

Hv-*CBF2A* overexpression in barley accelerates *COR* gene transcript accumulation and acquisition of freezing tolerance during cold acclimation

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Abstract C-Repeat Binding Factors (CBFs) are DNA-binding transcriptional activators of gene pathways imparting freezing tolerance. Poaceae contain three *CBF* subfamilies, two of which, HvCBF3/CBFIII and HvCBF4/CBFIV, are unique to this taxon. To gain mechanistic insight into HvCBF4/CBFIV CBFs we overexpressed Hv-*CBF2A* in spring barley (*Hordeum vulgare*) cultivar ‘Golden Promise’. The Hv-*CBF2A* overexpressing lines exhibited stunted growth, poor yield, and greater freezing tolerance compared to non-transformed ‘Golden Promise’. Differences in freezing tolerance were apparent only upon cold acclimation. During cold acclimation freezing tolerance of the

Hv-*CBF2A* overexpressing lines increased more rapidly than that of ‘Golden Promise’ and paralleled the freezing tolerance of the winter hardy barley ‘Dicktoo’. Transcript levels of candidate CBF target genes, *COR14B* and *DHN5* were increased in the overexpressor lines at warm temperatures, and at cold temperatures they accumulated to much higher levels in the Hv-*CBF2A* overexpressors than in ‘Golden Promise’. Hv-*CBF2A* overexpression also increased transcript levels of other *CBF* genes at *FROST RESISTANCE-H2-H2* (*FR-H2*) possessing CRT/DRE sites in their upstream regions, the most notable of which was *CBF12*. *CBF12* transcript levels exhibited a relatively constant incremental increase above levels in ‘Golden Promise’ both at warm and cold. These data indicate that Hv-*CBF2A* activates target genes at warm temperatures and that transcript accumulation for some of these targets is greatly enhanced by cold temperatures.

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Introduction

Many plants increase in freezing tolerance in response to low non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow 1999). During cold acclimation biochemical and physiological changes occur in the plant conditioning it to survive cellular dehydration caused by freezing temperatures (Thomashow 1999). Playing a key structural role in conferring some of these adaptive changes are the Cold Regulated (COR) and Dehydrin (DHN) proteins that function to stabilize membrane structure (Thomashow 1999; Koag et al. 2009). Transcripts for the *COR* and *DHN* genes accumulate to high levels at low temperatures and during dehydrating conditions (Thomashow 1999; Cattivelli et al. 2002). One of the key regulators affecting cold and dehydration induced expression of the *COR* and *DHN* genes is the family of C-Repeat Binding Factor/Dehydration Responsive Element Binding Factor proteins (CBF/DREB), which bind to the C-repeat/Dehydration Responsive Element (CRT/DRE) sites in the upstream regions of the *COR* and *DHN* genes, activating their expression (Stockinger et al. 1997; Liu et al. 1998; van Buskirk and Thomashow 2006). Transcripts for the CBFs are themselves induced by cold temperatures in a time frame that precedes that of *COR* and *DHN* transcript accumulation (Gilmour et al. 1998). In *Arabidopsis thaliana* many of the biochemical and physiological changes, and the increase in freezing tolerance that occur during cold acclimation, can be mimicked at normal warm growth temperatures through constitutive high level overexpression of the *CBFs* (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000; 2004; Vogel et al. 2005).

The *CBF* gene family of the temperate climate Triticeae cereals (barley, *Hordeum vulgare*; wheat, *Triticum* spp.; and rye, *Secale cereale*), is much larger than that of *Arabidopsis*, other dicots, and tropical cereals such as maize (*Zea mays*) and rice (*Oryza sativa*). The barley genome harbors more than 20 *CBF* coding sequences in a single genotype (Skinner et al. 2005). At least 13 of these colocalize in a cluster on the long arm of chromosome 5 (Skinner et al. 2005, 2006; Miller et al. 2006; Badawi et al. 2007; Francia et al. 2007; Knox et al. 2010). These *CBFs* are grouped into two phylogenetic subgroups—HvCBF3/CBFIII and HvCBF4/CBFIV, both of which are unique to the grasses (Skinner et al. 2005; Badawi et al. 2007). Additional *CBFs*, belonging to the HvCBF1/CBFI-CBFII

