Functional Identification of a C-repeat Binding Factor Transcriptional Activator from Blueberry Associated with Cold Acclimation and Freezing Tolerance

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ABSTRACT. Highbush blueberry (Vaccinium corymbosum) is susceptible to winter freezing injury and frost damage in the spring. As part of an ongoing project to understand the process of cold acclimation, we isolated a C-repeat binding factor (CBF) transcriptional activator gene-coding region from the highbush blueberry cultivar Bluecrop. Expression of the highbush blueberry CBF gene was compared in floral buds of the cold-tolerant northern highbush cultivar Bluecrop and the more cold-sensitive southern rabbiteye (V. virgatum) blueberry cultivar Tifblue. Relative gene expression was higher in ‘Bluecrop’ than in ‘Tifblue’. Expression in both cultivars was highest at the earliest time point in the fall (coincident with the first stage of cold acclimation), declined during the later fall and winter, and, in ‘Bluecrop’, increased again as buds deacclimated, when temperatures tend to fluctuate. To confirm the putative identity of the gene as a member of the CBF gene family, and to determine if expression in a heterologous system could enhance freezing tolerance, the blueberry gene coding sequence was overexpressed in transgenic Arabidopsis thaliana under the control of the cauliflower mosaic virus 35S promoter. Transgenic plants expressing the putative blueberry CBF gene exhibited induced expression of the A. thaliana cold-regulated (COR) genes COR78 and COR6.6, under non-inducing conditions (i.e., 23 °C); however, expression of two other COR genes was unaffected. Transgenic plants also exhibited enhanced freezing tolerance under non-acclimated conditions, but not to the level of acclimated control plants. Thus, the expression pattern in floral buds and the ability of the isolated gene to turn on a subset of COR genes and increase freezing tolerance in a heterologous system suggest it is a functional member of the CBF gene family in blueberry.

Enhanced cold tolerance, including tolerance to winter freezing and spring frosts, is needed for genetic improvement of current highbush blueberry cultivars (Moore, 1993). Freezing and cold injury not only cause direct crop loss through flower bud and shoot tip damage, but can cause indirect loss by predisposing the plants to pathogen infection such as stem blight caused by Botryosphaeria dothidea (Cline, 1995) and mummy berry caused by Monilinia vaccinii-corymbosi (Hildebrand and Braun, 1991). The response of plants to low temperatures is complex, requiring the activation of multiple genes and pathways (Fowler and Thomashow, 2002; Thomashow, 1999). The genes activated encode a diverse array of products, including enzymes required for the biosynthesis of osmoprotectants and lipid desaturases, protective proteins such as dehydrins and chaperones, and antioxidants and proteins involved in signal transduction such as transcription factors and protein kinases (Fowler and Thomashow, 2002; Seki et al., 2001; Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1999). Because one of the effects of freezing is cellular dehydration, many of the genes activated in response to cold temperatures are also induced under drought conditions (Stockinger et al., 1997; Wang et al., 1995). Expression of many genes in these overlapping pathways is induced by transcription factors called CBF (C-repeat or CRT binding factor). These transcription factors, best studied in Arabidopsis thaliana, are induced quickly in response to cold and drought stress (Fowler and Thomashow, 2002; Gilmour et al., 1998; Liu et al., 1998; Shinwari et al., 1998; Stockinger et al., 1997).

