman@unr.edu, Tel: +001-75-784-1918, Fax: +001-775-784-1419.

*These authors contributed equally to this work

[2]Current address: Department of Biology, Western Nevada College, Bently Hall 102, 1680 Bently Parkway South, Minden, NV 89423, USA.

million hectares of land worldwide, more than any other cultivated fruit (Vivier and Pretorius, 2002). *Vitis vinifera* cultivars grow well in temperate, semi-arid climates that can sometimes experience freezing or sub-freezing temperatures each winter.

As a deciduous perennial, *Vitis spp*. acquire freezing tolerance in advance of annual freezes, when shoots mature into overwintering canes and buds enter dormancy, cued by chilling temperatures and/or shortened day length (Sreekantan et al., 2010). Cooled gradually, *V. vinifera* cultivars can tolerate sustained winter temperatures as low as −15° C without injury, whereas wild North American and Asian species can tolerate exotherms of −35 to −40 °C (Fennell, 2004; Mullins et al., 1992). As vines exit dormancy each spring, freezing vulnerability returns quickly (Fennell, 2004). Breaking buds and newly emergent green tissues can suffer injury at just −2.5°C (Fuller and Telli, 1999). Moreover, damage to floral primordia of primary and secondary buds can drastically reduce crop yields (Fennell, 2004).

Cold acclimation, the process whereby plants sense low temperatures and activate mechanisms to increase tolerance to chilling or freezing stress, is a complex response involving multiple biochemical pathways (Nakashima and Yamaguchi-Shinozaki, 2006; Sreenivasulu et al., 2007). Central to the early cold-response pathway are the C-repeat binding factor (CBF)/dehydration-responsive element binding (DREB) family of transcription factors, of which the overexpression of a single Arabidopsis *CBF* gene family member is sufficient to impart an improved stress tolerance phenotype in *Arabidopsis* (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Stockinger et al., 1997), canola (*Brassica napus)* (Jaglo-Ottosen et al., 2001), tomato (Hsieh et al., 2002; Lee et al., 2003), and potato (Pino et al., 2007). In addition to cold tolerance, constitutive, ectopic expression of *AtCBF2* or *AtCBF3* has been shown to delay leaf senescence and extend plant longevity in *Arabidopsis* presumably to enable winter survival until spring for lifecycle completion (Sharabi-Schwager et al., 2010).

CBF/DREB genes have been identified in monocots and eudicots alike (Badawi et al., 2007; Benedict et al., 2006; Campoli et al., 2009; El Kayal et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2006), many of which have been characterised to have similar or conserved functions. For example, overexpression of *CBF* genes from cotton (Huang et al., 2009), rice (Dubouzet et al., 2003), birch (Welling and Palva, 2008), perennial ryegrass (Zhao and Bughrara, 2008), and wine grape (*V. vinifera*) cv. Koshu (Takuhara et al., 2011) or wild grape (*V. riparia*) (Siddiqua and Nassuth, 2011) in transgenic *Arabidopsis*, improved freezing tolerance in a manner similar to *AtCBF* overexpression. Ectopic expression of two *Eucalyptus CBF* genes in transgenic *Eucalyptus* plants resulted in improved cold tolerance (Navarro et al., 2011). Constitutive overexpression of *AtCBF1* (*AtDREB1b*) in transgenic *V. vinifera* cv. Centennial Seedless resulted in about a 2°C improvement in cold resistance as measured by electrolyte leakage and improved vine survival at −4°C (Jin et al., 2009). Lastly, constitutive overexpression of a peach (*Prunus persica*) *CBF* gene in apple (*Malus x domestica*) not only improved cold hardiness, but also resulted in short day length-related growth cessation and leaf senescence (Wisniewski et al., 2011).

The transcription factors encoded by the first three *CBF/DREB* family members described for *V. vinifera* and *V. riparia* were found to regulate genes that respond to low temperature,

drought stress, and exogenous ABA application (Xiao et al., 2006). These three transcription factor genes (*VvCBF1*, *VvCBF2*, and *VvCBF3*) showed increased mRNA expression in young compared with mature vegetative tissues upon exposure to freezing and drought stresses (Xiao et al., 2006). A fourth member of the *Vitis CBF/DREB1* gene family, *VvCBF4*, was also identified for both *V. vinifera* and *V. riparia* (Xiao et al., 2008). This transcription factor gene is unique among the *Vitis CBF* gene family in both its expression profile and sequence (Xiao et al., 2008). Expression of *VvCBF4* mRNA was sustained for several days following induction of a 4°C cold stress, in contrast to the transient expression of *VvCBF1–3* transcripts. In addition, *VvCBF4* was induced similarly in both young and mature tissues. More recently, *VvCBF4* transcripts have been shown to be induced after 4 h cold (4°C) stress in leaf, stem, and flower of *V. vinifera* cv. Koshu (Takuhara et al., 2011). *Vv*CBF4:green fluorescent protein (GFP) fusions localized to the nucleus and *VvCBF4* expression was shown to induce beta-glucuronidase (GUS) expression under the control of the *rd29a* promoter *in trans* when co-infiltrated in tobacco leaves (Xiao et al., 2008). *CBF* gene transcripts with similarly sustained cold-induction patterns have been reported in other woody perennial species, such as birch (Welling and Palva, 2008), poplar (Benedict et al., 2006), and *Eucalyptus* (Navarro et al., 2009). These CBF transcription factors and their target genes have been suggested to play a role in the freezing tolerance of overwintering woody perennials. Additional members of the *AP2/ERF* gene superfamily in *Vitis vinifera* display diverse expression patterns in both vegetative and reproductive tissues (Licausi et al., 2010).

In this report, the overexpression of the *VvCBF4* gene is shown to impart a reduced growth phenotype as well as confer improved freezing survival in non-acclimated vines, similar to the effects of CBF overexpression in *Arabidopsis* and other species. Microarray transcriptional profiling of a *VvCBF4* overexpressor identified 47 and 12 genes with either a greater than 1.5-fold increase or decrease in mRNA abundance, respectively, in young, unstressed shoots. These observed changes in mRNA expression suggest that the CBF regulon is widely conserved among woody and herbaceous species and that modulations in cell wall structure, lipid metabolism, epicuticular wax formation, and various stress-responses might participate in the acquisition of freezing tolerance in wine grape.

Results

VvCBF4 is most similar to CBFs of woody perennials

VvCBF4 was identified previously (Xiao et al., 2008) and so-named simply due to the order of its discovery. To identify its relationship with other *CBF* genes in *Vitis*, and eudicots in general, phylogenetic analyses were performed (Figure 1). Phylogenetic analysis of all four *Vitis CBF* genes with the full-length amino acid sequences of *CBFs* from other dicotyledonous plants (and a non-CBF, AP2-domain protein *AtERD10* as an outgroup gene) confirmed that, among the *Vitis* CBFs, the *VvCBF4* gene product shares the greatest similarity with *Arabidopsis* CBFs as well as two CBF gene products from another woody species, *Populus trichocarpa*, *PtDREB70* and *PtDREB71* (Figure 1A). The amino acid sequence alignment of the full-length CBF-encoding genes of *Arabidopsis*, *P. trichocarpa*, and *V. vinifera* (Figure 1B) showed that the *VvCBF4* gene product has 100% conservation

with the *Arabidopsis* CBF consensus sequences that flank the AP2 region (N-flanking: PKKR/PRAGRxKFxETHRP, C-flanking: DSAWR), which are required for CBF function (Canella et al., 2010). In contrast, the proteins encoded by the three other *Vitis CBF* genes (*VvCBF1*, *2*, and *3*) as well as those encoded by the *PtDREB66* and *67* genes share a variant of the established consensus between positions 1 and 4 of the N-flanking consensus (H/KKR/NK instead of PKKR/P). However, only the three other *Vitis CBF* (*VvCBF1*, *2*, and *3*) gene products contain a 21–25 amino acid stretch between the DSAWR motif and the C-terminus found in neither the *Arabidopsis* and *Populus* CBF gene products nor that of *VvCBF4*. Additionally, the gene products of *VvCBF1*, *2*, and *3* as well as those of the *PtDREB66* and *67* genes share a variation (e.g., LWN(E)D(H/E)) of the generally conserved LWSY amino acid sequence motif at their C-terminus.

Quantitative real-time RT-PCR (qRT-PCR)

To quantify expression of the CaMV 35S::*VvCBF4* transgene in three independently transformed lines of *Vitis* rootstock 'Freedom,' quantitative real-time, reverse-transcriptase-PCR (qRT-PCR) was performed. Whereas the ORFs of native and transgenic *VvCBF4* transcript are identical, the native 3'UTR was replaced by a 35S CaMV terminator region in the 35S::*VvCBF4* construct. Thus, primers were designed to detect either "native" or transgenic ("tg") *VvCBF4* transcripts exclusively from these regions (Supporting Information, Table S1, Figure S1).

qRT-PCR experiments demonstrated that both native and tg primers were specific to the mRNAs for which they were designed. In each of the three independent transgenic lines, 9-3, 9-12, and 9-1, tg transcripts were readily detected, whereas no amplification of tg could be detected in control line 8-6 (Figure 2). *VvCBF4* overexpression resulted in no observable changes in native transcript abundance. One-way ANOVA of native *VvCBF4* abundance showed no significant difference by genotype ($F >> 0.05$), which remained low regardless of the line tested (Figure 2).

In the 35S::*VvCBF4* transformed line 9-12, tg transcript accumulated to concentrations 20-fold greater than native transcript levels. Another line, 9-3, expressed 35S::*VvCBF4* less strongly than 9-12, with only 71% of the transgene abundance of 9-12, or 14-fold greater than native transcript. The tg mRNA abundance difference between 9-3 and 9-12 was statistically significant as determined by Student's *t* test with Bonferroni correction ($p = 0.048$). Transgene expression in line 9-1, however, was highly variable among biological replicates, with expression ranging between 72% and 360% of 9-12 transgene relative abundance (or between 14- and 72-fold of native transcript). Due to this large variability, comparison of expression in 9-1 with the other transgenic lines failed to reveal any significant expression differences in contrast to 9-3 or 9-12.

*Vv*CBF4 overexpression reduces shoot elongation

The constitutive overexpression of some CBF/DREB genes using the 35S-CaMV promoter is known to result in dwarfing effects in species such as *Arabidopsis thaliana* (Gilmour et al., 2000; Liu et al., 1998), *Medicago sativa* (Zhang et al., 2005), and *Lolium perenne* L. (Zhao and Bughrara, 2008). To assess the effect of 35S::*VvCBF4* transgene expression on

plant phenotype, the shoot elongation rate (SER) was measured at three-day intervals in young vines over an 18-day period for control line 8-6 and the three selected 35S::*VvCBF4*-overexpressing lines (Figure 3A,B). By one-way ANOVA, genotype accounted for highly significant observed differences in SER ($F < 0.0001$). Compared to control line 8-6, which had an average SER of 12.5 mm/d, SER was reduced in all 35S::*VvCBF4*-transformed vines. Line 9-3 SER was reduced slightly, although significantly, to 10.9 mm/d ($p < 0.05$). Lines 9-12 and 9-1 vines displayed even more dramatic reductions in SER, with rates of 7.4 and 4.5 mm/d, respectively (both were significantly different from control line 8-6, each $p < 0.001$) (Figure 3B).

In lines 9-12 and 9-1, lengths of stem sections, or internodes, were also reduced greatly (Figure 3C). Over the course 18 days of growth observation, the longest internodes on individual 9-12 vines (e.g., the single longest stem section from each of eight vines, averaged) were 36.8 mm, or 66% of the lengths achieved by 8-6 vines. Similarly, line 9-1 grew to maximum lengths of 33.1 mm (59% of control). These reduced node lengths were highly significant ($p < 0.01$).

The two most dwarfed lines, 9-12 and 9-1, differed in the number of nodes and the attachment site of leaves produced during this 18-day period (Figure 3D). Line 9-12 vines initiated 11.6 (SD ± 2.1) leaves on average, 86% as many leaves as did 8-6, which produced an average of 13.5 leaves (SD ± 1.3). This difference was not statistically significant. In contrast, the other dwarfed line, 9-1, produced an average of only 8.0 (SD ± 1.1) leaves in the same period. Genotypic differences were significant (one-way ANOVA by genotype, $P < 0.001$) and line 9-1 (and only line 9-1) differed from each of the other three lines (Bonferroni corrected pair wise tests, $p < 0.01$).

Taken together with the *VvCBF4* transgene expression data for each of these transformed lines, the growth data indicated that a dose-response relationship exists between *VvCBF4* transcript abundance and the dwarf phenotype, with high-overexpressing line 9-12 showing a more reduced SER than the medium-overexpressing line 9-3. Line 9-1 growth reduction was the most severe, although the high variability of *VvCBF4* transgene expression (Figure 2) limited the correlation with the growth phenotype in this line. Overall, these results are consistent with reports for other species in which constitutive overexpression of a CBF/DREB gene under the control of a constitutive promoter resulted in dwarf or reduced aerial biomass phenotypes (Achard et al., 2008a; Benedict et al., 2006; Gilmour et al., 2000; Kasuga et al., 1999; Navarro et al., 2011; Pino et al., 2007).

*Vv*CBF4 overexpression increases freezing survival

As *VvCBF4* transcripts were previously reported to undergo increased relative abundance during the application of 4°C chilling in Chardonnay (Xiao et al., 2008), the effect that *VvCBF4* overexpression had on freezing survival rates was investigated. Freezing stress resistance was assessed in 35S::*VvCBF4* transgenic vines by whole-plant survival analysis of non-acclimated vines. The 50% Lethal Temperature (LT_{50}) for control line 8-6 vines was determined experimentally to be at or near −2°C for 24 h (data not shown), so these conditions were chosen for survival testing between control and 35S::*VvCBF4* transgenic lines. Following six replicate experiments with 10 young vines/line in each experiment, the

freezing survival rate for control line 8-6 averaged only 29% (Figure 4). Survival of 35S::*VvCBF4* line 9-12 was significantly higher than control, at 52% survival ($p<0.01$). Survival of lines 9-1 and 9-3 were 39 and 43%, respectively, which was not significantly different from the control line ($p > 0.05$).

Cold acclimation in plants is a dynamic process that results from exposure to low non-freezing temperatures (Thomashow, 1999). Following a given cold acclimation period, electrolyte leakage assays are employed typically to assay any increased cold- or freezing-tolerance imparted to the cell by the numerous biochemical changes that occur during acclimation (Gilmour et al., 2000). Because overexpression of *CBF/DREB* genes might mimic the effects of cold acclimation, we performed electrolyte leakage assays on leaf discs cut from fully expanded leaves of non-acclimated control line 8-6 and *VvCBF4*-overexpressing vines. Line 9-3, which expressed ~70% as much *VvCBF4* mRNA as line 9-12 (Figure 2), exhibited a change in leakage only at −6°C (Figure 5A). Line 9-12 showed a 2°C greater resistance to electrolyte leakage than the control line with these differences appearing at −6 and −7°C (Figure 5B). At −8°C, line 9-12 leaf discs had begun leaking and no further differences could be observed. No significant changes between line 9-1 and control line 8-6 were observed (Figure 5C). The lack of measurably significant enhancement of survival or electrolyte leakage in line 9-1 might be related to the severity of growth retardation in this line (Figure 3) in combination with the propagation and assay conditions employed. Sterile cuttings from line 9-1 typically had shorter and fewer roots and had a lower survival rate when transplanted to soil compared with the other lines under identical propagation conditions, suggesting a lowered fitness for 9-1 vines propagated on the same time scale as faster-developing vines. Based upon freezing survival and electrolyte leakage assays, line 9-12 was selected for detailed genotypic evaluation.

Global changes in gene expression in 35S::*Vv*CBF4 overexpressor

To investigate the molecular mechanisms involved in the observed enhancement of freezing tolerance in *VvCBF4* overexpressor line 9-12, differences in relative mRNA abundance changes in this line were compared with those of empty vector control line 8-6 using microarray transcript profiling. RNA was extracted from whole aerial portions of young vines of line 9-12 and control line 8-6 (four biological replicates each), which had been propagated and harvested 30 d after transplantation to soil. RNA was verified to be of high quality with no degradation using an Agilent 2100 Bioanalyzer (Supporting Information, Figure S2). After cRNAs were hybridized to Affymetrix® *Vitis* GeneChip® microarrays and probeset intensities normalized by Robust Multi-Average (RMA), principal component analysis (PCA) of the RMA probe intensities found 99.4% of the variation among all samples to be explained by two components, p1 and p2 (Figure 6). Along these two axes, the microarray data can be seen to segregate by genotype, indicating that expression of the transgene is associated strongly with the observed phenotypic differences.