subgroup, occur dispersed on four other chromosomes (Skinner et al. 2005; Badawi et al. 2007). To date the chromosome 5 cluster has received the greatest attention because genes of this cluster are implicated in the molecular basis of *FROST RESISTANCE-2* (*FR-2*), one of two major quantitative trait loci affecting freezing tolerance and winter hardiness of Triticeae cereals (Vágújfalvi et al. 2003; Francia et al. 2004; Båga et al. 2007; Knox et al. 2008, 2010).

In vitro experiments and overexpression strategies suggest a complex relationship between the CBFs, their targets, and temperature. In vitro experiments using recombinant CBF proteins indicate HvCBF4/CBFIV proteins bind to oligonucleotides having CRT/DRE motifs if binding reactions are carried out at cold temperatures, but not at warm temperatures (Xue 2003; Skinner et al. 2005). In contrast, recombinant proteins of the HvCBF3/CBFIII and HvCBF1/CBFI-CBFII subgroups are not affected by temperature (Xue 2003; Skinner et al. 2005). Overexpression of HvCBF3/CBFIII *CBFs* in *Arabidopsis* also induces *COR* gene expression at normal growth temperatures and increases in freezing tolerance, whereas overexpression of HvCBF4/CBFIV *CBFs* does not induce *COR* gene expression at warm temperatures, nor does it have an effect upon freezing tolerance (Skinner et al. 2005). The reasons for the differences between HvCBF4/CBFIV and HvCBF3/CBFIII subgroup CBFs in *Arabidopsis* are not clear but it was considered that the low temperature-dependence observed in vitro might be indicative of a low temperature-dependence for activity in vivo, or simply, that the HvCBF4/CBFIV subgroup *CBFs* are unable to activate genes in *Arabidopsis* (Skinner et al. 2005). In rice, overexpression of barley *CBF4* increases tolerance to drought, salinity, and chilling temperatures and induces stress-related genes in the absence of stress (Oh et al. 2007; Lourenco et al. 2011), indicating that in a monocot, the overexpressed transgene is functionally active.

To further our mechanistic understanding of HvCBF4/CBFIV *CBFs*, we generated transgenic barley plants overexpressing Hv-*CBF2A* under control of the CaMV 35S RNA promoter in the spring cultivar ‘Golden Promise’. To date, ectopic expression of CBFs in Triticeae cereals has been carried out using wheat orthologs of barley HvCBF1/CBFI-CBFII subgroup *CBFs*, *CBF5* and *CBF7*, Ta-*DREB3* and Ta-*DREB2*, respectively (Pellegrineschi et al. 2004; Morran et al. 2011) and *Arabidopsis* *CBF3/DREB1A* but not with members of either the HvCBF3/CBFIII or HvCBF4/CBFIV subgroups. The Hv-*CBF2A* overexpressing plants, alongside non-transformed ‘Golden Promise’ and the winter-hardy facultative barley cultivar ‘Dicktoo’ were evaluated for freezing tolerance and for candidate target gene expression.