In woody perennials such as blueberry (Vaccinium spp.), cold acclimation is considered a two-step process (Weiser, 1970). The first stage is thought to be induced by a short photoperiod, while the second stage, characterized by a more pronounced increase in freezing tolerance, is induced by low
temperatures. In recent years, as part of our efforts to better understand the genetic basis of cold tolerance in blueberry, we have used approaches based on expressed sequence tags or ESTs (Dhanaraj et al., 2004), microarrays (Dhanaraj et al., 2007), and subtractive hybridization (Naik et al., 2007) to identify a variety of cold-responsive genes. We have focused primarily on genes expressed in floral buds during exposure to cold, as floral buds are susceptible to freeze/frost damage and, depending on the year, such damage can result in considerable yield losses. Transcription initiation factors such as CBF are desirable targets for further study because these activate the expression of a host of downstream genes that are associated with cold acclimation and freezing tolerance (Thomashow, 1999). Here, our objectives were to isolate a full-length CBF transcription factor from highbush blueberry, to compare expression of the putative CBF gene in floral buds of cold-tolerant and cold-sensitive blueberry cultivars throughout the dormant period from bud set to budbreak, to express the full-length clone in transgenic A. thaliana to confirm its function as an inducer of COR (cold-regulated) gene expression, and to determine if expression of the putative blueberry CBF gene enhances freezing tolerance in the transgenic A. thaliana.

**Materials and Methods**

**Isolation and sequencing of putative CBF clones from highbush blueberry.** Previously, forward and reverse subtracted blueberry cDNA libraries were prepared that enriched for transcripts expressed at higher levels in dormant ‘Bluecrop’ flower buds at 400 and 0 chill units, respectively (Naik et al., 2007). The highbush blueberry cultivar Bluecrop was chosen because it is quite cold hardy and is the industry standard of highbush cultivars. One chill unit, as defined here, equals 1 h of exposure to temperatures between 0 and 7 °C. The 0 and 400 chill unit time points corresponded to collection times on 29 Sept. 2003 and 8 Dec. 2003, respectively, and cold hardiness levels, expressed as lethal temperature50 (LT50) or temperature that kills 50% of the floral buds in a controlled freeze-thaw test, of –10 and –25 °C, respectively (Muthalif and Rowland, 1994; Naik et al., 2007).

Random clones were picked and sequenced from both libraries, and a search of the National Center for Biotechnology Information (NCBI) non-redundant protein database using BLASTX (Altschul et al., 1990) revealed that four of 167 (2.4%) randomly picked clones from the reverse subtracted library had significant homology to CBF gene products or the similar dehydration-responsive element binding (DREB) protein elements. Upon alignment of the four clones with CBF gene homologs from other plants, it appeared that one clone, RL82 (GenBank accession no. DW043077), represented the 5’ half of the gene, while the three other clones, RL6 (GenBank accession no. DW043065), RL44 (GenBank accession no. DW043054), and RL138 (GenBank accession no. DW043014), represented the 3’ half of the gene. Therefore, a forward primer (5’GGCGCAGTCTCCACATTTA) was designed near the 5’ end of the RL82 sequence and a reverse primer (5’CAAGTTC TCTCTGTTTATAT) was designed near the 3’ end of the RL6 sequence using the Primer3 program (Rozen and Skaltsky, 2000). These primers were used in PCR reactions with ‘Bluecrop’ genomic DNA as template in an attempt to amplify one fragment that would span the entire translated portion of the putative blueberry CBF-coding region (hereafter referred to as BB-CBF) plus about 150 bp on the 5’ and 3’ ends of the untranslated region (UTR). ‘Bluecrop’ genomic DNA isolation and polymerase chain reactions (PCR) were as previously described for the development of EST-PCR markers in blueberry (Rowland et al., 2003).

Using this approach, a fragment of the expected size (∼1 kb) was amplified and purified from a 1.4% agarose gel using the Ultrapure Gelspin Kit (MO BIO Laboratories, Solana Beach, CA) according to the manufacturer’s instructions. The purified fragment was cloned using the pGEM-T Vector System (Promega, Madison, WI) according to the directions provided. Recombinant plasmids were isolated using the Ultrapure Miniprep Kit (MO BIO Laboratories). Several isolates of the cloned fragment were cycle-sequenced using Cy5-labeled M13 forward and reverse primers and the SequiTherm EXCEL II Long-Read™ Kit (Epicentre Technologies, Madison, WI). Labeled primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Sequencing reactions were separated and detected on an ALF Express Sequencer (Amersham Pharmacia Biotech, Piscataway, NJ). Sequences were assembled using various modules of the Lasergene software package (DNASTAR, Madison, WI).