In *VvCBF4*-overexpressing line 9-12, 48 probesets were identified to have 1.5-fold or greater abundance than in control line 8-6, (significance of $p < 0.05$) using *t* statistics corrected for multiple comparisons (Benjamini and Hochberg, 1995). A total of 47 unique transcripts increased in abundance in the *VvCBF4* overexpressor, which included an acid

phosphatase class B (XP_002273448) that was specified by two independent probesets that showed 2.0-fold (probeset ID 1621892_a_at) and 1.6-fold (probeset ID 1617422_at) increase, respectively (Table 1). Changes in increased relative expression ranged from 1.5-fold to 5.6-fold. An additional 12 transcripts exhibited significantly decreased relative abundance in line 9-12, compared with control line 8-6 (Table 1). Changes in decreased relative expression ranged from −1.5-fold to −37.1-fold. The complete list of transcripts that displayed significantly different patterns of expression is presented in Supporting Information, Table S2.

Validation of transcript abundance by qRT-PCR

To validate the observed transcript abundance changes obtained using the *Vitis* GeneChip® microarray, qRT-PCR was performed on a set of nine genes that were selected at random from among those genes exhibiting either significantly increased or decreased transcript abundance using gene-specific primer pairs (Supporting Information, Table S1). Linear regression analysis of the $\log_2$-transformed values from the microarray analysis with those from qRT-PCR showed a goodness of fit (R^2) of 0.81, confirming that the observed changes in transcript abundance were accurate (Figure 7).

Comparison of woody CBF regulons

Transcriptome changes observed due to ectopic 35S::*VvCBF4* expression were compared with those identified in annual (leaf) and perennial (stem) tissues of 35S::*AtCBF1* regulons in the woody species *Populus* (Benedict et al., 2006). The closest protein homologues within previously reported CBF regulons were compared using BLAST homology searches. Comparing 35S::*VvCBF4* transgene expression with that in 35S::*AtCBF1* poplar revealed 10 genes with similar trends of increased and one gene, a chitinase class IV C gene, with decreased transcript abundance (Table 2). Three additional genes shared significant expression differences between the two transgenic woody species, but displayed varying directionality of expression (Table 2). These results indicate that at least part of the CBF-regulon is shared between these two diverse woody species.

Comparison of herbaceous CBF regulons

Next, the ectopic 35S::*VvCBF4* mRNA expression profile was compared with those from various independent reports of ectopic expression of *At*CBF or closely related genes including the 35S::*AtCBF1*, *2*, *3* regulons (Fowler and Thomashow, 2002; Maruyama et al., 2004; Maruyama et al., 2009; Sharabi-Schwager et al., 2010; Vogel et al., 2005) and the 35S::*DDF1* regulon (Magome et al., 2008) in the herbaceous species *Arabidopsis* (Table 3). A total of nine and four shared genes showed similar increases or decreases, respectively, in relative transcript abundance with two genes, a pectin methylesterase inhibitor (PMEI) and a GDSL lipase being common to four and five independent studies, respectively. Another five genes were shared among different experiments, but had only partially similar transcript expression trends between 35S::*VvCBF4* and the different *AtCBF* or *DDF1f* overexpressing *Arabidopsis* lines. Three genes showed consistent transcript expression trends with one or more independent experiments: a chitinase class IV gene, a thamatin-like gene, and a pepsin-like aspartic protease (Table 3). Lastly, six genes showed disparate transcript

expression trends between 35S::*VvCBF4* expression profiles and among the different *AtCBF* or *AtDDF1f* overexpressing Arabidopsis lines (Table 3). The amount of overlap in target genes observed for 35S::*VvCBF4* and these various *Arabidopsis* 35S::*AtCBF* (*DDF1f*) transgenic lines is comparable to the degree of overlap observed when 35S::*AtCBF1* driven expression in Poplar was compared with the *AtCBF3* regulon in *Arabidopsis*, wherein 12 genes showed similar increased transcript abundance (Benedict et al., 2006). These results indicate that CBF-regulons share common targets in both woody and herbaceous species.

2D-DIGE analysis

To quantify the effect of 35S::*VvCBF4* transgene expression on relative protein abundance, 2D-DIGE analysis was performed. Seventy-seven spots showed significant differences (one-way ANOVA, $p \leq 0.05$) in abundance in 35S::*VvCBF4* line 9-12 when compared with control line 8-6 across four biological replicates (data not shown). Of these, twenty-nine spots, of which 17 and 12 proteins were increased or decreased in protein abundance, respectively, were identified by MALDI-TOF/TOF analysis (Supporting Information, Table S4).

DISCUSSION

In this study, the role of a wine grape CBF transcription factor in conferring improved freezing tolerance was confirmed in the leaves of *V. vinifera* cv. Freedom. *VvCBF4* is unique among the four known *CBF/DREB* genes found in grapevine, sharing only 48, 45, and 45% homology with *VvCBF1*, *VvCBF2*, and *VvCBF3*, respectively (Xiao et al., 2008). In contrast, *VvCBF4* shares 58% sequence homology with *A. thaliana CBF1*, one of four known *Arabidopsis CBF/DREB1* genes. Among all genes found in the fully sequenced plant genomes, *VvCBF4* gene product has the greatest amino acid similarity the *Populus trichocarpa PtDREB70* and *PtDREB 71* gene products (Figure 1). *PtDREB70* and *PtDREB* 71, which were previously identified as *PtCBF2* and *PtCBF1* by Benedict et al. (2006), are cold-inducible *CBF* genes with peak mRNA expression at 9 h and 6 h after cold-exposure, respectively. Previously, transient expression assays of *VvCBF4* constructs in tobacco leaves confirmed that *VvCBF4* was capable of inducing the transcription of reporter genes via CRT cis-elements and that *Vitis* CBF4:GFP localized to the nucleus (Xiao et al., 2008). These observations, combined with the long duration of cold-induction (two-to-five days) of its transcript, which is even longer in the cold-hardy *Vitis riparia* species (Xiao et al., 2008), suggested that *VvCBF4* is likely to play an important role in adaptation to cold or freezing conditions. In *Eucalyptus*, another woody species, differences in the duration of cold induction of CBF genes between cold hardy and cold sensitive species have also been reported (Navarro et al., 2009).

The constitutive overexpression of *VvCBF4* enhanced freezing survival (Figure 4) and reduced electrolyte leakage under freezing conditions by about 2°C (Figure 5) in two of three *VvCBF4* expressing lines tested. The observed improvements in freezing tolerance are comparable to those observed in transgenic poplar ectopically expressing *AtCBF1*, which showed an improvement in freezing tolerance of 1.5°C in stems and 3°C in leaves, as assessed by electrolyte leakage assays (Benedict et al., 2006). These results were also

consistent with the improved freezing tolerance afforded by ectopic, constitutive expression of two endogenous *CBF* genes in transgenic *Eucalyptus* (Navarro et al., 2011). Overexpression of *AtCBF1* (*AtDREB1b*) under the control of the constitutive CaMV 35S promoter in transgenic *V. vinifera* cv. Centennial Seedless resulted in about a 2°C improvement in cold resistance as assessed by electrolyte leakage assays, reduced vine wilting, and improved vine survival at −4°C after 12 h (Jin et al., 2009). Ectopic expression of a *VvCBF4* gene from cv. Koshu under the control of the CaMV 35S promoter conferred cold tolerance in transgenic *Arabidopsis* (Takuhara et al., 2011). Lastly, these results were also consistent with the 2–3°C or 4–6°C increase in freezing tolerance exhibited by different transgenic apple trees expressing a peach *PpCBF1* gene compared with untransformed control trees as determined by electrolyte leakage assays (Wisniewski et al., 2011). Taken together, these results indicate that *Vitis spp*. employ CBF-mediated response regulons, in part, to modulate cold acclimation responses. If the protection against frost damage observed here for shoot tissues were pertinent to floral primordia of primary and secondary buds (Fennell, 2004), then this engineering strategy might serve to improve crop yields by reducing frost damage to fruit-bearing structures.

35S::*Vv*CBF4 overexpressor results in dwarf phenotypes

Interestingly, the 35S::*VvCBF4* expressing lines displayed a dwarf phenotype characterized by slower shoot elongation rates, shorter internodes, and fewer interodes per plant in a transgene dose-dependent manner (Figure 3). This observation is well known from constitutive, ectopic expression of *AtCBF1, 2,* and *3* genes in *Arabidopsis*, which results in dwarf phenotypes with smaller leaves (Gilmour et al., 2004; Kasuga et al., 1999; Liu et al., 1998; Sharabi-Schwager et al., 2010). Although direct measurements were not made to assess changes in either cell numbers or cell size in the transgenic *Vitis* lines, the dwarf phenotype observed here is likely to be the result of reduced cell expansion or elongation rather than cell division as observed in *EguCBF1a* overexpressing *Eucalyptus* (Navarro et al., 2011).

In the woody species poplar, ectopic *AtCBF1* expression resulted in a slowing of growth rate in plants less than six weeks of age (Benedict et al., 2006). After this age, tree growth rates returned to normal. Similarly, constitutive ectopic expression of two endogenous CBF genes in *Eucalyptus* resulted in reduced growth of microcuttings and leaf area, with *EguCBF1a* having a more pronounced effect than *EguCBF1b* (Navarro et al., 2011). Similarly, ectopic expression of a peach *CBF* gene in transgenic apple, a woody perennial species, resulted in reduced leaf size, but increased leaf dry weight, increased anthocyanin accumulation in cold-acclimated leaves, reduced shoot growth, and onset of dormancy as exhibited by terminal bud set and basipetal leaf senescence triggered by exposure to short day length (SD) or cold (4°C) conditions (Wisniewski et al., 2011). In contrast, constitutive expression of a *VvCBF4* gene from *cv*. Koshu in transgenic *Arabidopsis* apparently did not result in dwarfing, but only limited morphometric data were reported (Takuhara et al., 2011).

In wine grape, the observed reduction in growth rates observed for the 35S::*VvCBF4* transgenic lines might be desirable in a production setting because it would be expected to reduce vine vigor, thereby reducing labor costs normally associated pruning and leaf-

pulling, activities customarily practiced in vineyards to improve fruit and wine quality via increased sunlight exposure of the fruit (Matus et al., 2009). *CBF* gene overexpression is also known to delay flowering in some instances depending upon the *CBF* gene family member (Gilmour et al., 2004; Sharabi-Schwager et al., 2010). Delays in flowering time in wine grape might help avoid late spring frost damage to floral primordial and improve berry yield in some areas (Fennell, 2004). However, such delays might also limit berry-ripening potential in areas with short growing seasons.

Gene expression changes in the 35S::*Vv*CBF4 overexpressor

The 47 transcripts with significantly increased relative abundance in *Vv*CBF4-overexpressing grape vines spanned a wide range of molecular functions (Table 1). Five transcripts encoded proteins that catalyze the polymerization or depolymerization of cell wall structural components. For example, increased expression of the proteinaceous pectin methylesterase inhibitor (PMEI; 5.6-fold; 1606429_at) might act to reduce pectin depolymerization-based cell wall loosening leading to growth inhibition consistent with a dwarf phenotype (Figure 3), although the exact *in vivo* role of this inhibitor remains unclear (Jolie et al., 2010). In another example, increased transcript abundance of a leucine-rich repeat extensin (LRX; 2.4-fold; 1620976_at), which are thought to reinforce and stabilize cell wall polysaccharide structure by cross-linking (Baumberger et al., 2003; Ringli, 2010), might also be consistent with dwarfing as increased activity of this enzyme would be expected to limit cell size. Increased expression of an xyloglucan endotransglycosylase (XET; 2.0-fold; 1617739_at), which can participate in cell wall strengthening and reduce cell wall extension *in vitro* (Maris et al., 2009), might also be in agreement with the observed dwarf phenotype. However, the exact role of this gene product is difficult to predict on the basis of sequence information alone given the large size of this gene family and should be investigated by direct experimentation as other xyloglucan-specific enzymes have been reported to stimulate cell expansion (Maris et al., 2009). Increased abundance of two DUF1070 transcripts predicted to encode short arabinogalactosylated proteins (AGP; 1.7- and 1.9-fold; 1622530_at, 1619401_at), might also contribute to dwarfing as overexpression of an *Arabidopsis* arabinogalactan protein gene *AtAGP18* resulted in plants with smaller rosettes and shorter stems and roots (Zhang et al., 2011). Poplar, acclimated to cold for seven days, or overexpressing *AtCBF1*, also displayed increased abundance of an *AGP31* gene (Table 2) (Benedict et al., 2006). The elevated mRNA expression of LRX and EXGT genes was also observed in *AtCBF1*-overexpressing Poplar (Table 2), and that of the PMEI was conserved in *AtCBF2* and *AtCBF3* overexpressing *Arabidopsis* (Table 3) indicative of conservation of *CBF* gene regulons in both woody and herbaceous species. Lastly, a gibberellin-regulated gibberellic acid-stimulated Arabidopsis (GASA) *GASA5*-like gene (1617881_at) increased in abundance 2.27-fold (Table 1). In *Arabidopsis*, *GASA5* gene expression is stimulated by GA signaling and subsequent GASA5 activity negatively regulates GA-related gene expression and influorescence stem growth, making it both GA-regulated and GA-regulating (Zhang et al., 2009). Transcripts for a related *GASA1* gene have also been observed to be elevated in *AtCBF2* overexpressing *Arabidopsis* (Table 3), however, the exact role of this gene remains unclear at this time. Additional experiments are required to determine whether or not the expression of the *Vitis* GASA5-like gene is influenced by GA and if its product is involved in GA-signaling.

Another class of genes showing increased relative transcript abundance in the *VvCBF4* gene overexpressing line included those involved in lipid metabolism and epicuticular wax formation or modification. A homolog of the lipid transfer protein 3 (*LTP3*-like) gene increased in abundance 2.2-fold (1608175_at). Although the function of this gene is unclear, various functions have been assigned to lipid transfer genes including lipid exchange between membranes or as lipid sensors or chaperones (D'Angelo et al., 2008). The *Vitis LTP3*-like gene is most similar to the *Arabidopsis LTP3* (At5g59320) gene, which encodes a PR-14 family lipid transfer protein that is located in the apoplast and cell wall and whose transcript abundance is regulated by water deficit and ABA (Huang et al., 2008).

Three GDSL-motif lipase genes increased in abundance between 1.7- and 1.9-fold (1607744_at, 1607341_at, 1620618_at). In plants, members of the GDSL-lipase family are known to be involved in various types of lipid and acyl-group metabolism, including lipase, lysophospholipase, esterase, thioesterase, and arylesterase activities (Akoh et al., 2004). GDSL-lipases comprise a large gene family in land plant species, including grapevine, which contains more than 90 GDSL-lipase genes (Volokita et al., 2010). Some GDSL-lipase genes are required for proper cuticle formation. For example, in rice, the GDSL-lipase gene *Wilted Dwarf and Lethal* 1 (*WLD1*) is required for correct cuticle formation; *wld1* knockouts contain abnormal wax crystals and exhibit rapid water loss as a result of aberrant cuticle (Park et al., 2010). GDSL-lipase genes might also participate in bacterial and fungal pathogen resistance through eliciting local and systemic resistance (Kwon et al., 2009). Homologues of three GDSL-motif lipase genes also exhibited increased transcript abundance in *AtCBF1* and *CBF*-overexpressing Poplar and *Arabidopsis*, respectively (Table 2, Table 3).

The relative mRNA expression of genes involved in biosynthesis of the epicuticular wax also increased, including a glycerol-3-phosphate acyltransferase 4-like gene (*GPAT4*; 1.8-fold; 1612479_at), a LACERATA-like CYP450 (1.6-fold; 1621973_at), and a glucose-methanol-choline (GMC) oxidoreductase (1.5-fold; 1622345_at). The best matches in *Arabidopsis* for each of these transcripts were elevated in *A. thaliana* transgenic plants overexpressing the wax-inducing *WIN1/SHN1* AP2 domain-containing ERF transcription factor genes (Kannangara et al., 2007). The increased expression of epicuticular wax biosynthetic genes is consistent with the observed enhancement of epicuticular wax deposition in transgenic *Eucalyptus* (*E. urophylla* × *E. grandis*) overexpressing an endogenous *CBF1a* gene (Navarro et al., 2011). Although additional experiments are needed to determine if and how ectopic expression of *VvCBF4* might have affected cuticle formation in grapevine, transgenic vines retained a normal appearance.