Materials and methods

Plasmid construction

Hordeum vulgare cv. 'Dicktoo' CBF2A clone (AY785841) encompassing the coding sequence and flanking untranslated regions was PCR amplified using primers 5'-CCAC AACGCACTCTCGACGC-3' and 5'-GCATATTCATGG TTTGAGATTG-3' by *Pfu* DNA polymerase (Promega Corporation; www.promega.com). As *Pfu* produces blunt-ended PCR products, A-overhangs were added using a *Taq* polymerase incubation, and these were then cloned into a T-overhang *Xcm*I site between the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) and 7'T-5'T terminator elements of pGA643 assembled in the pLITMUS28 vector (New England Biolabs, www.neb.com) yielding a CaMV 35S:CBF2A:7'T-5'T expression cassette. This construct was sequenced to confirm errors were not introduced. The CaMV 35S:CBF2A:7'T-5'T cassette was excised from the pLITMUS28 vector with *Age*I and *Bsi*WI, subcloned into identically-cut pGEM/HAB, and then sequenced. (pGEM/HAB was constructed by subcloning a synthetic *Hind*III-*Age*I-*Bsi*WI (HAB) adapter having A-overhangs into pGEM-T-Easy.) The CaMV 35S:CBF2A:7'T-5'T cassette in pGEM/HAB was excised using *Not*I and subcloned into *Not*I-cut binary vector pWBVec10a (Wang et al. 1998). [pWBVec10a contains hygromycin resistance (*hygromycin phosphotransferase*, *hph*) and GUS (β -glucuronidase, *uidA*) marker genes internal to the T-DNA border sequences and flanking the CaMV 35S:CBF2A:7'T-5'T cassette; both *hph* and *uidA* expression cassettes contain introns so that their expression occurs only in the transformed eukaryotic cells (Wang et al. 1998)]. The final construct, pWBVec10a/CaMV 35S-CBF2A, was transformed into *A. tumefaciens* AGL1 (Lazo et al. 1991) according to the procedure described (Walkerpeach and Velten 1994).

Plant material and transformation

Hordeum vulgare L. cv. 'Golden Promise' plant material, spike collection, sterilization and excision of immature embryos were carried out using established methods (Chang et al. 2003).

Transformation of 'Golden Promise' was carried out using established methods (Horvath et al. 2002) with the following modifications. *A. tumefaciens* AGL1 harboring pWBVec10a/CaMV 35S-CBF2A binary vector was grown in YEP medium containing spectinomycin and carbenicillin (100 μ g mL⁻¹ each) at 28 °C overnight. Bacterial cultures were centrifuged and the harvested bacterial pellets were resuspended in two volumes (relative to the culture medium volume) of infection medium (IM; MS,

30 g L⁻¹ maltose, pH 5.2) containing 100 μ M acetosyringone. 1 mL aliquots were then transferred to 1.5 mL tubes. After excision, each immature embryo was immediately transferred into a 1.5 mL tube containing the *A. tumefaciens* suspension. Immature embryos from a single spike were collected into the same tube. The tube contents were gently mixed by inverting 4–5 times. All immature embryos were then allowed to settle to the bottom of the tube. After collecting immature embryos from 3 to 5 spikes per round of transformations, tubes were again mixed by inverting for 1 min and then incubated at room temperature. After 15 min the IM containing the majority of *A. tumefaciens* cells was removed by pipetting. The immature embryos were then placed scutellum-side down onto co-cultivation medium: callus induction medium (CIM) containing 100 μ M acetosyringone, pH5.2. The immature embryos from each tube were plated on their own plates where they were co-cultured for 2–3 days at 25 °C in complete darkness. After co-cultivation, immature embryos were collected and washed 3–5 times with liquid MS medium containing 30 g L⁻¹ maltose and 250 mg L⁻¹ Timentin (pH5.8).

Selection on CIM, regeneration on shoot generation medium, and rooting on root generation medium were carried out using established methods (Horvath et al. 2002). The three media contained 50, 20, and 50 mg L⁻¹ hygromycin, respectively, and 150 mg L⁻¹ Timentin. Ten seeds from each T₀ plant were then germinated in individual square (5.7 cm) bio-degradable plant containers (Jiffypots, www.jiffypot.com) filled with vermiculite. Each seedling was screened for β -glucuronidase activity (Jefferson 1987). The β -glucuronidase-positive seedlings were transplanted to soil in 15 cm-diameter pots and grown to maturity. Spikes were harvested from each plant separately and stored in paper bags until further use. Each line was carried forward to the T₄ or T₅ generation by single seed descent through each generation.

Evaluation of freezing tolerance

Freezing tolerance was determined using two independent tests previously established to assay freezing tolerance levels of Triticeae cereals (Veisz and Sutka 1989; Gusta et al. 2001; Limin and Fowler 2006). One method consisted of isolating the crown region from plants and then subjecting the excised crowns to the target freezing temperatures. The second method involved subjecting the whole plants in soil to target freezing temperatures. Both methods utilized regrowth assays, providing a robust means of assaying freezing tolerance (Olien 1964).