The identity of the putative BB-CBF clone was confirmed by comparison with sequences posted in GenBank using nucleotide BLAST (Altschul et al., 1990). To further confirm the identity of the gene product, the translated sequence was compared with the non-redundant NCBI protein database using blastp (protein-protein Blast). Several similar sequences were downloaded for comparison. Alignment of the predicted amino acid sequences was performed using the M-Coffee web server (Moretti et al., 2007). Gaps were manually removed from the alignment and reintroduced into the dataset as coded characters. A midpoint-rooted phylogenetic tree was constructed in PAUP* (Swofford, 2000) using maximum parsimony performed with TBR branch swapping and a branch-and-bound search strategy. Bootstrap values supporting the branches were calculated using 1000 replicates.

The deduced amino acid sequence of the BB-CBF was aligned with several other CBF/DREB proteins published in GenBank using ClustalW. Graphic display was generated using the Geneious Pro software package (version 4.6.5; Biomatters, Auckland, New Zealand). Amino acid numbering is relative to a deduced consensus sequence.

**Expression analysis of the BB-CBF gene in floral buds of the northern highbush blueberry cultivar Bluecrop and the southern rabbiteye blueberry cultivar Tifblue.** To study the expression pattern of the BB-CBF gene in floral buds from bud set to budbreak, a time course experiment was performed using field-grown plants (Beltsville, MD) of the relatively cold-hardy highbush cultivar Bluecrop and the relatively cold-sensitive rabbiteye cultivar Tifblue. RNA was extracted from floral buds of ‘Bluecrop’ and ‘Tifblue’ field plants that had accumulated 0 (collected 29 Sept. 2003), 67 (20 Oct. 2003), 399 (8 Dec. 2003), 779 (2 Feb. 2004), and 1234 (13 Mar. 2004) chill units (defined as hours of exposure to temperatures between 0 and 7 °C). RNA was extracted from ∼600-ng frozen samples using the hot borate protocol described by Wilkins and Smart (Wilkins and Smart, 1996). To remove any genomic DNA contamination, total RNA was treated with RNase-free DNase I (Promega). Total RNA (5 µg/lane) from each time point was separated on 1% agarose/formaldehyde gels, visualized, and photographed to confirm quality and concentration.
cDNA was synthesized from RNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for the qPCR reactions. Primers for qPCR were designed using Primer Express 3.0 software (Applied Biosystems) and are listed in Table 1. Reactions were run on an Applied Biosystems 7500 real-time PCR machine. Assays were normalized using expression of a blueberry metallothionein gene, which does not change with cold acclimation based on previous northern blot (Dhanaraj et al., 2004) and microarray data (Dhanaraj et al., 2007). There were three replicates of each cultivar at each time point and each experiment was repeated twice with similar results. Relative expression levels were calculated by the ΔΔCT method using the Applied Biosystems 7500 software package (version 2.0.1).

**VECTOR CONSTRUCTION AND A. THALIANA TRANSFORMATION.**

The sequenced isolates of the putative BB-CBF gene from blueberry were found to contain an EcoRI recognition site near the 3’ end (nt 954–955). To facilitate downstream cloning, this site was removed by digestion with EcoRI, filling in the overhang, and ligating the blunt ends, using standard techniques (Sambrook et al., 1989). This construct was digested with HindIII and EcoRI, yielding the BB-CBF fragment downstream of the cauliflower mosaic virus 35S promoter and enhancer and ended with the same enzymes (pCAMBIA 2301 (GenBank accession no. AF234316) digested with HindIII enzymes. The resulting plasmid was digested with vector (Timmermans et al., 1990) digested with the same vector (Promega Corporation) multiple cloning region and the site was removed by digestion with SphI (pMP90) (Koncz and Shell, 1986) using the freeze-thaw protocol. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for the qPCR reactions. All of the primers used for qPCR were designed using the Primer Express 3.0 software (Applied Biosystems). Sequences for primer design were downloaded from GenBank, with the exception of the BB-CBF, which was based on our own sequence. All reactions were run on an Applied Biosystems 7500 real-time PCR machine. Assays were normalized using expression of eukaryotic translation initiation factor 4A-2 (eIF4A-2) (Gilmour et al., 2000; Metz et al., 1992). There were three technical replications in each run. Primers used are listed in Table 1.