A set of stress-responsive genes also showed increased transcript abundance in the *VvCBF4* overexpressing line including genes encoding the RESPONSIVE TO DESICCATION 22 (*VvRD22*) gene (1.9-fold; 1621818_at), a polyketide cyclase/dehydratase (major latex protein 28-like) (1.7-fold; 1607196_at), thaumatin-like *VvTL1* (1.7-fold; 1614746_at), stress-related basic secretory protein (1.5-fold; 1615434_at), and an unknown stress-regulated transcript (2.1-fold; 1613368_at) genes, which have each been reported previously to increase in mRNA abundance under abiotic stress conditions either in grape or *Arabidopsis* (Fowler and Thomashow, 2002; Hanana et al., 2008; Kuwabara et al., 1999;

Lytle et al., 2009). Transcripts coding for the DNA repair enzyme DNA-3-methyladenine glycosidase I (1.9-fold; 1621976_at) and the abiotic stress-responsive methylglyoxal detoxification enzyme lactoylglutathione lyase/glyoxalase I (2.1-fold; 1619235_at) also increased (Santerre and Britt, 1994; Yadav et al., 2008). Two other stress-regulated transcripts with increased mRNA expression with likely roles in signal transduction included a calmodulin (CaM)-like gene (2.6-fold; 1617516_at), which has also been shown to increase in expression in cold-stressed *Arabidopsis* (Fowler and Thomashow, 2002), and a calcium-binding EF-hand-containing, *MSS3*-like transcript (1.6-fold; 1612996_at). Lastly, the mRNA expression an AAA-type ATPase domain (chaperone-like) gene (1610816_at) increased in abundance 4.7-fold (Table 1). A homolog of this gene is known to respond to salt stress in *Arabidopsis* (Fujita et al., 2005).

In contrast to the relatively large numbers of genes with elevated transcript abundance, only twelve genes showed a significant decrease in mRNA abundance in the *VvCBF4* overexpressor. Most notable was a BAG-domain (**B**CL-2 **a**ssociated athanogene) gene that showed a 37-fold decrease in relative transcript abundance (Table 1). This nuclear localized, calmodulin-binding domain containing gene is most similar to *AtBAG6*, which when overexpressed induces programmed cell death in transformed yeast and *Arabidopsis* (Kang et al., 2006). *AtBAG6* is also thought to coordinate stress-induced hormone signaling and might play a role in limiting pathogen colonization (Doukhanina et al., 2006). Additional experiments will be necessary to determine the exact role of this gene in *Vitis*.

Lack of correlation between mRNA and protein expression

Interestingly, none of the proteins identified as being significantly overexpressed in the *VvCBF4*-overexpressing line 9-12 (Supporting Information, Table S4) matched the differentially expressed transcripts described here. This result is in contrast to the correlation observed between transcript and protein abundance changes described for heat-stressed, 35S::*DREB2C* overexpressing *Arabidopsis* plants, wherein a total of 10 different proteins showed significant changes in protein abundance (6 and 4 proteins showed increased or decreased abundance, respectively) compared with wild type plants (Lee et al., 2009). However, no significant differences were apparent if the transgenic plants were not heat-treated. One possible explanation for these different results is that heat stress might be necessary for transcription factor activation as described for *DREB2A*, which was activated by dehydration, high salinity, ABA, or cold treatments, presumably by posttranslational modification (Liu et al., 1998). Indeed, phosphorylation of a stress-inducible *DREB2A* transcription factor from *Pennisetum glaucum* was shown to prevent DNA binding (Agarwal et al., 2007). However, such posttranslational modifications are not always necessary as in the case of *DREB1A*-related transcription factors (Liu et al., 1998). In another example, comparison of non-transgenic and transgenic potato expressing 35S::*AtDREB1A* using 2D-DIGE showed increased expression of only patatin, a major storage protein and decreased expression of lipoxygenase and starch synthase (Nakamura et al., 2010). Thus, there is precedent for ectopic expression of *CBF*-family transgenes having a relatively small effect on the proteome.

One possible explanation for the lack of correlation between transcript and protein abundance is that *VvCBF4* might require posttranslational modification for activation. Additional experiments would be required to test this hypothesis. Studies in various plant species, including wine grape berries, have shown that there are only moderate to weak correlations (r = 0.50 or less) between mRNA and protein depending on the study (Faurobert et al., 2007; Gion et al., 2005; Grimplet et al., 2009b; Liu et al., 2006; Watson et al., 2003). Additional possible explanations for this weak correlation include the observation that mRNA abundance changes are typically presented as fold-changes and not in absolute terms, so this might favor the reporting of low abundance transcripts. Also, alternatively spliced transcripts might not be detected by microarray or qRT-PCR analytical methods, however, such events might result in changes in relative protein expression.

Conclusions

In conclusion, ectopic expression of the *VvCBF4* resulted in improved freezing survival in non-acclimated vines to a degree comparable to that observed for *CBF* overexpression in *Arabidopsis* and other woody species within *Eucalyptus*, *Malus*, *Populus*, and *Vinifera* genera. The gene expression profiling results presented here clearly show that wine grape possesses an evolutionarily conserved CBF regulon that is widely conserved among cold-adapted herbaceous species as well as other woody species. Improvement in freezing tolerance should be useful in reducing late spring frost damage to floral primordia of primary and secondary buds in *Vitis*. The observed dwarf phenotype might be advantageous to improving berry ripening by reducing canopy vigor and allowing more sun exposure to developing berries. However, to avoid possible undesirable effects of dwarfing, such as reductions in sink tissue (i.e., berries) production typically associated with constitutive expression of CBF transcription factors, future experiments should focus on the development of transgenic vines expressing this or other *VvCBF* gene family members under the control of abiotic stress-inducible promoters. *VvCBF4*-overexpressing lines might also be predicted to be more tolerant to salinity and water deficit stress. Additional experiments are in progress to verify these possibilities.

EXPERIMENTAL PROCEDURES

*Vv*CBF4 sequence analysis

Phylogenetic analysis, based on minimum evolution, was conducted on the full-length protein sequences of *CBF* gene family members from eudicots for which complete genome drafts are available. The polypeptides aligned included four *CBF* genes from *Arabidopsis*: *AtCBF1* (NP_567721.1), *AtCBF2* (NP_567719.1), *AtCBF3* (NP_567720.1), *AtCBF4* (NP_200012.1); four CBFs from *Glycine max*: *GmCBF1* (AAQ02703.1), *GmCBF2* (ACB45077.1), *GmCBF3* (ACA63936.1), and *GmDREB7* (ABQ42206.1); six CBFs from *Populus trichocarpa*: *PtDREB66* (XP_002313656.1), *PtDREB67* (XP_002328068.1), *PtDREB68* (XP_002299565.1), *PtDREB69* (XP_002298067.1), *PtDREB70* (XP_002318846.1), and *PtDREB71* (XP_002321877.1); four *CBF* genes from *Medicago truncatula*: *MtCBF1* (ABX80062.1), *MtDREB1A* (ABG75914.1), *MtCBF2* (ABX80063.1), and *MtDREB* (ABB72792.1); and four *CBF* genes from *Vitis vinifera*: *VvCBF1* (AAR28673.1), *VvCBF2* (AAR28677.1), *VvCBF3* (XP_002267961.1), and *VvCBF4*

(XP_002280097.1). The *Arabidopsis* AP2-AE *ERF10* (NP_171876.1) was included as a non-CBF/DREB rooting outlier. Alignments were generated in the MacVector (ver. 12.0, MacVector, Inc., Cary, NC) software suite, using ClustalW alignment. The phylogeny tree was constructed using neighbor-joining tree building, with Poisson-corrected distances, 1000 bootstrap replicates, and post-build outlier rooting.

CBF4 overexpression construct

The full-length *VvCBF4* open reading frame (ORF, GenBank accession: DQ497624) was PCR-amplified from genomic DNA using the forward primer 5'-CACCATGAATACTACTTCTCCACCATATTCC-3' and reverse primer 5'-CTAAATAGAGTAACTCCATAATGACATGTC-3'. The PCR product was cloned into pENTR/D-TOPO and then into the pH2GW7 GATEWAY-type expression vector using LR clonase to recombine the *VvCBF4* ORF between sites *attr1* and *attr2*, downstream of the Cauliflower Mosaic Virus (CaMV) 35S constitutive promoter. Empty vector control line (e.g., "8-6") vectors were constructed by the removal of the GATEWAY cassette from pH2GW7 with ApaI/SstI digestion, blunt-end formation via T4 polymerase 3' exonuclease activity, and religation with T4 ligase (see Supporting Information, Figure S1 for details). Both the *VvCBF4* expression vector and the empty vector control were verified by DNA sequencing and restriction digestion (Tattersall, 2006). Expression constructs were then introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Vitis vinifera cv. Freedom transformation

Grape vine transformation was performed by the Ralph M. Parsons Plant Transformation Facility, University of California, Davis, CA. Briefly, immature anthers from the *Vitis* hybrid rootstock cv. 'Freedom' were used as a source of tissue for embryogenic callus. The embryogenic callus was inoculated with an overnight suspension of *Agrobacterium tumefaciens* containing the expression cassettes outlined above and adjusted to an $O.D._{600}$ of 0.075. Callus tissue was plated onto a sterile No. 1 Whatman filter paper, which was placed on top of co-culture medium consisting of Woody Plant Media (WPM) (Lloyd and McCown, 1981) supplemented with 10 mg/l picloram, 2 mg/l thidiazuron (TDZ), 500 mg/l activated charcoal, 1000 mg/l casein and 200 μM acetosyringone. *Agrobacterium* was added dropwise to the callus until thoroughly moistened. After 15 min, the callus was blotted dry using a second piece of sterile No. 1 Whatman filter paper, and incubated in the dark at 23°C. After 48–72 h the callus was transferred to WPM supplemented with 10 mg/l picloram, 2 mg/l thidiazuron (TDZ), 500 mg/l activated charcoal, 1000 mg/l casein, 400 mg/l carbenicillin, 250 mg/l cefotaxime and 25 mg/l hygromycin sulfate. Callus was incubated in the dark at 26°C and transferred to fresh medium every 21–28 days. Hygromycin-resistant embryogenic callus colonies formed after 3–4 months. These colonies were harvested and transferred to germination medium consisting of WPM medium supplemented with 20 g/l sucrose 500 mg/l activated charcoal, 1000 mg/l casein, 150 mg/l timentin, 0.5 mg/l benzylaminopurine (BAP) and 0.1 mg/l naphthalene acetic acid (NAA). Elongating embryos were transferred to rooting media consisting of one-half strength Murashige and Skoog minimal organics medium (Murashige and Skoog, 1962) supplemented with 15 g/l sucrose, 0.01 mg/l naphthalene acetic acid (NAA), 150 mg/l timentin and 25 mg/l hygromycin. Rooted plantlets were acclimated to soil in a growth

chamber under 16 h photoperiod at 26°C and then transferred to greenhouse for further development.

Vine propagation

Green cuttings (2–3 internodes in length) from well-established, woody vines were sterilized with 50% (v/v) household bleach and 0.02% (v/v) Triton X-100 for 30 sec and placed in autoclaved, sterile 77 mm × 77 mm × 97 mm (W × L × H) Magenta GA-7 boxes (Magenta Corp., Chicago, IL) containing 80 ml of 0.8% (w/v) Plant Tissue Culture Agar (#A111, Phytotechnology Laboratories, Shawnee Mission, KS) with Murashige and Skoog modified basal medium w/Gamborg vitamins (#M404, Phytotechnology Laboratories), 1.5% sucrose, pH 5.6–5.7 and 300 μM indoleacetic acid (IAA) to promote rooting (Gamborg et al., 1968; Murashige and Skoog, 1962). Vines were allowed to develop roots in a Percival Scientific growth chamber (Model # CU-32L; Perry, IA) under 18 h of fluorescent light (200 μmol m^{-2} sec^{-1} PPFD) at 25°C and 6 h darkness at 20°C. Upon development of root primordia (~14 days), individual vines were transferred to a second Magenta box containing media prepared as above, except without IAA, to promote normal plant growth for about 10 d. Upon establishment of normal growth, cuttings from these vines were further propagated in Magenta boxes (one cutting/box) for subsequent use in stress phenotyping assays, electrolyte-leakage assays, mRNA and protein expression assays and growth phenotyping. These cuttings were handled identically as the “mother vines” (omitting the bleaching step, as they were already sterile), and were allowed ~24 d to develop roots in Magenta boxes, followed by transplantation to 4” square plastic pots (Part # TSD4, McConkey Co., Garden Grove, CA) containing Metromix® 200 soil (Sun Gro Horticulture, Bellevue, WA). Vines were allowed an additional two weeks to adapt to soil and low humidity before abiotic stress assays were performed. Acclimation was accomplished by placing the newly potted vines in a tray covered with a clear plastic dome to maintain high humidity. After 5 d, a dome having a 50% vented surface area replaced the full dome. After an additional 5 d, the vented dome was removed completely. Twice during the two-week time period, vines were given ¼ strength modified Hoagland’s solution with full-strength iron and micronutrients (600 μM KNO_3, 400 μM $Ca(NO_3)_2 \cdot H_2O$, 200 μM $NH_4H_2PO_4$, 100 μM MgSO4·$7H_2O$, 280 μM Fe-EDTA, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4 \cdot H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.5 μM $CuSO_4 \cdot 5H_2O$, and 0.5 μM H_2MoO_4) (Hoagland and Arnon, 1950).

Freezing stress assays

For freezing stress assays, groups of vines (n=10) from the empty vector control line (8-6) and three 35S::*VvCBF4* overexpressor lines (9-1, 9-3, and 9-12) were placed in a pre-chilled Percival Scientific growth chamber (model # AR75L) for 24 h at −2°C. The built-in humidifier was turned off and water drained from the reservoir to avoid ice buildup and damage to the growth chamber. Twenty kg of frozen ice packs and a small fan were added to the growth chamber to maintain maximum temperature equilibrium throughout the chamber during stress application. Plant position within the growth chamber was randomized. Assays began and ended at 10 AM, with 18 h light (200 μmol • m^{-2} • sec^{-1} light) beginning at 6 AM and 6 h dark beginning at 10 PM. Vines were watered two days post-stress and allowed to recover under normal growth conditions (25°C day / 20°C night temperature) under the same light conditions as above for two weeks, at which point

survival was scored by the presence of new growth. Freezing stress assays were performed on multiple groups of vines, as described, and Student's *t* test corrected for multiple comparisons was used to identify statistically significant genotypic differences in freezing tolerance (adjusted *p*-value < 0.05).

Electrolyte leakage assay

The freezing tolerance of the three transgenic *V. vinifera* cv. 'Freedom' lines overexpressing *VvCBF4* and one control line was also assayed by leaf electrolyte leakage. Transgenic *V. vinifera* lines 9-1, 9-3, 9-12, and the empty vector control line, 8-6, were grown in pots for 6 weeks (150–200 μmol • m^{-2}• sec^{-1} PPFD, 16 h light at 25°C/ 8 h dark at 20°C) without any cold acclimation. Identical-sized leaf discs (6 mm diameter) were punched from individual fully expanded leaves and were equilibrated in 200 μl of Nanopure water (Millipore, Inc., Bedford, MA) in 1.5 ml microfuge tubes for 1 h. For freezing treatment, we manually adjusted a Neslab RTE-4 (Thermo Fisher Scientific, Inc., Waltham, MA) refrigerated circulating antifreeze bath to achieve 1 °C/30 min decrement from 0°C to −16°C. Tubes were floated in the refrigerated water bath and exposed to the freezing conditions. At each 1°C/30 min interval, tubes were removed and gently thawed at 4°C for 18 h. Leaf discs and incubation solutions were transferred to another tube containing 4 ml of Nanopure water. After shaking overnight, conductivity was measured using an Orion 4Star Portable Conductivity Meter with the Orion 013010MD Conductivity Cell (Thermo Fisher Scientific, Inc.). Samples were autoclaved for 20 min at 121°C and the conductivity was re-measured. The level of electrolyte leakage induced by freezing was determined as the percentage of conductivity before autoclaving *versus* conductivity due to total leakage after autoclaving. Student's *t* test corrected for multiple comparisons was used to identify significant genotypic differences in electrolyte leakage (adjusted *p*-value < 0.05).

Growth phenotyping

The shoot elongation rate (SER) of two-week-old soil-rooted plantlets of *VvCBF4* overexpressor lines and empty vector control lines (n=8) was measured to identify possible alterations (reductions) in growth. The shoots were marked with acrylic nail polish just below the first above-soil internode, and all internodal lengths were measured using dial calipers (Mitutoyo America Corp., model no. 505-675-66, Aurora, IL) every third day. Total shoot length and SER were determined over 21 days. Student's *t* test corrected for multiple comparisons was used to identify significant genotypic differences in growth rates and internode length (adjusted *p*-value < 0.05).

RNA extraction

Entire aerial portions (stems and young leaves) of grapevines that had been growing in soil for two weeks were collected in 50 ml conical tubes and immediately frozen in liquid nitrogen. Total RNA was isolated using a three-step process. First, total RNA was extracted and applied to an RNeasy Midi column (Qiagen, Inc., Valencia, CA) with 2% Polyethylene Glycol (PEG, mw = 20,000, Sigma Aldrich, Inc., St. Louis, MO) added to the RLT buffer, followed by on-column DNase digestion. The resultant RNA was then subjected to phenol:chloroform:isoamyl-alcohol (25:24:1) extraction (Sambrook and Russell, 2001).