For freezing experiments using isolated crowns, barley seeds were sown in 10 cm square plastic pots, 15 seeds/pot, in Sunshine SB40 mix (Sun Gro Horticulture Inc., www.sungro.com), and grown in a growth chamber at 18 °C

using 16 h/8 h light/dark photoperiod, $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD provided by Very High Output CW/VHO fluorescent lamps (Phillips Lighting, <http://www.usa.lighting.philips.com>) for 2 weeks. To cold acclimate, the plants were transferred to a growth room maintained at $3 \pm 1^\circ\text{C}$ using similar lighting conditions for the defined periods. The crowns of the barley plants were then harvested for the freezing experiments. The crowns consisted of the region approximately 2 mm below and 5 cm above the apex. Five crowns per genotype at each sampling were placed on moistened paper that was then placed into 16×120 mm test tubes. Test tubes were placed into a low temperature Neslab bath (Model LT-50DD, www.thermo.com) set at -1°C , and incubated for 1 h. After 1 h, ice nucleation was initiated by adding an ice chip to each tube. Test tubes with the samples remained in the -1°C temperature bath overnight to ensure complete freezing of plant tissues. The following day the temperature was decreased at a rate of 2°C h^{-1} . Sample tubes were removed at either -3°C or -6°C for percent survival determination, or at 2°C intervals in the temperature range from -2°C to -12°C for LT_{50} determination, and then placed at 4°C , at which they were allowed to thaw overnight. Three replicates were performed per genotype, per treatment. After thawing, crowns were planted into soil in pots for regrowth under normal growth conditions. Freezing tolerance was expressed as either LT_{50} values (temperature at which 50 % of the crowns failed to regrow determined 3 weeks after replanting them in soil) or percent survival (number of plants surviving/total number of plants tested) at -3°C or -6°C .

Freezing whole plants in soil utilized plants grown in wooden boxes ($38 \text{ cm} \times 26 \text{ cm} \times 11 \text{ cm}$) in which there were nine rows of plants per box, 20 plants per row. Plants were grown in a Conviron PGR-15 climatic chamber (Conviron, www.conviron.com) for 3 weeks at a constant 18°C , 16 h illumination, $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. Wooden boxes were transferred to a Conviron C-812 climatic chamber and the temperature was lowered at the rate of 0.5°C h^{-1} to -3°C , or to -6°C . The minimum temperatures were maintained for 24 h before being returned to 18°C at the rate of 0.5°C h^{-1} . Plants were allowed to recover at normal growth conditions for 3 weeks before determining survival.

DNA blot hybridization

High MW DNAs from barley were isolated using established methods (Stockinger et al. 1996). Approximately $10 \mu\text{g}$ DNA was digested separately with each *Bam*HI, *Bgl*II, and *Xho*I restriction endonucleases. Enzyme-digested DNAs were electrophoresed on 0.8 % TAE-agarose gels, transferred to Hybond N (GE Healthcare, www.gehealthcare.com), and UV crosslinked to Hybond N

membranes using standard procedures (Ausubel et al. 1993). Hybridization and washes were carried out as described (Knox et al. 2010). The same filter was sequentially hybridized starting with a *CBF2A* gene specific probe encompassing the *CBF2A* 3' region, followed by coding sequence (CDS) probes of *hph*, *CBF2A*, and *uidA*. After each round of hybridization the probe was stripped from the filter in 0.1 % SDS at 65°C .