**COLD ACCLIMATION AND FREEZING TOLERANCE IN WILD-TYPE (WT) AND TRANSGENIC (OVEREXPRESSED BB-CBF) A. THALIANA PLANTS.** For cold-acclimation treatment, 3-week-old seedlings from the same lines used above (S3–7 and S3–11) were transferred from the growth chamber to a cold room at 3 ± 1 °C and 50 μmol·m⁻²·s⁻¹ light intensity for 6 d. Freezing tolerance (FT) tests were performed by subjecting plants to a controlled freeze-thaw regime (in a glycol bath) followed by the assessment of injury (and FT as LT₅₀) by measuring electrolyte leakage.

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**Table 1. Plant sources, target gene designations, GenBank accession numbers, and primer sequences used for real-time PCR assays.**

| Plant source and gene designation* | Accession no. | Primer sequences* |
|-----------------------------------|---------------|-------------------|
| Vaccinium corymbosum             | FJ222601      | F-TCGGAGCGAGGAGCTGATGA  |
| Blueberry CBF                     |               | R-TCCGGCGTCGCTTCTCTC  |
| V. corymbosum                     |               | F-ACCTGACATGACCTTCCTGC |
| Blueberry metallothionein         | CF811253      | R-ACCCAAATCTCTCGTCTG   |
| Arabidopsis thaliana              |               | F-TGATGTGTCAACGGAAAGTCTCA |
| COR78                             | NM_124610     | R-ACAGGCGCAGCCTCTCACA  |
| A. thaliana                       |               | F-CCAGGTGAACCTTGGCTACCA |
| COR414-TM1                        | NM_102679     | R-TGATATGGCGCCACAATACCA |
| A. thaliana                       |               | F-GGATGGCCAGCTTTGTGAT  |
| CBF-1                             | NM_118681     | R-AGACGGCGCGCCGTTAAAG  |
| COR86                             | X55053        | F-GGCCGCGTCAGGCAAGCT   |
| A. thaliana                       |               | R-GGACGACCTCTGTTGGCAGTC |
| COR15a                            | AY057640      | F-CGTTGATCATCCGAGGGCTAAC |
| A. thaliana                       |               | R-TGGGACCCTGAGGAGTCTCTC |
| EIF4A-2                           | NM_104305     | F-CGTTGACCTGAGGAGCATTCA |
|                                   |               | R-ACACGACGCGGAGCTTCCCA |

*Plant source (species) and target gene designations.
*aAll primer sequences are given 5’ to 3’. Forward and reverse primers are abbreviated F and R, respectively.
from freeze-thaw–injured tissues as described (Jaglo-Ottosen et al., 1998; Peng et al., 2007). Three replicates (individual plants) per treatment temperature were used in FT tests, which were repeated three times. Freezing injury (and L1₅₀) data presented are the mean of three independent experiments. Basically, non-acclimated (NA) and cold-acclimated (CA) WT and transgenic BB-CBF plants were placed in test tubes in a glycol bath for 1.5 h at –1 °C in a completely randomized design, after which ice chips were added to initiate ice-nucleation. After an additional hour of incubation at –1 °C, the temperature was lowered at a rate of 1 °C/h until it reached –8 °C (for NA plants) or –12 °C (for CA plants). Frozen samples at each treatment temperature were removed from the glycol bath and thawed on ice overnight followed by incubation in 20 mL of distilled water, vacuum infiltration (three times for 3 min each at 172.4 kPa), and shaking for 1 h at 250 rpm. Initial electrical conductivity (EC) was measured for each sample with a conductivity meter. Total conductivity for the same sample was determined after autoclaving at 121 °C for 20 min. Initial leakage was expressed as the percentage of the final conductivity, and the percentage of leakage for each treatment temperature was converted to percentage of injury (Lim et al., 1998). The temperature at which 50% injury occurred (LT₅₀) was defined as the FT.