Lastly, the RNA extract was further purified using an RNeasy mini column (Qiagen, Inc.) with 2% PEG (Tattersall et al., 2005). Total RNA was quantified and 260/280 ratios determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA integrity was confirmed by electrophoresis on formaldehyde-containing 1.5% agarose gels.

Quantitative real-time (reverse transcription)-PCR (qRT-PCR)

cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to manufacturer's instructions, using uniform 2 μg RNA per reaction volume reverse-transcription reactions. Gene-specific primers for qRT-PCR reactions were selected using the Primer-BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) using RefSeq *V. vinifera* transcripts as input, screened against all other *V. vinifera* RefSeq sequences, and the following Primer3 (Rozen and Skaletsky, 2000) settings: T_m range 58–60°C, product size = 50–150 bp, primer size = 13–25 nt, max poly-X = 3, and G/C content = 30–80%. Primer pair selection against a GC clamp, such that no more than two of the last five 3' nucleotides were G or C, was conducted per qRT-PCR instrument recommendations. qRT-PCR reactions were prepared using Fast SYBR® Green Master Mix and performed using the ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Inc.) using four biological replicate samples and normalized to an endogenous actin 7 control gene (NCBI locus ID, LOC 100232968) (Reid et al., 2006). Gene-specific primer pairs and products used are summarized in Supporting Information, Table S1. Relative quantitation of qRT-PCR outputs was performed using the Pfaffl method, an elaborated ΔΔCt method that employs observed primer efficiencies (rather than assuming 2^-ΔΔCt) when comparing different primer pairs/products (Pfaffl, 2001).

Microarray-based mRNA expression profiling

For microarray experiments, RNA was re-quantified using RiboGreen® fluorescent nucleic acid stain (Invitrogen Life Technologies, Inc., Carlsbad, CA) and read using a Labsystems Fluoroskan Ascent fluorescence plate reader (Thermo Fisher Scientific, Inc.). Sample RNA integrity was determined with an Agilent 2100 Bioanalyzer microfluidics station (Agilent Technologies, Inc., Santa Clara, CA). Total RNA (300 ng) was loaded onto a capillary electrophoretic column to determine major (rRNA) band sizes and quality (see Supporting Information, Figure S2). Complementary DNA (cDNA) was generated from mRNA using a GeneChip® T7-Oligo(dT) Promoter Primer Kit containing the T7 polymerase promoter sequence and oligo(dT) priming region (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3') (Affymetrix®, Santa Clara, CA) and SuperScript™ II Reverse Transcriptase (Invitrogen Life Technologies, Inc.) according to the manufacturer's instructions (Affymetrix, 2009). Biotinylated complementary RNAs (cRNAs) were synthesized *in vitro* from four biological replicates using T7 RNA polymerase in the presence of biotin-labeled UTP/CTP, then purified, fragmented and hybridized to GeneChip® 16K *Vitis vinifera* (Grape) Genome Arrays ver. 1.0 (Affymetrix®). The hybridized arrays were washed and stained with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody using an Affymetrix® 3000 7G

Scanner, Hybridization, and Fluidics System. Scanner and image data was collected and processed on a GeneChip® Workstation using Affymetrix® GCOS software.

The images of all arrays were examined, and no obvious scratches or spatial variations were observed. The 'present' call rates were also consistent across the eight arrays, ranging from 66% to 70% (mean rate = 69%). Raw hybridization intensity values were processed by Robust Multi-Array Average (RMA) (Irizarry et al., 2003), using the R package *affy* (Gautier et al., 2004). Specifically, expression values were extracted from raw *CEL* files by first applying the RMA model of probe-specific correction to PM (perfect match) probes. Corrected probe values were then normalized via quantile normalization, and a median value computed for the PM probeset. Resulting RMA expression values were $\log_2$-transformed. Distributions of the RMA-expression values of all arrays (four biological replicates) were visualized by two-dimensional Principle Component Analysis (PCA).

A *t* test was performed for each RMA-PM probeset to determine which genes were differentially expressed between the transgenic line (9–12) and empty vector control-transformed (8-6) genotypes. Multiple testing correction was performed on the *t* statistic of each probeset to minimize false discovery rate (Benjamini and Hochberg, 1995). Genes that were differentially expressed (±1.5-fold at adjusted *p*-value < 0.05) were identified and assigned functional annotation derived from *Vitis*Net (Grimplet et al., 2009a) and the PlexDB microarray annotations http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Grape (Wise et al., 2007) and presented in Table 1. The complete list of significantly differentially expressed genes is presented in Supporting information, Table S2. In this table, the light red shading indicates increased abundance, light green shading indicates decreased abundance, and genes not significant by false discovery were shaded in light gray. Microarray data were deposited in the NCBI GEO database under series GSE29948 viewable at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29948.

Genes identified by microarray analysis that showed significantly increased or decreased transcript abundance in the 35S::*VvCBF4* overexpressor were compared by sequence homology against other *CBF/DREB1f* regulon genes from previous studies as listed in Tables 2 and 3. The translated protein sequences of the genes or transcripts associated with each increased/decreased probeset were compared using Basic Local Alignment Search Tool (BLAST), with the NCBI BLAST+ software (Camacho et al., 2009) using the command line BLASTP algorithm, word size 3, open penalty 11, extend penalty 1, window size 40 and maximum e-value cutoff 1×10^{-15}.

Protein extraction

Whole aerial portions of young vines grown in soil for 3 weeks (control line 8-6 plant height of ~16 cm; 35S::*VvCBF4*, line 9-12 plant height of ~10 cm) were collected and frozen immediately in liquid nitrogen and stored at −80°C until further use. Four biological replicates from each treatment were individually ground in liquid nitrogen with a mortar and pestle, followed by extraction of total proteins with a phenol-based protocol optimized for grape (Vincent et al., 2006), based upon protocols previously developed for recalcitrant plant tissues (Hurkman and Tanaka, 1987; Saravanan and Rose, 2004). From each of the

eight samples, 5 g of frozen, ground tissue were added to 10 ml of protein extraction buffer (0.5 M Tris-HCl pH 7.5, 0.7 M sucrose, 50 mM EDTA, 0.1 mM KCl, 2 mM PMSF, and 2% (v/v) β-mercaptoethanol in water) containing 1 Complete™ Protease Inhibitor Cocktail Tablet per 10 ml of buffer (Roche Applied Science, Inc., Indianapolis, IN) in a 50 ml BD Falcon™ tube and vortexed for 30 sec followed by a 10 min incubation at 4°C. Next, 10 ml of Tris-saturated phenol (pH 7.9) was added to the mixture vortexed for 30 sec, followed by a 30 min incubation at 4°C with inversion of the samples every 10 min. Samples were then centrifuged at 3650 × *g* and 4°C for 30 min in a Beckman Allegra™ 6R centrifuge (Beckman Coulter Inc., Brea, CA). The upper phenol phase was then removed to a new 50 ml BD Falcon™ tube and an equal volume of fresh protein extraction buffer added. This was followed by vortexing for 30 sec and incubation for 30 min at 4°C with inversion of the samples every 10 min. Samples were again centrifuged at 3650 × *g* and 4°C for 30 min, and the phenol phase for each sample was again removed to a new 50 ml BD Falcon™ tube. To precipitate proteins, five volumes of cold (−20°C) methanolic ammonium acetate (0.1 M ammonium acetate in methanol) were added to each Falcon tube, followed by placement of samples for 3 h at −20°C with inversion every 10 min. Next, the 50 ml BD Falcon™ tubes were spun at 3650 × *g* at 4°C for 30 minutes using the Beckman Allegra™ centrifuge to pellet the proteins. Following centrifugation the supernatants were discarded and 5 ml cold (−20°C) methanol was added to each tube to wash the pellet. The samples were vortexed for 30 sec and placed for 1 h at −20°C with inversion every 10 min, followed by centrifugation at 3650 × *g* at 4°C for 30 min. The supernatant was discarded, and three additional wash, vortex, and centrifugation steps were performed in the same manner as the methanol wash, all with ice-cold (−20°C) acetone. Following the final acetone wash, wet pellets were transferred to 2 ml Eppendorf tubes and placed on ice until 2D-DIGE analysis.

Two-dimensional-Difference in Gel Electrophoresis (2D-DIGE) analysis

The acetone-wetted protein pellets were centrifuged at 13,000 × *g* at 0°C for 10 min and washed an additional two times with acetone/water (4:1) before being chilled to −20°C. The final pellets were allowed to dry in open tubes for 10 min, including 1 min of drying at 3,000 × *g* in a Speed Vac System (Thermo Fisher Scientific Inc.). Individual dried pellets were resuspended in 200 μL DIGE Reaction Buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.74). The tubes were vortexed frequently a minimum of 10 times and then sonicated for a total of 10 min using 30 sec pulses in a water bath sonicator (model no. FS30, Fisher Scientific, Pittsburgh, PA) over a period of 2 h. Samples were then centrifuged at 13,000 × *g* at 22°C for 10 min. The supernatant from each sample was removed to a clean 1.7 ml microfuge tube and assayed for protein concentration using an EZQ™ Protein Quantification Kit with ovalbumin as the standard (Bio-Rad Laboratories, Inc., Hercules, CA). Samples were stored overnight at −20°C.

Following thawing on ice, additional aliquots of DIGE reaction buffer were added to each sample to bring the final protein concentration to 1.33 mg/ml. Sixty μl of each sample (80 μg protein) was then pipetted into a 0.5 ml microfuge tube. Each of three Cy-dyes (e.g., Cy2, Cy3, Cy5; GE Healthcare, Inc., Piscataway, NJ), which were solutions of 5 nmoles dye in 5 μL dimethylformamide (DMF), were diluted 5× (1μl Cy dye in 4μl DMF) prior to use. Samples were then subjected to a random dye-swap scheme for normalization of differences

in Cy-dye fluorescence intensity (Supplemental Information, Table S3). A 1.15 µl aliquot of Cy3 and Cy5 were added to each sample. For an internal control, 30 µl of resuspended protein from each sample was mixed in a single tube and 4.6 µl Cy2 was added. At this point, each of the 8 sample tubes contained approximately 80 µL total protein and 230 pmoles total Cy dyes (Cy3 and Cy5), whereas the pooled sample control tube contained 331 µg protein and 920 pmoles Cy2. The tubes were quickly vortexed and then placed on ice, centrifuged briefly, then placed back on ice. The ice bucket was then covered with aluminum foil and stored in a dark cabinet for 30 min for Cy dye/protein binding. At the end of the 30 min incubation, 2 µl of 10 mM aqueous lysine was added to each tube. The tubes were vortexed and then put back on ice in the dark for an additional 10 min. Four 1.7 ml microfuge tubes were numbered gel 1 – 4, corresponding to the gel numbers given in Supporting Information, Table S3. The contents of the incubated protein/Cy dye mixes were then transferred to the labeled "gel" tubes, completed by washing each 0.5 ml tube with 50 µl 2X DIGE Reaction buffer (7 M urea, 2 M thiourea, 4% CHAPS). To each of the “gel” tubes, the following were added: 60 µl pooled sample, 137 µl 2× DIGE Reaction Buffer, 4 µl 0.1% bromophenol blue, 2.35 µl carrier ampholytes pI = 3–10 (Bio-Rad Laboratories, Inc.), and 47.0 µl of 10.5 M DTT solution. The mixtures were then centrifuged at 13,000 × *g* (13,000 rpm) and 22°C for 10 min. From each of the four gel tube mixtures, 450 µl of supernatant was applied to a 24 cm 4–7 immobilized pH gradient (IPG) strip (GE Healthcare, Inc.). The strip was rehydrated passively overnight (for 22 h) at 20°C. Isoelectric focusing (IEF) was performed as follows: active rehydration at 50 V for 4 h, a linear increase to 200 V in 1 h, a linear increase to 500 V in 1 h, a linear increase to 1000 V in 1 h, a linear increase to 10,000 V in 2 1/2 h, and maintenance of steady voltage from 10,000 V to 70,000 Vh (Volt-hours) were conducted (~7 ½ h). IPG strips were removed and stored at −80°C until the second dimension was run.

IEF strips were thawed at room temperature (10 min) followed by equilibration in Equilibration Base Buffer (6 M urea, 30% v/v glycerol, 2 M Tris-HCl pH 8.8, and 2% w/v SDS) containing 1% w/v DTT for 20 min to reduce proteins, and then 2.5% w/v iodoacetamide for 20 min to alkylate proteins. The strips were then loaded onto 12.0% (v/v) 26 × 20 × 0.1 cm polyacrylamide gels (Jule Inc., Milford, CT). The gels had been treated with Bind-Silane (γ-methacryloxypropyltrimethoxysilane) and had reference markers attached on the short plate. Electrophoresis was performed using a Protean® Plus Dodeca Cell (Bio-Rad Laboratories, Inc.) with standard Tris-Glycine-SDS Buffer (25 mM Tris-base, 0.5 M Glycine, 0.1% v/v SDS) under the following conditions: 40 V for 2 h followed by 100 V for 24 h at 10°C.

Prior to imaging, the larger glass plate from each of the gel cassettes was removed. A Typhoon Trio™ Imaging System (part no. 63-0055-87, GE Healthcare, Inc.) was used for image acquisition using the Blue (488 nm) laser. Immediately following imaging, each gel was placed in destain solution (7% v/v acetic acid, 10% v/v methanol) and shaken gently for 72 h. Images captured with the Typhoon were analyzed using DeCyder™ 2D Software ver. 7.0 (GE Healthcare, Inc.) for protein quantification. Sample labeling and CyDye swapping schema are summarized in Supporting Information, Table S3.

Protein identification

For spot picking and protein identification, two gels (replicates #1 and #4) were selected for Sypro® Ruby staining. The destain solution was removed from the gels and ~110 ml of Sypro® Ruby Staining solution (Invitrogen, Inc., Carlsbad, CA) in ~960 ml of fresh destain solution was added. Gels were gently shaken for 48 h followed by washing once with fresh destain solution and once with water. Spot excision was performed using the EXQuest™ spot cutter (Bio-Rad Laboratories, Inc.) followed by trypsin digestion according to the protocol developed by Rosenfeld et al. (Rosenfeld et al., 1992) using the Investigator™ ProPrep™ protein digestion kit (Genomic Solutions, Ann Arbor, MI, USA). The trypsin-digested fragments were analyzed using an ABI 4700 Proteomics Analyzer™ (Applied Biosystems, Foster City, CA, USA) matrix-assisted laser desorption/ionization (MALDI) Time-of-flight/time-of-flight (TOF/TOF) mass spectrometer (MS). A 0.5 mL aliquot of matrix solution with 5 mg/ml alpha-Cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich, Inc.) and 10 mM ammonium phosphate (Sigma-Aldrich, Inc.) in 0.2% formic acid was co-spotted with 0.5 ml of sample (Zhu and Papayannopoulos, 2003). The data were acquired in reflector mode from a mass range of 700–4000 Da, and 1,200 laser shots were averaged for each mass spectrum. Each sample was internally calibrated if both the 842.51 and 2211.10 Da ions from trypsin autolysis were present. The eight most intense ions from the MS analysis not present on the exclusion list were subjected to MS/MS analysis. To this end, the mass range was from 70 to precursor ion size with a precursor window of 1–3 Daltons using an average of 2500 laser shots for each spectrum. The resulting mass data were then used to search the NCBI *nr* database (ver. 10_09_2009; 9,694,989 sequences) and the contigs from *Vitis* Gene Index (ver. 18_9_2009, 23,493 sequences) using automated MASCOT V.2.1 software (http://www.matrixscience.com/). Peptide tolerance was 20 ppm, one missed cleavage was allowed, and MS/MS tolerance was 0.8 Da. The possibility of matching multiple translated isoforms was examined by manual analysis of peptides present within the sequences. All MS analyses were performed in cooperation with the Nevada Proteomic Center at the University of Nevada, Reno. The complete list of differentially expressed proteins is presented in Supporting Information, Table S4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by funding from the National Science Foundation NSF (DBI-0217653) and the University of Nevada Agricultural Experiment Station (to GRC and JCC). The authors would like to thank David Tricoli and Kim Carney of the Ralph M. Parsons Plant Transformation Facility, Davis, CA for performing the *Vitis* transformations. The authors thank Craig Osborn of the Nevada Genomic Center for performing microarray services, Rebecca Woolsey, Kathy Schegg, and David Quilici of the Nevada Proteomics Center for support and for performing MS analyses, and Rebecca Albion and Kitty Spreeman for invaluable technical support. We would also like to thank Mary Ann Cushman for her critical reading of the manuscript. This publication was also made possible by NIH Grant Number P20 RR-016464 from the INBRE Program of the National Center for Research Resources through its support of the Nevada Genomics, Proteomics and Bioinformatics Centers.

References

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P. The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell. 2008a; 20:2117–2129. [PubMed: 18757556]

Affymetrix. GeneChip® Expression Analysis Technical Manual. 2009

Agarwal P, Agarwal P, Nair S, Sopory S, Reddy M. Stress-inducible DREB2A transcription factor from *Pennisetum glaucum* is a phosphoprotein and its phosphorylation negatively regulates its DNA-binding activity. Mol. Genet. Genomics. 2007; 277:189–198. [PubMed: 17089163]

Akoh C, Lee G, Liaw Y, Huang T, Shaw J. GDSL family of serine esterases/lipases. Prog. Lipid Res. 2004; 43:534–552. [PubMed: 15522763]

Arroyo-Garcia R, Ruiz-Garcia L, Bolling L, Ocete R, Lopez M, Arnold C, Ergul A, Soylemezoglu G, Uzun H, Cabello F, Ibanez J, Aradhya M, Atanassov A, Atanassov I, Balint S, Cenis J, Costantini L, Goris-Lavets S, Grando M, Klein B, McGovern P, Merdinoglu D, Pejic I, Pelsy F, Primikirios N, Risovannaya V, Roubelakis-Angelakis K, Snoussi H, Sotiri P, Tamhankar S, This P, Troshin L, Malpica J, Lefort F, Martinez-Zapater J. Multiple origins of cultivated grapevine (*Vitis vinifera* L. ssp. *sativa*) based on chloroplast DNA polymorphisms. Molec. Ecol. 2006; 15:3707–3714. [PubMed: 17032268]

Badawi M, Danyluk J, Boucho B, Houde M, Sarhan F. The CBF gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal CBFs. Mol. Genet. Genomics. 2007; 277:533–554. [PubMed: 17285309]

Baumberger N, Doesseger B, Guyot R, Diet A, Parsons R, Clark M, Simmons M, Bedinger P, Goff S, Ringli C, Keller B. Whole-genome comparison of leucine-rich repeat extensins in *Arabidopsis* and rice. A conserved family of cell wall proteins form a vegetative and a reproductive clade. Plant Physiol. 2003; 131:1313–1326. [PubMed: 12644681]

Benedict C, Skinner J, Meng R, Chang Y, Bhalerao R, Huner N, Finn C, Chen T, Hurry V. The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp. Plant Cell Environ. 2006; 29:1259–1272. [PubMed: 17080948]

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Royal Stat. Soc. Ser. B. 1995; 57:289–300.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden T. BLAST+: architecture and applications. BMC Bioinform. 2009; 10:421.

Campoli C, Matus-Cádiz M, Pozniak C, Cattivelli L, Fowler D. Comparative expression of Cbf genes in the Triticeae under different acclimation induction temperatures. Mol. Genet. Genomics. 2009; 282:141–152. [PubMed: 19421778]

Canella D, Gilmour S, Kuhn L, Thomashow M. DNA binding by the *Arabidopsis* CBF1 transcription factor requires the PKKP/RAGRxKFxETRHP signature sequence. Biochim. Biophys. Acta. 2010; 1799:454–462. [PubMed: 19948259]

D'Angelo G, Vicinanza M, De Matteis M. Lipid-transfer proteins in biosynthetic pathways. Curr. Opin. Cell Biol. 2008; 20:360–370. [PubMed: 18490149]

Doukhanina E, Chen S, van der Zalm E, Godzik A, Reed J, Dickman M. Identification and functional characterization of the BAG protein family in *Arabidopsis thaliana*. J. Biol. Chem. 2006; 281:18793–18801. [PubMed: 16636050]

Dubouzet J, Sakuma Y, Ito Y, Kasuga M, Dubouzet E, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J. 2003; 33:751–763. [PubMed: 12609047]

El Kayal W, Navarro M, Marque G, Keller G, Marque C, Teulieres C. Expression profile of CBF-like transcriptional factor genes from *Eucalyptus* in response to cold. J. Exp. Bot. 2006; 57:2455–2469. [PubMed: 16816002]

Faurobert M, Mihr C, Bertin N, Pawlowski T, Negroni L, Sommerer N, Causse M. Major proteome variations associated with cherry tomato pericarp development and ripening. Plant Physiol. 2007; 143:1327–1346. [PubMed: 17208958]

Fennell A. Freezing tolerance and injury in grapevines. J. Crop. Improv. 2004; 10:201–235.

Fowler S, Thomashow MF. *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell. 2002; 14:1675–1690. [PubMed: 12172015]

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K. AREB1 Is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. Plant Cell. 2005; 17:3470–3488. [PubMed: 16284313]

Fuller M, Telli G. An investigation of the frost hardiness of grapevine (*Vitis vinifera*) during bud break. Ann. Appl. Biol. 1999; 135:589–595.

Gamborg O, Miller R, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 1968; 50:151–158. [PubMed: 5650857]

Gautier L, Cope L, Bolstad B, Irizarry R. affy—Analysis of Affymetrix GeneChip data at the probe level. Bioinform. 2004; 20:307–315.

Gilmour S, Fowler S, Thomashow M. *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Molec. Biol. 2004; 54:767–781. [PubMed: 15356394]

Gilmour S, Sebolt A, Salazar M, Everard J, Thomashow M. Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 2000; 124:1854–1865. [PubMed: 11115899]

Gilmour S, Zarka D, Stockinger E, Salazar M, Houghton J, Thomashow M. Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J. 1998; 16:433–442. [PubMed: 9881163]

Gion J, Lalanne C, Le Provost G, Ferry-Dumazet H, Paiva J, Chaumeil P, Frigerio J, Brach J, Barre A, de Daruvar A, Claverol S, Bonneu M, Sommerer N, Negroni L, Plomion C. The proteome of maritime pine wood forming tissue. Proteomics. 2005; 5:3731–3751. [PubMed: 16127725]

Grimplet J, Cramer G, Dickerson J, Mathiason K, Van Hemert J, Fennell A. VitisNet: "Omics" integration through grapevine molecular networks. PLoS ONE. 2009a; 4:e8365. [PubMed: 20027228]

Grimplet J, Wheatley M, Jouira H, Deluc L, Cramer G, Cushman J. Proteomic and selected metabolite analysis of grape berry tissues under well-watered and water-deficit stress conditions. Proteomics. 2009b; 9:2503–2528. [PubMed: 19343710]

Hanana M, Deluc L, Fouquet R, Daldoul S, Leon C, Barrieu F, Ghorbel A, Mliki A, Hamdi S. Identification and characterization of '*rd22*' dehydration responsive gene in grapevine (*Vitis vinifera* L.). *C. R.* Biologies. 2008; 331:569–578. [PubMed: 18606386]

Hoagland, D.; Arnon, D. Circular No 347. Berkeley, California, USA: California Agricultural Experiment Station; 1950. The water culture method of growing plants without soil.

Hsieh T, Lee J, Yang P, Chiu L, Charng Y, Wang Y, Chan M. Heterology expression of the *Arabidopsis* C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiol. 2002; 129:1086–1089. [PubMed: 12114563]

Huang D, Wu W, Abrams S, Cutler A. The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. J. Exp. Bot. 2008; 59:2991–3007. [PubMed: 18552355]

Huang J, Yang M, Liu P, Yang G, Wu C, Zheng C. *GhDREB1* enhances abiotic stress tolerance, delays GA-mediated development and represses cytokinin signalling in transgenic *Arabidopsis*. Plant Cell Environ. 2009; 32:1132–1145. [PubMed: 19422608]

Hurkman W, Tanaka C. The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiol. 1987; 83:517–524. [PubMed: 16665281]

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 4:249–264. [PubMed: 12925520]

Jaglo-Ottosen K, Gilmour S, Zarka D, Schabenberger O, Thomashow M. *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. Science. 1998; 280:104–106. [PubMed: 9525853]

Jaglo-Ottosen K, Kleff S, Amundsen K, Zhang X, Haake V, Zhang J, Deits T, Thomashow M. Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol. 2001; 127:910–917. [PubMed: 11706173]

Jin W, Dong J, Hu Y, Lin Z, Xu X, Han Z. Improved cold-resistant performance in transgenic grape (*Vitis vinifera* L.) overexpressing cold-inducible transcription factors *AtDREB1b*. HortScience. 2009; 44:35–39.

Jolie R, Duvetter T, Van Loey A, Hendrickx M. Pectin methylesterase and its proteinaceous inhibitor: a review. Carbohydr. Res. 2010; 345:2583–2595. [PubMed: 21047623]

Kang C, Jung W, Kang Y, Kim J, Kim D, Jeong J, Baek D, Jin J, Lee J, Kim M, Chung W, Mengiste T, Koiwa H, Kwak S, Bahk J, Lee S, Nam J, Yun D, Cho M. AtBAG6, a novel calmodulin-binding protein, induces programmed cell death in yeast and plants. Cell Death Differ. 2006; 13:84–95. [PubMed: 16003391]

Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Hofte H, Pauly M, Riechmann J, Broun P. The transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis thaliana*. Plant Cell. 2007; 19:1278–1294. [PubMed: 17449808]

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. 1999; 17:287–291. [PubMed: 10096298]

Kuwabara C, Arakawa K, Yoshida S. Abscisic acid-induced secretory proteins in suspension-cultured cells of winter wheat. Plant Cell Physiol. 1999; 40:184–191. [PubMed: 10202814]

Kwon S, Jin H, Lee S, Nam M, Chung J, Kwon S, Ryu C, Park O. GDSL lipase-like 1 regulates systemic resistance associated with ethylene signaling in *Arabidopsis*. Plant J. 2009; 58:235–245. [PubMed: 19077166]

Lee J, Prasad V, Yang P, Wu J, Ho TD, Charng Y, Chan M. Expression of *Arabidopsis* CBF1 regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. Plant Cell Environ. 2003; 26:1181–1190.

Lee K, Han K, Kwon Y, Lee J, Kim S, Chung W, Kim Y, Chun S, Kim H, Bae D. Identification of potential DREB2C targets in *Arabidopsis thaliana* plants overexpressing DREB2C using proteomic analysis. Mol. Cells. 2009; 28:383–388. [PubMed: 19830397]

Licausi F, Giorgi F, Zenoni S, Osti F, Pezzotti M, Perata P. Genomic and transcriptomic analysis of the AP2/ERF superfamily in *Vitis vinifera*. BMC Genomics. 2010; 11:719. [PubMed: 21171999]

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell. 1998; 10:1391–1406. [PubMed: 9707537]

Liu Y, Lamkemeyer T, Jakob A, Mi G, Zhang F, Nordheim A, Hochholdinger F. Comparative proteome analyses of maize (*Zea mays* L.) primary roots prior to lateral root initiation reveal differential protein expression in the lateral root initiation mutant *rum1*. Proteomics. 2006; 6:4300–4308. [PubMed: 16819721]

Lloyd G, McCown B. Commercially feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by the use of shoot tip culture. Int. Plant Prop. Soc. Proc. 1981; 30:421–427.

Lytle BL, Song J, de la Cruz NB, Peterson FC, Johnson KA, Bingman CA, Phillips GN Jr, Volkman BF. Structures of two *Arabidopsis thaliana* major latex proteins represent novel helix-grip folds. Proteins. 2009; 76:237–243. [PubMed: 19326460]

Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K. The DDF1 transcriptional activator upregulates expression of a gibberellin-deactivating gene, GA2ox7, under high-salinity stress in *Arabidopsis*. Plant J. 2008; 56:613–626. [PubMed: 18643985]

Maris A, Suslov D, Fry S, Verbelen J, Vissenberg K. Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis* and their effect on root growth and cell wall extension. J. Exp. Bot. 2009; 60:3959–3972. [PubMed: 19635745]

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K. Identification of cold-inducible downstream genes of the *Arabidopsis*

DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J. 2004; 38:982–993. [PubMed: 15165189]

Maruyama K, Takeda M, Kidokoro S, Yamada K, Sakuma Y, Urano K, Fujita M, Yoshiwara K, Matsukura S, Morishita Y, Sasaki R, Suzuki H, Saito K, Shibata D, Shinozaki K, Yamaguchi-Shinozaki K. Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. Plant Physiol. 2009; 150:1972–1980. [PubMed: 19502356]

Matus J, Loyola R, Vega A, Peña-Neira A, Bordeu E, Arce-Johnson P, Alcalde J. Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. J. Exp. Bot. 2009; 60:853–867. [PubMed: 19129169]

Mullins, M.; Bouquet, A.; Williams, L. Biology of the grapevine. Cambridge, New York: Cambridge University Press; 1992.

Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 1962; 15:473–497.

Nakamura R, Satoh R, Nakamura R, Shimazaki T, Kasuga M, Yamaguichi-Shinozaki K, Kikuchi A, Watanabe K, Teshima R. Immunoproteomic and two-dimensional difference gel electrophoresis analysis of *Arabidopsis dehydration response element-binding protein 1A* (*DREB1A*)-transgenic potato. Biol. Pharm. Bull. 2010; 33:1418–1425. [PubMed: 20686241]

Nakashima K, Yamaguchi-Shinozaki K. Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. Physiol. Plant. 2006; 126:62–71.

Navarro M, Ayax C, Martinez Y, Laur J, El Kayal W, Marque C, Teulieres C. Two EguCBF1 genes overexpressed in *Eucalyptus* display a different impact on stress tolerance and plant development. Plant Biotech. J. 2011; 9:50–63.

Navarro M, Marque G, Ayax C, Keller G, Borges J, Marque C, Teulieres C. Complementary regulation of four *Eucalyptus* CBF genes under various cold conditions. J. Exp. Bot. 2009; 60:2713–2724. [PubMed: 19457981]

Park J, Jin P, Yoon J, Yang J, Jeong H, Ranathunge K, Schreiber L, Franke R, Lee I, An G. Mutation in Wilted Dwarf and Lethal 1 (WDL1) causes abnormal cuticle formation and rapid water loss in rice. Plant Mol. Biol. 2010; 74:91–103. [PubMed: 20593223]

Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

Pino M, Skinner J, Park E, Jeknic Z, Hayes P, Thomashow M, Chen Y. Use of a stress inducible promoter to drive ectopic AtCBF expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotech. J. 2007; 5:591–604.

Reid K, Olsson N, Schlosser J, Peng F, Lund S. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol. 2006; 6:27. [PubMed: 17105665]

Ringli C. The hydroxyproline-rich glycoprotein domain of the *Arabidopsis* LRX1 requires Tyr for function but not for insolubilization in the cell wall. Plant J. 2010; 63:662–669. [PubMed: 20545889]

Rosenfeld J, Capdevielle J, Guillemot J, Ferrara P. In-gel digestion of proteins for internal sequence-analysis after 1-dimensional or 2-dimensional gel-electrophoresis. Anal. Biochem. 1992; 203:173–179. [PubMed: 1524213]

Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 2000; 132:365–386. [PubMed: 10547847]

Sambrook, J.; Russell, D. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory; 2001.

Santerre A, Britt A. Cloning of a 3-methyladenine-DNA glycosylase from *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. 1994; 91:2240–2244. [PubMed: 8134381]

Saravanan R, Rose J. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics. 2004; 4:2522–2532. [PubMed: 15352226]

Sharabi-Schwager M, Lers A, Samach A, Guy C, Porat R. Overexpression of the CBF2 transcriptional activator in *Arabidopsis* delays leaf senescence and extends plant longevity. J. Exp. Bot. 2010; 61:261–273. [PubMed: 19854800]

Siddiqua M, Nassuth A. *Vitis* CBF1 and *Vitis* CBF4 differ in their effect on *Arabidopsis* abiotic stress tolerance, development and gene expression. Plant Cell Environ. 2011 Apr 12. [Epub ahead of print].

Sreekantan L, Mathiason K, Grimplet J, Schlauch K, Dickerson J, Fennell A. Differential floral development and gene expression in grapevines during long and short photoperiods suggests a role for floral genes in dormancy transitioning. Plant Mol. Biol. 2010; 73:191–205. [PubMed: 20151315]

Sreenivasulu N, Sopory S, Kavi Kishor P. Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene. 2007; 388:1–13. [PubMed: 17134853]

Stockinger E, Gilmour S, Thomashow M. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA. 1997; 94:1035–1040. [PubMed: 9023378]

Takuhara Y, Kobayashi M, Suzuki S. Low-temperature-induced transcription factors in grapevine enhance cold tolerance in transgenic *Arabidopsis* plants. J. Plant Physiol. 2011; 168:967–975. [PubMed: 21185622]

Tattersall, E. Reno, NV: University of Nevada; 2006. Changes in gene expression in response to abiotic stress in grapevine (*Vitis vinifera*); p. 154

Tattersall E, Ergul A, AlKayal F, Deluc L, Cushman J, Cramer G. Comparison of methods for isolating high-quality RNA from leaves of grapevine. Am. J. Enol. Vitic. 2005; 56:400–406.