Results

Confirmation of the BB-CBF Sequence as a CBF Gene. A putative CBF gene fragment (about 1 kb) was amplified from ‘Bluecrop’ highbush blueberry genomic DNA using PCR primers based on EST clones of what appeared to be the 5’ and 3’ ends of a CBF-like gene. The fragment was purified, cloned, and sequenced. The putative highbush BB-CBF nucleotide sequence (GenBank accession no. FJ222601) was compared with other sequences in the GenBank database and had a high similarity (e-values <10⁻²⁰⁻) to numerous CBF/DREB sequences, with the greatest identity associated with the conserved AP2 binding domain-coding region (data not shown). To further confirm the identity of the gene, the translated sequence was compared with the non-redundant NCBI protein database, and several similar sequences were downloaded for comparison. The phenogram resulting from a PAUP* analysis (Fig. 1) supports the identity of the blueberry sequence as a CBF/DREB gene and shows that similarity is highest to the Vaccinium vitis-idea (lingonberry) DREB1 deduced amino acid sequence (accession no. ACJ54953). Of the sequences downloaded for comparison, the BB-CBF was least similar to the A. thaliana CRT/DRE binding factor 2 (used as the out-group in the phenogram).

The BB-CBF protein has a predicted molecular weight of 24.7 kDa and an isoelectric point of ≈7. The deduced amino acid sequence of the BB-CBF protein was found to contain the ERF/AP2 DNA-binding domain (Fig. 2). The CBF signatures (Jaglo et al., 2001) ETRHP/DSAWR are also present, but the DSAWR signature has been modified from DSAWR to DSVWR in the blueberry protein. This amino acid substitution (A to V) is also present in the V. vitis-idea DREB1. Phylogenetic analysis of the downloaded CBF/DREB proteins generally shows groupings by accepted taxonomic relationships rather than specific gene function.

Expression of the BB-CBF Gene in Floral Buds of Cold-Tolerant and Cold-Sensitive Blueberry Cultivars. The expression pattern of the BB-CBF gene was compared in floral buds of field-grown plants of the relatively cold-hardy highbush cultivar Bluecrop and the relatively cold-sensitive rabbiteye cultivar Tifblue using real-time PCR (Fig. 3). A time-course experiment was performed to monitor expression from bud set to budbreak (about the end of September through the middle of March). ‘Bluecrop’ flower buds generally reach a maximum midwinter cold hardiness level of about –27 °C, whereas ‘Tifblue’ is generally cold hardy to about –20 °C (Arora et al., 2004; Dhanaraj et al., 2007; Rowland et al., 2005). Expression of BB-CBF was found to be higher in the cold-hardy ‘Bluecrop’ than in the cold-sensitive ‘Tifblue’. Additionally, in both cultivars, expression was highest at the earliest time point in the fall, at the first stage of cold acclimation, and declined during the later fall and winter. In ‘Bluecrop’, expression increased slightly by the last time point as buds were deacclimating, when temperatures tend to fluctuate. The largest difference in CBF-mRNA levels between the two cultivars was at the first time point, in late September, when CBF-mRNA levels were almost 4-fold higher in ‘Bluecrop’ than in ‘Tifblue’.