This P, Lacombe T, Thomas M. Historical origins and genetic diversity of wine grapes. Trends Genet. 2006; 22:511–519. [PubMed: 16872714]

Thomashow M. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 1999; 50:571–599. [PubMed: 15012220]

Vincent D, Wheatley M, Cramer G. Optimization of protein extraction and solubilization for mature grape berry clusters. Electrophoresis. 2006; 27:1853–1865. [PubMed: 16586412]

Vivier M, Pretorius I. Genetically tailored grapevines for the wine industry. Trends Biotech. 2002; 20:472–478.

Vogel J, Zarka D, Van Buskirk H, Fowler S, Thomashow M. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Plant J. 2005; 41:195–211. [PubMed: 15634197]

Volokita M, Rosilio-Brami T, Rivkin N, Zik M. Combining comparative sequence and genomic data to ascertain phylogenetic relationships and explore the evolution of the large GDSL-lipase family in land-plants. Mol. Biol. Evol. 2010; 28:551–565. [PubMed: 20801908]

Watson B, Asirvatham V, Wang L, Sumner W. Mapping the proteome of barrel medic (*Medicago truncatula*). Plant Physiol. 2003; 131:1104–1123. [PubMed: 12644662]

Welling A, Palva E. Involvement of CBF transcription factors in winter hardiness in birch. Plant Physiol. 2008; 147:1199–1211. [PubMed: 18467468]

Wise R, Caldo R, Hong L, Shen L, Cannon E, Dickerson J. BarleyBase/PLEXdb. Methods Mol. Biol. 2007; 406:347–363. [PubMed: 18287702]

Wisniewski M, Norelli J, Bassett C, Artlip T, Mascarisin D. Ectopic expression of a novel peach (*Prunus persica*) CBF transcription factor in apple (*Malus x domestica*) results in short-day induced dormancy and increased cold hardiness. Planta. 2011; 233:971–983. [PubMed: 21274560]

Xiao H, Siddiqua M, Braybrook S, Nassuth A. Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. Plant Cell Environ. 2006; 29:1410–1421. [PubMed: 17080962]

Xiao H, Tattersall E, Siddiqua M, Cramer G, Nassuth A. CBF4 is a unique member of the CBF transcription factor family of *Vitis vinifera* and *Vitis riparia*. Plant Cell Environ. 2008; 31:1–10. [PubMed: 17971068]

Yadav SK, Singla-Pareek S, Sopory S. An overview on the role of methylglyoxal and glyoxalases in plants. Drug Metabol. Drug Interact. 2008; 23:51–68. [PubMed: 18533364]

Zhang J, Broeckling C, Blancaflor E, Sledge M, Sumner L, Wang Z. Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular

wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). Plant J. 2005; 42:689–707. [PubMed: 15918883]

Zhang S, Yang C, Peng J, Sun S, Wang X. GASA5, a regulator of flowering time and stem growth in *Arabidopsis thaliana*. Plant Mol. Biol. 2009; 69:745–759. [PubMed: 19190987]

Zhang Y, Yang J, Showalter A. AtAGP18 is localized at the plasma membrane and functions in plant growth and development. Planta. 2011; 233:675–683. [PubMed: 21165646]

Zhao H, Bughrara S. Isolation and characterization of cold-regulated transcriptional activator LpCBF3 gene from perennial ryegrass (*Lolium perenne* L.). Mol. Genet. Genomics. 2008; 279:585–594. [PubMed: 18351391]

Zhu X, Papayannopoulos I. Improvement in the detection of low concentration protein digests on a MALDI TOF/TOF workstation by reducing alpha-cyano-4-hydroxycinnamic acid adductions. J. Biomolec. Tech. 2003; 14:298–307.

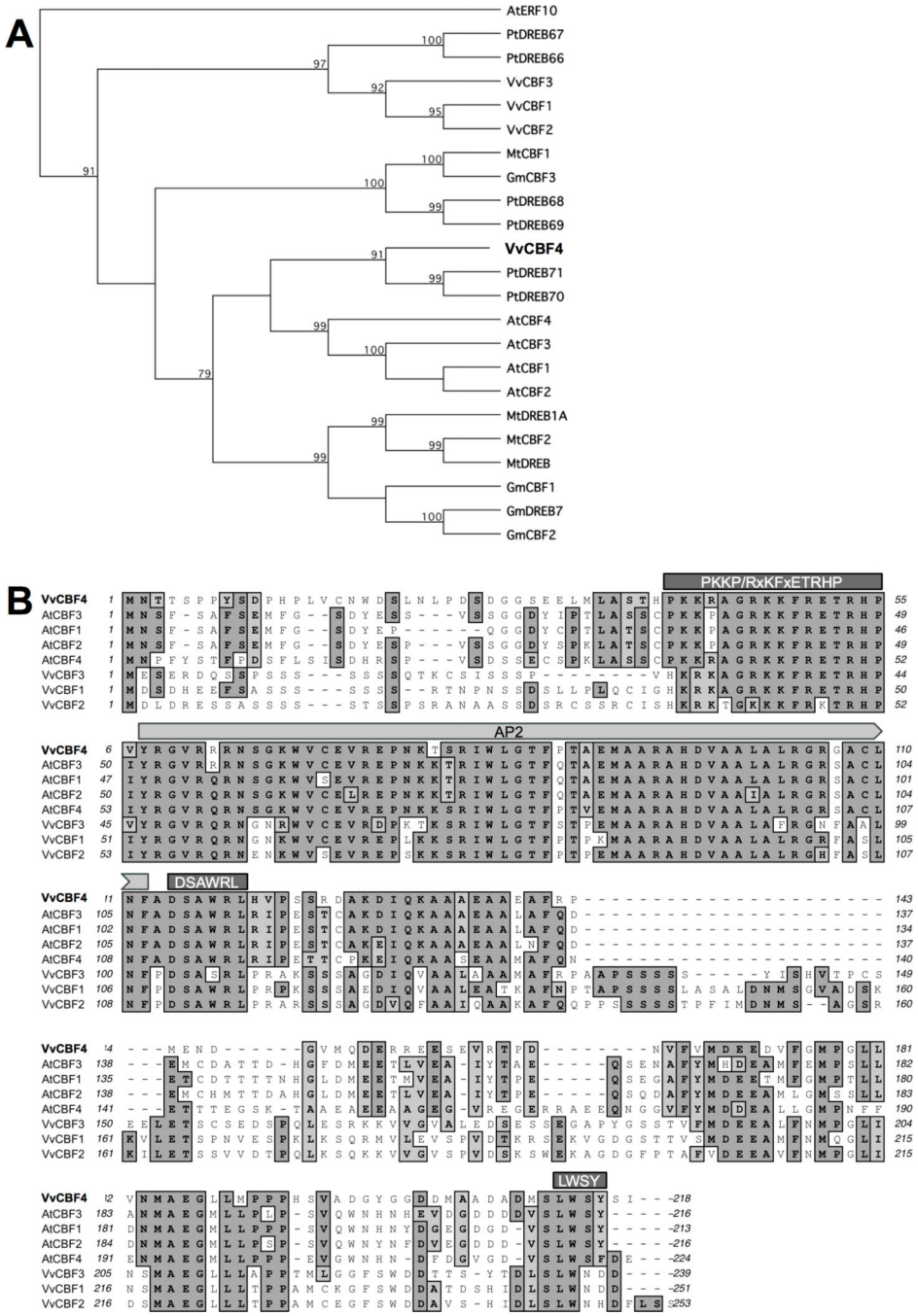

Figure 1.

Comparative sequence analysis of C-repeat binding factor (CBF) protein family members in eudicot species. (A) Phylogenetic analysis of CBF/DREB proteins found in eudicot species. CBF proteins were identified from *A. thaliana*, *V. vinifera*, *Populus trichocarpa*, *Glycine max* and *Medicago truncatula* – species with published draft genomes. *AtERF10* is included as an outgroup root. (B) Amino acid alignment of *A. thaliana*, *P. trichocarpa*, and *V. vinifera CBF* transcription factors. Identical and similar amino acids are colored dark and light gray respectively. The conserved APETALA 2 (AP2) domain and the CBF-conserved

AP2 N-flanking PKKP/RAGRxKFxETRHP, AP2 C-flanking DSAWRL and LWSY motifs are labeled with boxes above the alignment. Accession numbers are listed in the Experimental Procedures section.

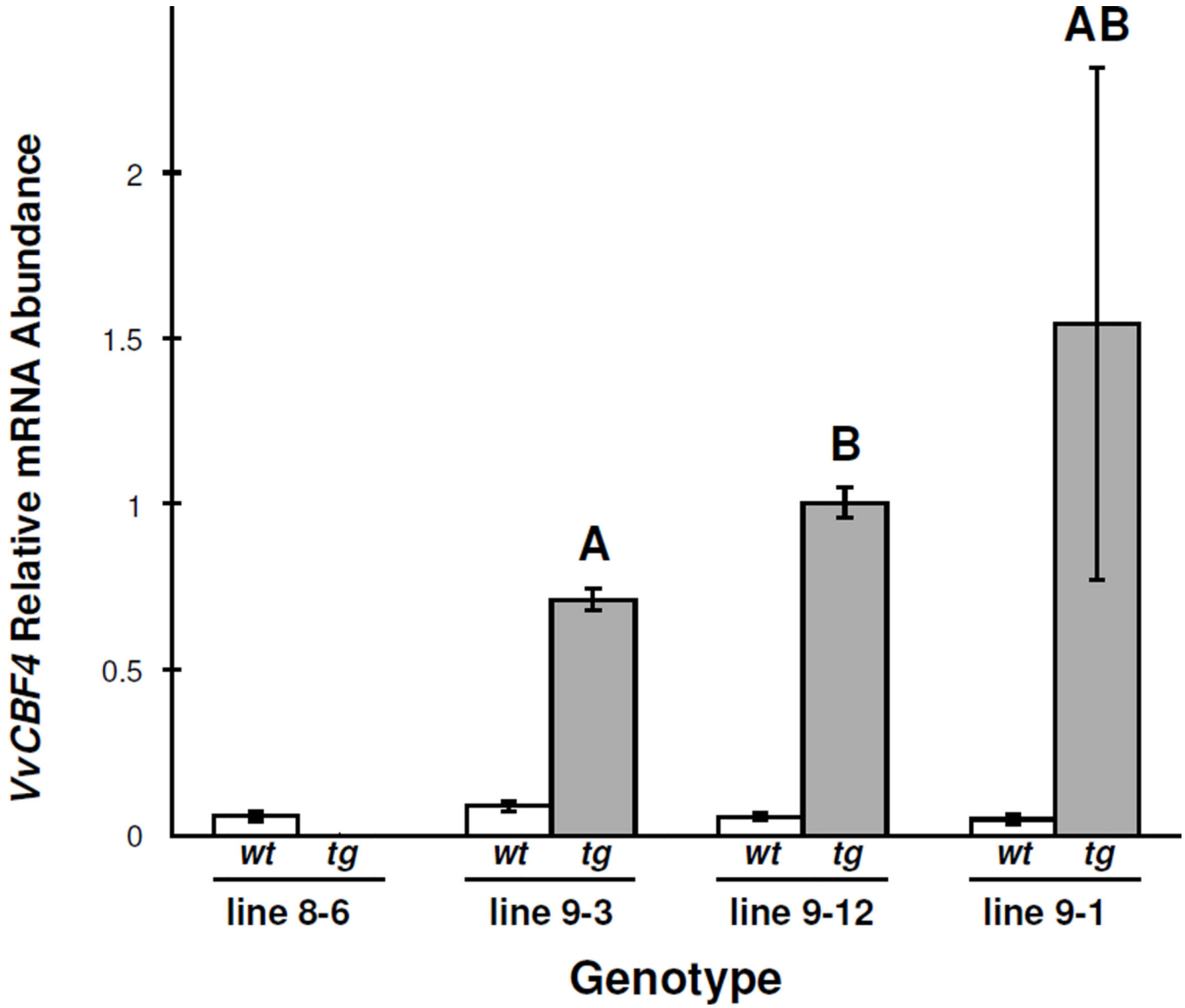

Figure 2.

Overexpression of 35S::*VvCBF4* in transformed *Vitis* cv. 'Freedom' shoots. qRT-PCR of native and transgenic *VvCBF4* transcripts for control (line 8-6) and three independently transformed 35S::*VvCBF4*-overexpressing lines (9-3, 9-12, 9-1). Wild type *VvCBF4* (wt) transcript relative abundance; transgenic *VvCBF4* (tg) transcript relative abundance. Transcript abundances were normalized to an actin reference gene. Error bars indicate ± Standard Error (SE). Significant pair-wise differences in tg expression are indicated with letters (A, B). Significantly different abundance was determined using the Student's t-test with Bonferroni correction, $p < 0.05$; n=3).

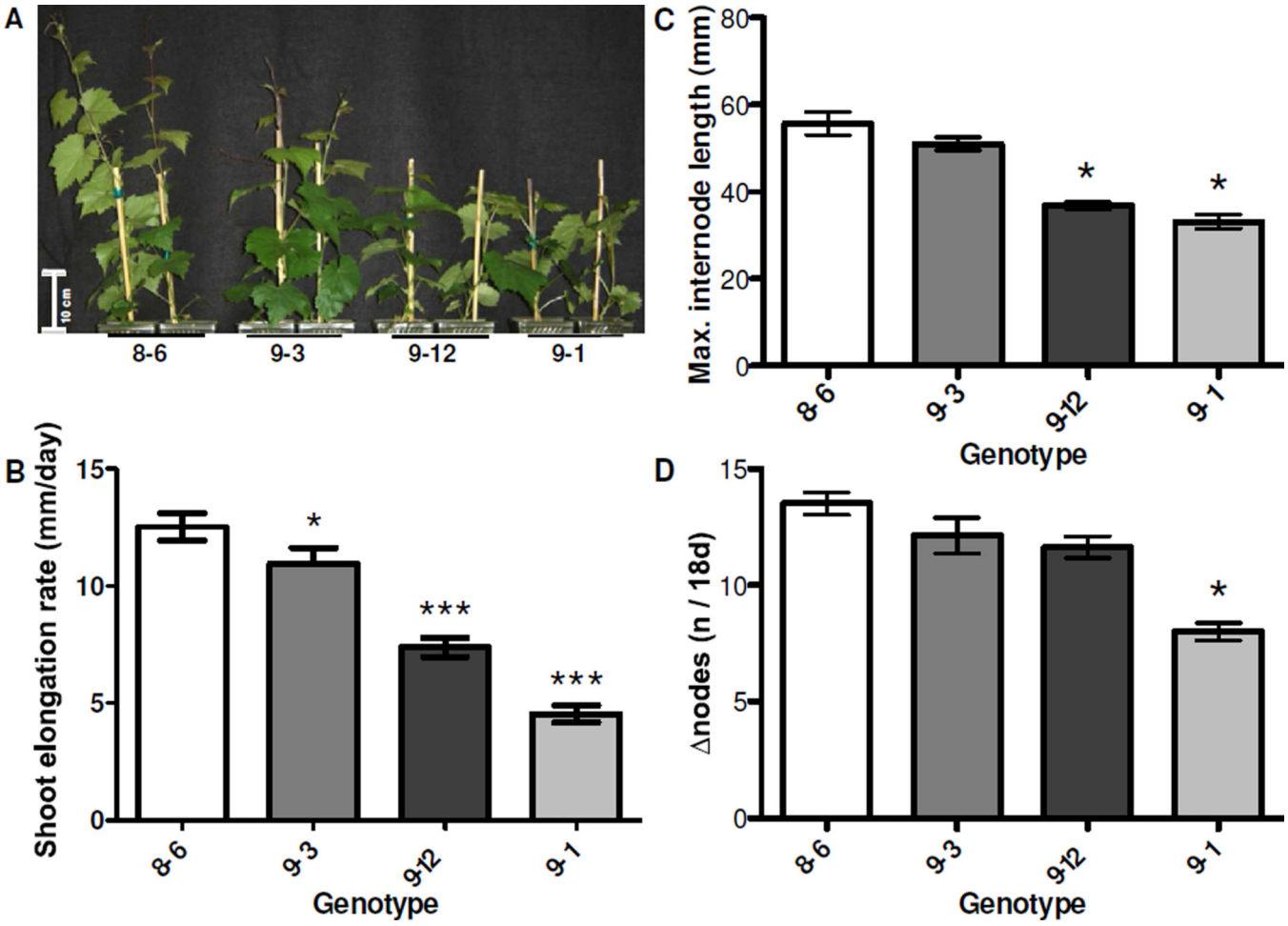

Figure 3.

The CBF-induced dwarfing effect is dependent on relative level of *VvCBF4* overexpression in 35S::*VvCBF4 Vitis* cv. 'Freedom.' A) Image showing two representative vines each of control line 8-6 and 35S::*VvCBF4* overexpressing lines 30 days after transplantation to soil. B) Shoot elongation rate for line 8-6 (control) and 35S::*VvCBF4* overexpressing lines (9-3, 9-12, 9-1). Each bar represents the mean shoot elongation rate of eight vines measured over 18 days, every third day. Error bars indicate Standard Error of the mean; n=8. Growth for each 35S::*VvCBF4* overexpressing line was significantly different from control line 8-6 (Repeated Measures ANOVA). * denotes $p<0.05$; *** denotes $p<0.001$. C) Average length of the maximum (Max.) internode length per plant. D) Total increase in number of nodes (Δ nodes) after 18 days of observation for control line 8-6 and 35S::*VvCBF4* overexpressing lines (n=8). Error bars represent ±SE. * Indicates significant difference between control (line 8-6) and *VvCBF4* overexpressing lines based on the Student's t-test with Bonferroni correction, $p < 0.05$; n=8).

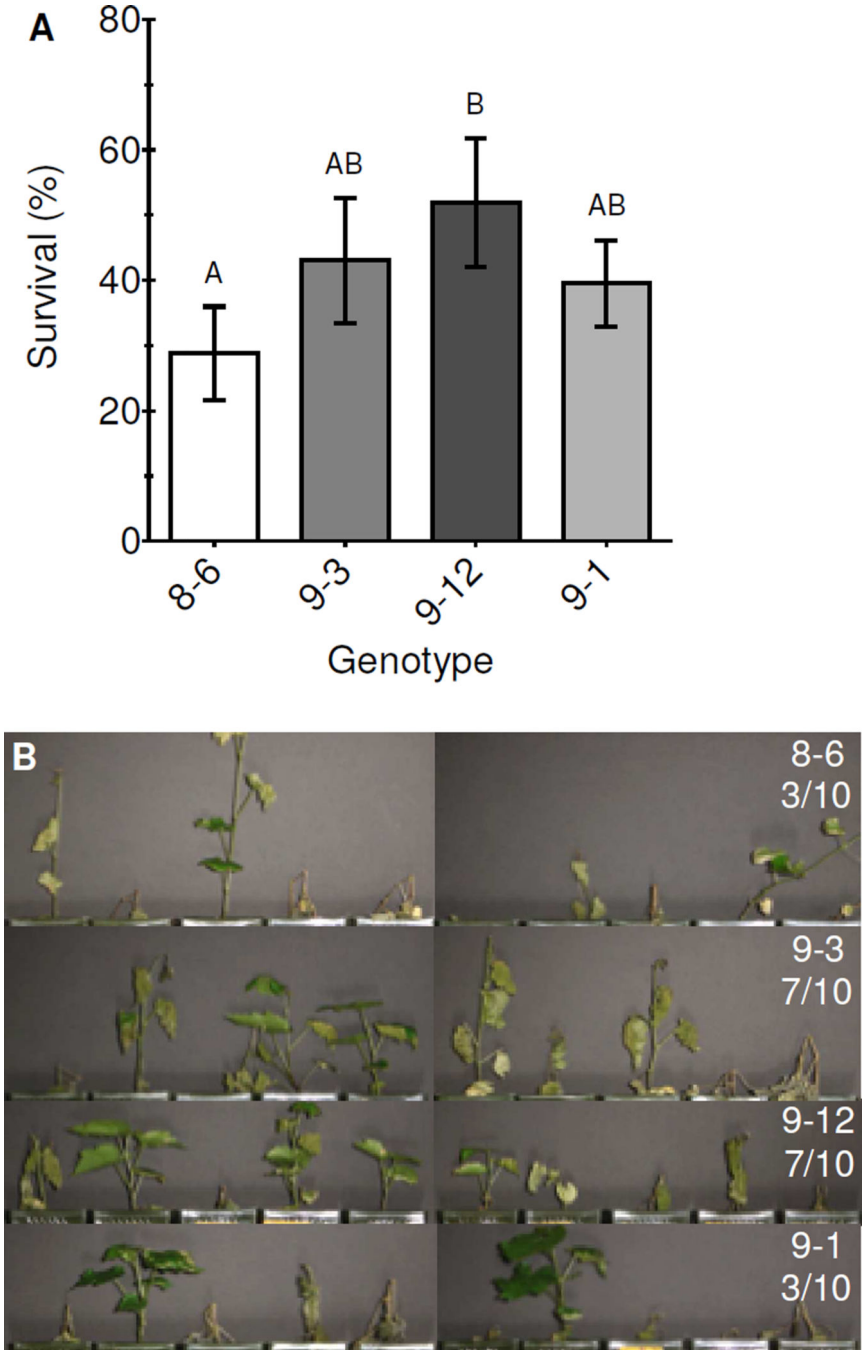

Figure 4.

35S::*VvCBF4* overexpression enhances freezing survival in transformed line 9-12. A) Percentage survival for mock-transformed line 8-6 (control) and 35S::*VvCBF4* overexpressing lines (9-3, 9-12, 9-1) following exposure to freezing at -2°C for 24 h with 14 d recovery at 22°C. Each bar represents the mean of nine replicate experiments with 10 individual vines used for each genotype in each experiment. Error bars indicate ±SE. The results indicated by different letters are significantly different based on the Student's t-test

with Bonferroni correction; ($p < 0.01$). B) Exemplar images of freezing survival after recovery from one of the nine experimental trials.

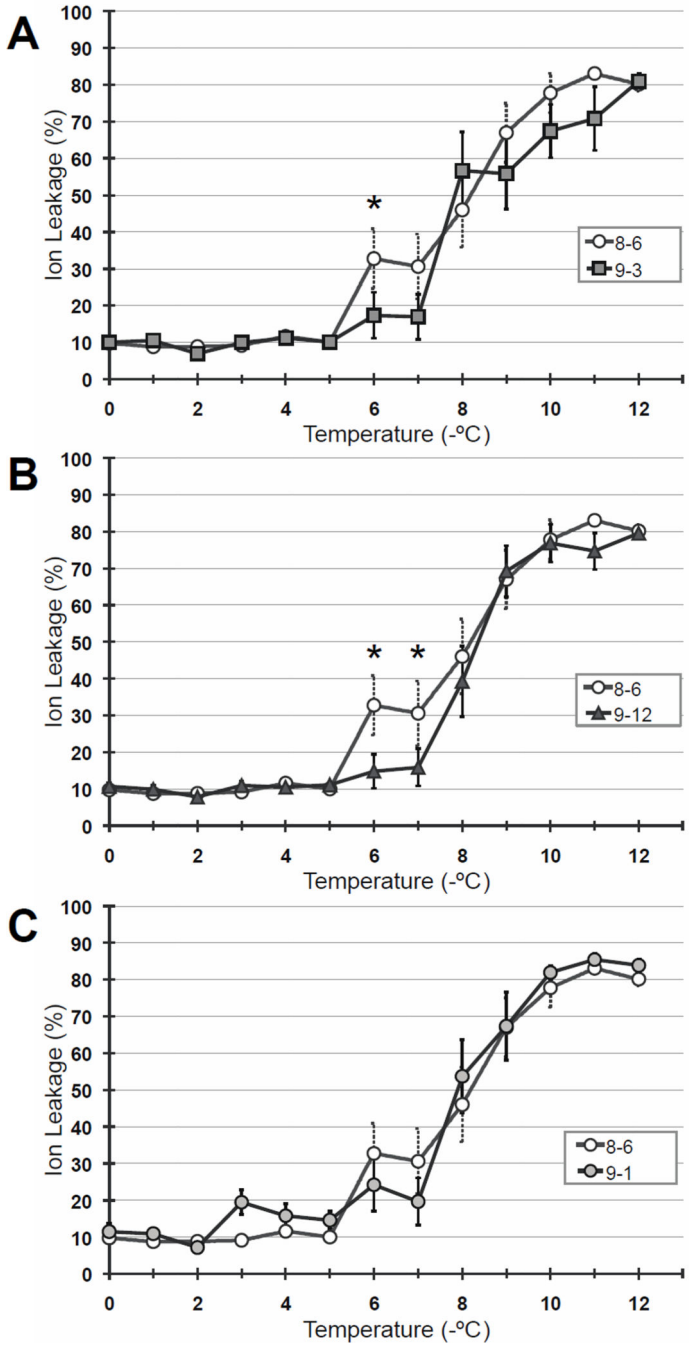

Figure 5.

35S::*VvCBF4* transformed 'Freedom' lines exhibit less electrolyte leakage upon freezing stress. Percentage leaf-disc electrolyte leakage for line 8-6 (control) and 35S::*VvCBF4* overexpressing lines A) 9-3, B) 9-12, and C) 9-1 following exposure to sub-freezing temperatures as indicated. Each point represents the mean electrolyte leakage of leaf discs from 11. Error bars indicate ±SE. * Indicates a significant difference between the 35S::*Vv*CBF4 transformed line and control line 8-6 at the marked temperature (one-way ANOVA with post-hoc Student's t-test with ordered differences test, $p < 0.05$; n=11).

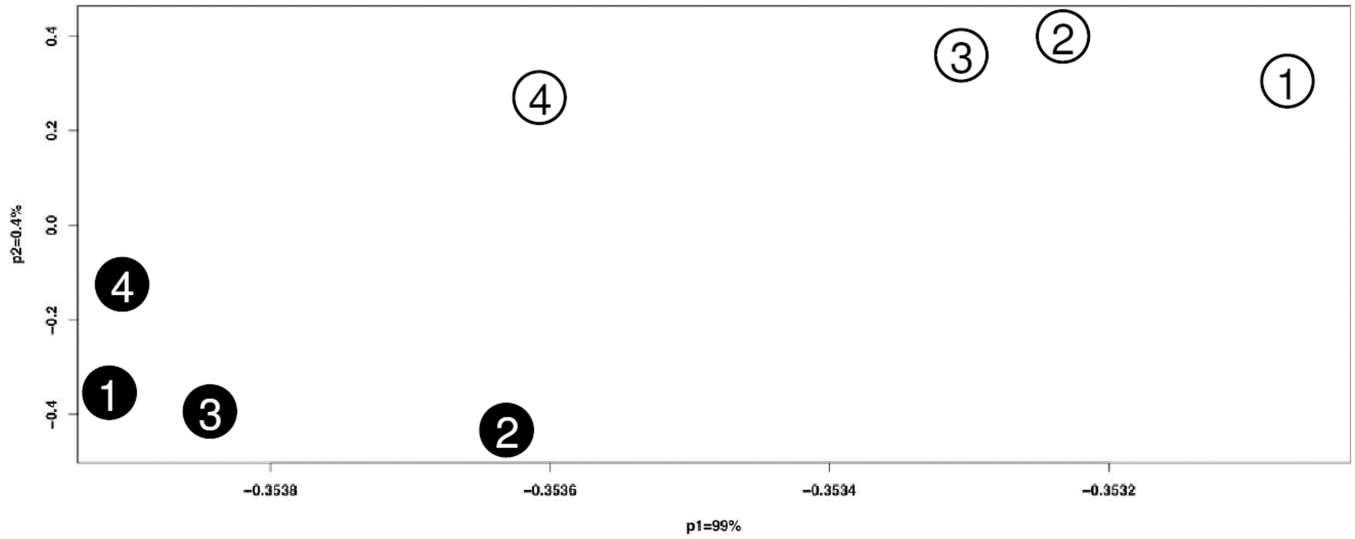

Figure 6.

Principal Component Analysis (PCA) of microarray probeset variation. Robust multi-average (RMA) normalized intensities were analyzed with ~16,000 probesets on Affymetrix® *Vitis* GeneChip® microarrays for the RNA transcripts of 35S::*VvCBF4* overexpressing line 9-12 (black) and empty vector-transformed *Vitis* rootstock 'Freedom' control line 8-6 (white). Whole aerial portions (stem + five leaves) of non-stressed young vines were used for RNA extraction, and tissue from three different vines was pooled for RNA isolation and microarray hybridization (n = 4 arrays per genotype). Principal components 1 and 2 (p1, p2) account for 99% and 0.4% of the variation between microarrays of control and 35S::*VvCBF4* overexpressor lines, respectively.

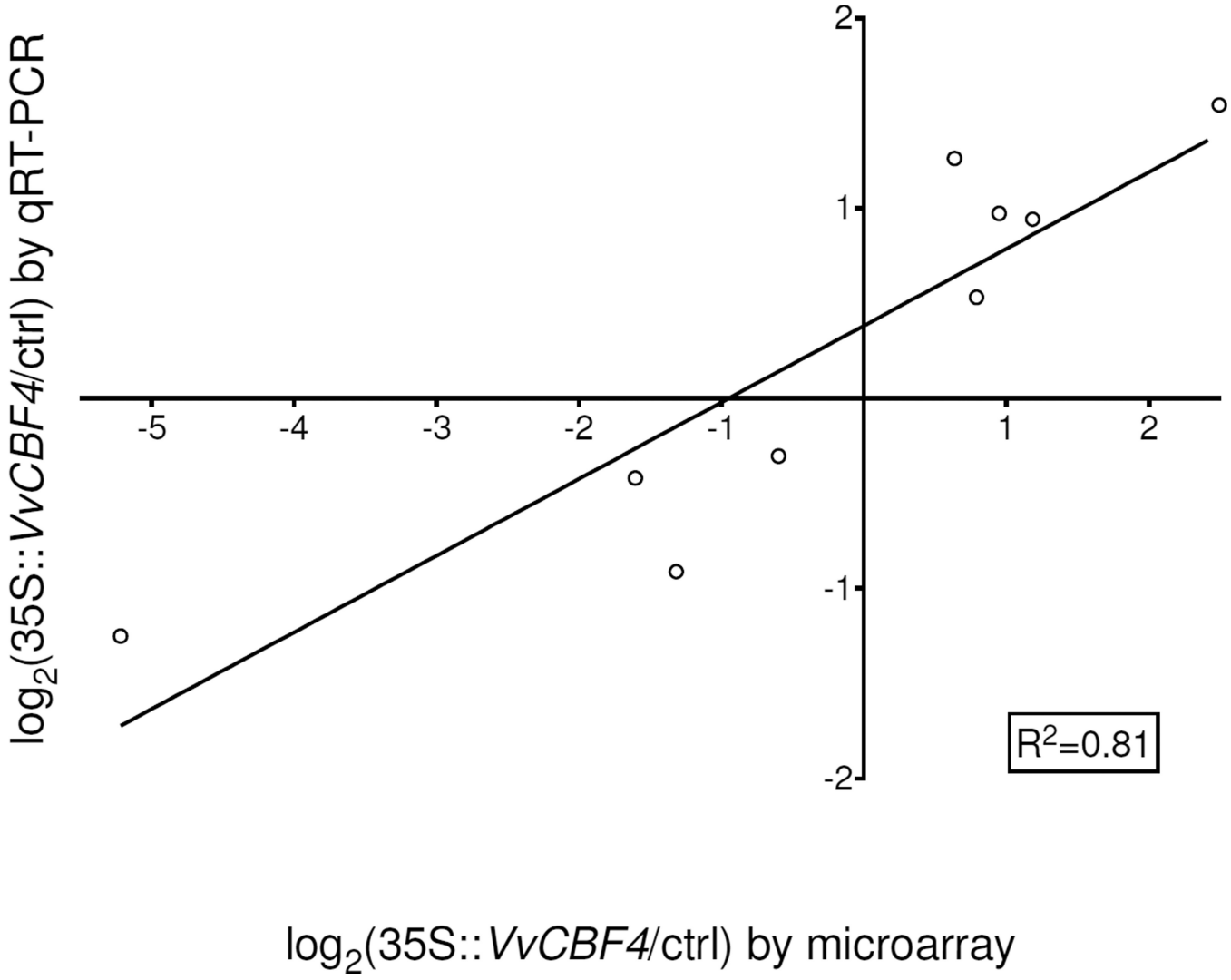

Figure 7.

Verification of microarray results by real-time qRT-PCR. Log_2-transformed values of Affymetrix® *Vitis* GeneChip® signal intensities (x-axis) and real-time PCR expression ratios (y-axis) for (35S::*VvCBF4* / control) of 9 differentially expressed microarray probe sets (open circles). The linear regression had a goodness of fit $R^2 = 0.81$.

Table 1

Transcripts with significantly different expression in *VvCBF4* overexpressor line (9–12) compared with control line (8-6).