COR Gene Activation in Transgenic A. thaliana. To determine if the BB-CBF gene product could activate cold-regulated (COR) genes known to be part of the CBF regulon, the relative expression levels of several COR genes were monitored using real-time PCR in transgenic (expressing the BB-CBF gene) and WT A. thaliana under normal (non-inducing temperature) and cold-treated conditions (Fig. 4). The two lines of transgenic plants (S3–7 and S3–11) always exhibited similar expression levels of the genes tested under the same treatments (i.e., the same expression levels when cold treated and also the same expression levels under non-inducing conditions). Therefore, the data from both lines were combined to generate the graph (Fig. 4). The relative expression levels of
Fig. 2. Multiple alignment of the amino acid sequences of C-repeat binding factor/dehydration responsive element binding factor (CBF/DREB) proteins. Alignment numbers are relative to a deduced consensus sequence. Shaded regions show high similarity (60%–100%), based on the Blosum62 matrix. Unshaded regions are less than 60% identical. The ethylene-responsive element binding factor/APETELA2 (ERF/AP2) DNA-binding domain is underlined and the CBF signatures [ETRH and DS(A/V)WR] are indicated by **** and are in bold. A neighbor-joining tree based on the alignment is shown in the left margin. Abbreviations (species followed by the GenBank accession numbers) are as follows: Bp CBF/DREB1 = Betula pendula ABP98987, Eg CBF1a = Eucalyptus gunnii DQ241820, Sl CBF3 = Solanum lycopersicum AAS77819, At CBF1 = Arabidopsis thaliana AY667247, At CBF2 = Arabidopsis thaliana AAC99371, Rc DREB1A = Ricinus communis EEF51089, Mb CBF/DREB1 = Malus baccata ABQ59086, Pa DREB1 = Prunus avium BAC20184, Pt CBF1 = Populus tomentosa ABC79626, Vv-i DREB1 = Vaccinium vitis-idea ACJ54953, and Vc CBF/DREB = Vaccinium corymbosum AC145245.

Fig. 3. Relative expression of the highbush blueberry C-repeat binding factor (BB-CBF) gene in the cold-hardy northern highbush blueberry cultivar Bluecrop (BC) and the less cold-hardy rabbiteye blueberry cultivar Tibblue (TB) at various stages of chilling-hours accumulation (0, 67, 399, and 799 chilling hours), which is defined as hours of exposure to temperatures between 0 and 7 °C. Error bars are 95% confidence intervals. DA = deacclimating and corresponds to exposure to about 1234 chilling hours.

Fig. 4. Relative expression of cold-responsive (COR) genes COR6.6 (solid bars) and COR78 (hatched bars) in the highbush blueberry C-repeat binding factor (BB-CBF) overexpressing (Transgenic) and untransformed (WT) Arabidopsis thaliana under cold-treated (exposed to 4 °C for 12 h) and non-induced (maintained at 23 °C) conditions. Error bars are 95% confidence intervals.
the endogenous *A. thaliana* *CBF1* and the BB-CBF transgene were also monitored to confirm expression of the transgene in the appropriate plants and to determine if expression of the blueberry transgene affected expression of the endogenous *A. thaliana* *CBF1* gene. The results indicated that the blueberry transgene was very highly expressed in the transgenic *A. thaliana* under all conditions (data not shown). Following cold treatment, all tested *COR* genes (*COR78*, *COR6.6*, *COR15A*, and *COR414-TM1*) were highly expressed in WT and transgenic plants. The endogenous *A. thaliana* *CBF1* gene was also expressed at similar levels in the WT and transgenic plants (data not shown). Under non-inducing conditions, *COR78* and *COR6.6* were upregulated in the transgenic plants, presumably due to activation by the BB-CBF gene product. The other *COR* genes tested (*COR15A* and *COR414-TM1*) were not activated under non-inducing conditions in all plants tested (data not shown). Transgenic plants overexpressing the BB-CBF gene appeared phenotypically normal (i.e., the same as WT) under all conditions tested (Fig. 5, A and B).

**Freezing tolerance of transgenic *A. thaliana*.** The FT (LT50) of NA WT and transgenic (BB-CBF) S3–7 and S3–11 seedlings were about –3.99, –4.73, and –4.84 °C, respectively (Fig. 6A, Table 2). Moreover, the relative level of freeze-thaw injury of WT plants at individual subfreezing treatments (–3, –3.5, –4, –4.5, and –5 °C) was on average ≈35%, 50%, 75%, 32%, and 38%, respectively, higher than that of both the transgenic BB-CBF lines (Fig. 6B). These results indicated that transgenic BB-CBF-expressing *A. thaliana* plants had higher constitutive (i.e., under NA conditions) freezing tolerance than the WT plants.

After 6 d of cold acclimation, the LT50 of WT, S3–7, and S3–11 plants were about −7.32, −6.23, and −6.37, respectively (Fig. 6C, Table 2); consequently, the gain in FT during cold acclimation (∆LT50 = CA FT − NA FT) by these genotypes was 3.33, 1.5, and 1.59 °C, respectively. The relative freeze-thaw injury...
injury of CA BB-CBF lines at various subfreezing temperatures (−4, −5, −6, −7, and −8 °C) were on average ≈60%, 85%, 115%, 55%, and 25%, respectively, greater than the freezing injury of corresponding WT controls (Fig. 6D). Differences in comparative injury response at NA and CA states for the BB-CBF lines and WT (as gleaned from results above) and their CA-derived ΔLT50 values indicate that BB-CBF lines had lower cold acclimation ability than the WT *A. thaliana* plants.

### Discussion

The reverse subtracted library we created previously was enriched for transcripts that were expressed at higher levels in dormant ‘Bluecrop’ flower buds at 0 chill units (the end of September) than at 400 chill units (early December) (Naik et al., 2007). This library yielded fragments of a putative CBF-like gene. Higher expression of the blueberry CBF-like gene at 0 chill units than at 400 chill was confirmed with real-time PCR (discussed in greater detail in the following paragraph). Sequence analysis suggested that the CBF/DREB fragments might represent the 5′ and 3′ ends of the same CBF gene. Therefore, we used the sequence data to design primers in an attempt to amplify the full coding sequence of the putative CBF gene from genomic DNA of the highbush blueberry cultivar Bluecrop. We successfully amplified the coding region of the putative BB-CBF and confirmed identity by comparison of the deduced amino acid sequence to CBF/DREB proteins from other plants. From a PAUP* analysis, it was not surprising that the most amino acid sequence to CBF/DREB proteins from other plants and confirmed identity by comparison of the deduced genomic DNA of the highbush blueberry cultivar Bluecrop. We

| Treatment | WT | LT50 [mean ± sd (°C)] | S3-7 | S3-11 |
|-----------|----|------------------------|------|-------|
| NA        | −3.99 ± 0.06 | −4.73 ± 0.38 a | −4.84 ± 0.42 a |
| CA        | −3.72 ± 0.26 | −6.23 ± 0.58 b | −6.37 ± 0.48 b |

*Letters indicate significantly different versus WT as determined by Student’s *t* test at the following probability levels: a) *P* < 0.01 and b) *P* < 0.05. Where letters are not listed, the values are not significantly different from WT.

### Table 2. Freezing tolerance of non-acclimated (NA) and cold-acclimated (CA) wild-type (WT) and transgenic (BB-CBF overexpressing) *Arabidopsis thaliana* seedlings (lines S3–7 and S11) as assessed by a laboratory freeze-thaw stress test. Data are expressed as the temperature at which 50% of the floral buds are killed (LT50) in a controlled freeze-thaw test.
In conclusion, the ability of the isolated BB-CBF gene to turn on certain downstream COR genes and increase freezing tolerance in NA *A. thaliana* plants, its temporal expression pattern in floral buds, and the relationship of its expression level with cold hardiness level in cultivars with different freezing tolerances all suggest that it is a functional member of the CBF/DREB gene family in blueberry.

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