Annotation	Fold Ratio	adj. p-value	Probeset ID	Protein ID	Gene ID
Increased abundance					
Pectin methylesterase Inhibitor (PMEI)	5.629	0.026	1606429_at	XP_002264167	LOC100251832
AAA-type ATPase domain (chaperone-like)	4.724	0.004	1610816_at	XP_002275572	LOC100252698
Calmodulin-like	2.621	0.019	1617516_at	XP_002284268	LOC100252031
Sulfite exporter TauE/SafE	2.532	0.048	1609884_at	XP_002267318	LOC100253061
Leucine-rich repeat extensin	2.413	0.026	1620976_at	XP_002277227	LOC100252056
Hypothetical protein	2.321	0.045	1612536_s_at	XP_002283137	LOC100255628
Gibberellin-regulated protein (GASA5)	2.272	0.049	1617881_at	XP_002280219	LOC100259439
Phytepsin aspartyl protease	2.252	0.048	1616668_at	XP_002276363	LOC100266485
Lipid transfer protein (LTP3)	2.193	0.049	1608175_at	XP_002275107	LOC100265591
Lactoylglutathione lyase / glyoxalase I	2.148	0.048	1619235_at	XP_002271396	LOC100266132
Flavonoid 3',5'-hydroxylase (F3'5'H) / CYP96A10	2.089	0.026	1612325_at	XP_002279531	LOC100266173
Serine carboxypeptidase III	2.069	0.05	1620729_at	XP_002271855	LOC100242723
Unknown protein	2.052	0.031	1613368_at	XP_002283053	LOC100243457
Acid phosphatase class B	2.024	0.01	1621892_a_at	XP_002273448	LOC100246316
Xyloglucan endo-transglycosylase (XET)	2.008	0.038	1617739_at	XP_002274520	LOC100232906
Rho GDP-dissociation inhibitor 2	1.931	0.05	1611669_at	XP_002263904	LOC100260088
RD22-C	1.931	0.048	1621818_at	XP_002284286	LOC100264522
DUF1070 domain AGP41-like	1.927	0.05	1622530_at	XP_002283086	LOC100249036
Lipase GDSL	1.88	0.039	1607341_at	XP_002283363	LOC100242887
DNA-3-methyladenine glycosidase I	1.876	0.044	1621976_at	XP_002263612	LOC100256507
Lipase GDSL	1.829	0.049	1620618_at	XP_002271851	LOC100263626
Stellacyanin-like	1.824	0.019	1613509_at	XP_002280885	LOC100246223
Pepsin-like aspartic protease	1.808	0.045	1611138_s_at	XP_002265771	LOC100246744
Glycosyl hydrolase 17-like	1.784	0.031	1622656_at	XP_002285661	LOC100257244
Glycerol-3-phosphate acyltransferase 4-like	1.775	0.031	1612479_at	XP_002275348	LOC100243093
BSL1-like serine/threonine phosphoesterase	1.772	0.031	1615748_at	XP_002270638	LOC100249353
Pollen proteins Ole e I	1.761	0.027	1610746_at	XP_002272595	LOC100265200

Annotation	Fold Ratio	adj. p-value	Probeset ID	Protein ID	Gene ID
Embryo-specific 3	1.757	0.026	1614771_at	XP_002266473	LOC100242179
Lipase GDSL	1.752	0.031	1607744_at	XP_002272970	LOC100249332
Zinc finger (C3HC4-type RING)	1.729	0.031	1612240_at	XP_002262825	LOC100263579
Polyketide cyclase/dehydratase (MLP28-like)	1.724	0.045	1607196_at	XP_002284578	LOC100262819
Pectate lyase	1.717	0.048	1616158_at	XP_002285340	LOC100242302
Unknown protein	1.696	0.031	1617384_at	XP_002281002	LOC100261133
Similar to thaumatin-like VVTL1	1.689	0.049	1614746_at	XP_002284403	LOC100259225
Exordium-like 3	1.689	0.048	1614951_at	XP_002264723	LOC100266367
Acid phosphatase class B	1.686	0.046	1617433_at	XP_002273448	LOC100246316
DUF1070 domain AGP20-like	1.655	0.019	1619401_at	XP_002280494	LOC100250031
FRA8-like	1.632	0.048	1608896_at	XP_002275679	LOC100243163
ROP6-like	1.569	0.031	1615962_at	XP_002269907	LOC100256456
LACERATA-like CYP450	1.564	0.048	1621973_at	XP_002275806	LOC100247907
GDP-fucose protein O-fucosyltransferase	1.562	0.045	1613171_at	XP_002267185	LOC100260861
Calcium-binding EF-hand-containing MSS3-like	1.554	0.048	1612996_at	XP_002266359	LOC100254364
Tubulin beta-1 chain	1.536	0.045	1607001_at	XP_002275306	LOC100259087
Glucose-methanol-choline(GMC) oxidoreductase	1.535	0.05	1622345_at	XP_002282510	LOC100266705
Unknown protein	1.529	0.035	1610862_at	XP_002274279	LOC100260563
Ferredoxin-related	1.52	0.049	1610753_at	XP_002281459	LOC100257849
Plant Basic Secretory Protein	1.518	0.031	1615434_at	XP_002283729	LOC100257403
Protein transport Sec61 subunit beta	1.507	0.048	1622732_at	XP_002276029	LOC100247887
Decreased abundance					
Chlorophyllase 2 (CHL2)	−1.514	0.035	1616275_at	XP_002279285	LOC100252835
Unknown protein (DUF1279, thylakoid)	−1.521	0.045	1619538_at	XP_002271410	LOC100258627
Galactinol synthase 4-like	−1.618	0.048	1608907_s_at	XP_002265947	LOC100260266
Copper amine oxidase	−1.675	0.045	1608650_at	XP_002263349	LOC100257527
ABC transporter MRP4-like	−1.703	0.049	1617849_at	XP_002265012	LOC100253698
Secretory peroxidase	−2.095	0.048	1621431_at	XP_002269918	LOC100257005
Chitinase class IV C (CHI4C)	−2.205	0.048	1617192_at	XP_002275516	LOC100232911
Ubiquitin-protein ligase (PUB23-like)	−2.256	0.04	1606741_at	XP_002267438	LOC100250551
Nicotinamidase 1	−2.264	0.048	1610169_at	XP_002270896	LOC100250570

Plant Biotechnol J. Author manuscript; available in PMC 2015 March 12.

Annotation	Fold Ratio	adj. p-value	Probeset ID	Protein ID	Gene ID
CYP94 family protein	−2.491	0.026	1609552_at	XP_002278009	LOC100267102
Alpha-glucan phosphorylase (PHS2-like)	−3.04	0.043	1614707_at	XP_002280732	LOC100251865
AtBAG6-like	−37.14	0.049	1614614_at	XP_002279584	LOC100256846

Table 2

Comparison of changes in gene expression in *VvCBF4*-overexpressing grapevine with *AtCBF1*-overexpressing Poplar.

In 35S::*Vv*CBF4-grapevine			**In 35S::*AtCBF1*-Poplar (as reported by Benedict *et al*., 2006)**				
***V. vinifera* gene description**	***V. vinifera* RefSeq ID**	***Vitis* Shoot FC**	**Poplar Leaf FC**	**Poplar Stem FC**	**Poplar gene ID (best *At* match)[a]**	**Poplar gene description**	**BLAST *e*-value**
Reported transcript changes similar in Vitis and Poplar CBF overexpressors							
Exordium-like 3	XP_002264723	1.7	−1.4	1.8	At2g17230.1	Exordium-like 5	3.4E-92
Glycosyl hydrolase 17-like	XP_002285661	1.8	2.3	−1.1	At5g55180.2	O-Glycosyl hydrolase family 17	2.0E-18
Calcium-binding EF-hand MSS3-like	XP_002266359	1.6	1.0	1.8	At5g39670.1	Calcium-binding EF-hand family protein	9.0E-16
Xyloglucan endo-transglycosylase (XET)	XP_002274520	2.0	1.6	2.0	At3g23730.1	Xyloglucan endo-transglucosylase/hydrolase 16	2.6E-87
Unknown protein	XP_002283053	2.1	1.1	1.9	At4g35320.1	unknown protein	4.7E-30
Leucine-rich repeat extensin	XP_002277227	2.4	1.8	−1.1	At1g28290.1	Arabinogalactan protein 31 (AGP31)	9.4E-30
Rare Cold-Inducible 2B (RCI2B)	XP_002279333	1.4	1.9	1.9	At3g05880.1	Low temperature and salt responsive protein (LTI6A)	5.0E-22
Lipase GDSL (1)	XP_002271851	1.8	2.3	1.1	At5g45950.1	GDSL-like Lipase/Acylhydrolase (1)	2.3E-70
Lipase GDSL (2)	XP_002272970	1.8	2.3	1.2	At1g29660.1	GDSL-like Lipase/Acylhydrolase (2)	1.1E-56
Lipase GDSL (3)	XP_002283363	1.9	2.8	1.1	At1g71691.2	GDSL-like Lipase/Acylhydrolase (3)	6.1E-55
Chitinase class IV C (CHI4C)	XP_002275516	−2.2	−1.2	−2.0	At3g54420.1	Chitinase (ATEP3, ATCHITIV)	1.1E-111
Reported transcript changes differ between Vitis and Poplar CBF overexpressors							
Pectate lyase	XP_002285340	1.7	−2.0	−2.1	At4g24780.1	Pectin lyase-like	7.1E-202
Lactoylglutathione lyase/glyoxalase I	XP_002271396	2.1	−2.5	1.1	At1g15380.1	Lactoylglutathione lyase/glyoxalase I	9.0E-45
ABC transporter MRP4-like	XP_002265012	−1.7	−1.2	2.4	At3g21250.2	Multidrug resistance-associated protein 6 (MRP6)	3.7E-297

FC, fold change.

[a] *Arabidopsis* gene locus identifiers used as the sole designators for UPSC-KTH 13K Poplar microarray probes from Benedict *et al*. 2006 (Supplemental Tables 1a–c).

Table 3

Comparisons of changes in gene expression in *VvCBF4*-overexpressing grapevine with multiple *CBF/DREB1f*-overexpressing *Arabidopsis*

In 35S::*Vv*CBF4-grapevine			**In CBF-overexpressing *Arabidopsis***							
***V. vinifera* RefSeq ID**	***V. vinifera* gene description**	***Vitis CBF4***	***CBF1*[a] FC**	***CBF2*[b] FC**	***CBF2*[c] FC**	***CBF3*[d] FC**	***DDF1*[e] FC**	***Arabidopsis* Gene ID**	***Arabidopsis* gene description**	**BLAST e-value**
Transcript abundance trends similar between 35S::VvCBF4-grapevine and CBFs in Arabidopsis										
XP_002264167	Pectin methylesterase Inhibitor (PMEI)	5.6		5.6	2.9	7.3		At5g62350.1	Invertase/pectin methylesterase inhibitor	1.9E−52
				4.8	3.9	4.6		At5g62360.1	Invertase/pectin methylesterase inhibitor	6.9E−42
			4.4			3.9		At3g47380.1	Invertase/pectin methylesterase inhibitor	1.7E−35
XP_002280219	Gibberellin-regulated protein (GASA5)	2.3		3.9				At1g75750.1	GASA1	1.6E−19
XP_002271855	Serine carboxypeptidase III	2.1		2.5				At2g22980.1	Serine carboxypeptidase-like 13	2.3E−39
XP_002283363	Lipase GDSL (#1)	1.9		3.0				At1g29660.1	GDSL-like Lipase/Acylhydrolase (#2)	1.1E−56
XP_002271851	Lipase GDSL (#2)	1.8	32.4	11.0	258.5		64.0	At2g24560.1	GDSL-like Lipase/Acylhydrolase (#3)	1.0E−68
XP_002272970	Lipase GDSL (#3)	1.8		3.8		2.1		At1g29670.1	GDSL-like Lipase/Acylhydrolase (#4)	1.7E−59
						2.3		At4g30140.1	CDEF1 | GDSL-like	5.4E−42
XP_002280885	Stellacyanin-like	1.8				2.2		At4g27520.1	Early nodulin-like 2	1.5E−16
XP_002285340	Pectate lyase	1.7		2.5	4.5			At3g09540.1	Pectin lyase-like superfamily	1.4E−39
XP_002275806	LACERATA-like CYP450	1.6					4.0	At2g27690.1	CYP94C1	1.0E−84
XP_002279285	Chlorophyllase 2 (CHL2)	−1.5		−9.8	−5.1	−2.8	−5.3	At1g19670.1	Chlorophyllase 1 (CHL1)	3.2E−70
XP_002263349	Copper amine oxidase	−1.7		−2.7				At4g12280.1	Copper amine oxidase	3.6E−129
XP_002269918	Secretory peroxidase	−2.1					−8.6	At5g06730.1	Peroxidase superfamily	2.7E−85
XP_002278009	CYP94 family protein	−2.5					−5.3	At5g63450.1	CYP94B1	1.3E−118
Transcript abundance trends partially similar between 35S::VvCBF4-grapevine and CBFs in Arabidopsis										
XP_002275516	Chitinase class IV C (CHI4C)	−2.2					−4.0	At2g43590.1	Chitinase family protein	2.2E−91

In 35S::*Vv*CBF4-grapevine			In CBF-overexpressing *Arabidopsis*							
***V. vinifera* RefSeq ID**	***V. vinifera* gene description**	***Vitis CBF4***	***CBF1*[a] FC**	***CBF2*[b] FC**	***CBF2*[c] FC**	***CBF3*[d] FC**	***DDF1*[e] FC**	***Arabidopsis* Gene ID**	***Arabidopsis* gene description**	**BLAST e-value**
			52.1	29.0	40.4	19.7	3.0	At2g43620.1	Chitinase family protein	1.0E−69
XP_002284403	Similar to thaumatin-like VVTL1	1.7				−4.2	−3.2	At1g75040.1	Pathogenesis-related gene 5	1.5E−64
				4.5	5.0	2.0		At4g36010.1	Pathogenesis-related thaumatin	1.1E−59
XP_002265771	Pepsin-like aspartic protease	1.8				−2.7		At5g10770.1	Eukaryotic aspartyl protease family	7.8E−41
					−3.6	−4.6		At5g10760.1	Eukaryotic aspartyl protease family	5.4E−34
				5.5				At3g52500.1	Eukaryotic aspartyl protease family	2.1E−30
				10.0	3.0		3.7	At3g54400.1	Eukaryotic aspartyl protease family	1.9E−26
XP_002265947	Galactinol synthase 4-like	−1.6	17.7		6.1		4.6	At1g60470.1	Galactinol synthase 4	5.5E−158
				3.5				At2g47180.1	Galactinol synthase 1	3.6E−149
				−4.4		18.3		At1g56600.1	Galactinol synthase 2	3.2E−142
			88.4	62.7	346.5	50.6	21.1	At1g09350.1	Galactinol synthase 3	1.3E−138
XP_002284578	Polyketide cyclase/dehydratase (MLP28-like)	1.7				2.4	−3.2	At1g70850.3	MLP34 | MLP-like protein 34	5.2E−44
							−17.1	At1g70880.1	Polyketide cyclase/dehydrase	8.4E−34
Transcript abundance trends dissimilar between 35S::VvCBF4-grapevine and CBFs in Arabidopsis										
XP_002265012	ABC transporter MRP4-like	−1.7				2.2		At3g62700.1	Multidrug resistance-associated protein 10	0.0E+00
XP_002273448	Acid phosphatase class B	2.0		−13.3			−5.7	At5g24780.1	Vegetative storage protein (VSP1)	8.0E−42
XP_002279531	Flavonoid 3',5'-hydroxylase CYP96A10	2.1				−2.4	−19.7	At5g52320.1	CYP96A4	2.5E−138
XP_002280732	Alpha-glucan phosphorylase (PHS2-like)	−3.0				3.2		At3g29320.1	Glycosyl transferase, family 35	0.0E+00
						3.9		At3g46970.1	Alpha-glucan phosphorylase 2 (PHS2)	0.0E+00
XP_002266359	Calcium-binding EF-hand MSS3-like	1.6				−2.2		At2g43290.1	Calcium-binding EF-hand (MSS3)	4.5E−76
						−2.2		At3g59440.1	Calcium-binding EF-hand family	3.7E−70
XP_002274520	Xyloglucan endo-transglycosylase (XET)	2.0				−2.5		At5g65730.1	XTH6	1.0E−87
						−2.9		At4g30270.1	XTH24	1.8E−79

In 35S::*Vv*CBF4-grapevine			In CBF-overexpressing *Arabidopsis*							
V. vinifera **RefSeq ID**	***V. vinifera*** **gene description**	***Vitis CBF4***	***CBF1***[a] **FC**	***CBF2***[b] **FC**	***CBF2***[c] **FC**	***CBF3***[d] **FC**	***DDF1***[e] **FC**	***Arabidopsis*** **Gene ID**	***Arabidopsis*** **gene description**	**BLAST e-value**
							−11.3	At2g18800.1	XTH21	1.6E−75
				−2.8		−2.4		At3g44990.1	XTH31	7.6E−54

FC, fold change.

[a] 35S::AtCBF1 in *Arabidopsis* (Fowler and Thomashow, 2002);

[b] 35S::AtCBF2 (Sharabi-Schwager et al., 2010);

[c] 35S::AtCBF2 (Vogel et al., 2005);

[d] 35S::AtCBF3 (Maruyama et al., 2009);

[e] 35S::DDF1 (Magome et al., 2008).