RNA blot hybridization

Five seeds each of 'Dicktoo', 'Golden Promise', and the CaMV 35S-*CBF2A* lines were sown in $9 \text{ cm} \times 9 \text{ cm}$ pots (Kord Products, www.kord.ca) in eight replicates and transferred to Conviron growth chambers after germination. Seedlings were grown at 18°C on a 8 h light/16 h dark photoperiod for 3 weeks, then the growth chamber temperature was decreased to 6°C at daybreak of day 21. Samples were harvested at 2 h intervals starting at daybreak through the 12 h time point. The temperature was maintained at 6°C for two additional days and samples were harvested at the 6 h time point each day. The 6 h time point was chosen to avoid circadian clock effects, and because unpublished data showed peak expression of *CBF* genes occurs at approximately 6–8 h during the subjective day. At each harvest time point, crown tissue of five seedlings was pooled and flash frozen in liquid nitrogen. Total RNA was isolated using RNeasy Plant Mini Kits (Qiagen, www.qiagen.com). $7 \mu\text{g}$ was electrophoresed on a 1.2 % MOPS-agarose gel and transferred to Hybond N membrane (GE Healthcare, www.gehealthcare.com) using standard procedures (Ausubel et al. 1993). All subsequent steps were as described under 'DNA blot hybridization' above, except Denhardt's solution concentration in the hybridization buffer was 2X instead of 5X. The same RNA blot was sequentially hybridized with barley CDS probes of radiolabeled *CBF2A*, *COR14B*, *DHN5*, and *DHN8*, and subsequently with each of the *CBF* gene probes.

Real-time quantitative PCR (RT-qPCR)

RT-qPCR analysis of the early generation plants utilized RNA extracted using TRIzol Reagent (Invitrogen, www.invitrogen.com) and standard methods (Ausubel et al. 1993). RNA was treated with DNase using RQ1 RNase-Free DNase (Promega, www.promega.com). cDNA was produced using the OligodT₂₀ primer (Invitrogen, www.invitrogen.com) and the M-MLV Reverse Transcriptase kit (Promega, www.promega.com). For cDNA quality analysis, PCR amplification of the barley 'housekeeping' gene *cyclophilin* was carried out using $1 \mu\text{l}$ of a tenfold dilution of cDNA template as described (Dobbin et al. 2009). RT-qPCR analysis of the later generation plants utilized RNA extracted using the RNeasy

Plant Mini Kits (Qiagen, www.qiagen.com). These cDNA populations were generated using the QuantiTect Reverse Transcription kit (Qiagen, www.qiagen.com) following the manufacturer's instructions, including removal of genomic DNA.

RT-qPCR analysis of *CBF2*, *COR14b*, *DHN8*, and *DHN5* transcript levels was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, www.appliedbiosystems.com) as described (Burton et al. 2004). Values were normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The PCR product sizes, and forward (F) and reverse (R) primer sequences were: *CBF2* (128 bp. F:5'-CGGATCAAGTTGCAGGAGACGC-3'; R:5'-GTGCCGAGCCAGAGCCTGGAGTA-3'), *COR14B* (103 bp. F:5'-TTGAGGATGTGAGCAAATGAG-3'; R:5'-TACATCGTCAATGACGAGACC-3'), *DHN5* (106 bp. F:5'-TGGCGAAGTTCCACCGTATGC-3'; R:5'-ACGAAACTGTTGCCACACTG-3'), *DHN8* (144 bp. F:5'-GCTCCAGCTCCAGCTCGTCTA-3'; R:5'-CAGCCTCGTTGTCCTTGTGGCCG-3'), *GAPDH* (198 bp. F:5'-GTGAGGCTGGTGCTGATTA-3'; R:5'-CGTGGTGCAGCTAGCATTGAGAC-3'). The forward (F) and reverse (R) primer sequences and PCR product sizes used for analyses of *CBF2* and *DHN8* in the early generation plants differed from those used for the later generation plants and are as follows: *CBF2* (274 bp. F:5'-CCATCACCTCAAGCGACCTATCG-3'; R:5'-GCCTGACGCCTGGTGAAGAAG-3'), *DHN8* (278 bp. F:5'-GCTCCAGCTCCAGCTCGTCTA-3'; R:5'-CTTCTCCTCCTCGGCACTG-3'). The primer binding sites for both sets of *CBF2* primers are 100 % conserved across the three different *CBF2* paralogs (*CBF2B/A* in 'Golden Promise', *CBF2A* and *CBF2B* in 'Dicktoo' and thus are in regions 100 % identical between the endogenous and transgene *CBF2*. The first set of *CBF2* primers are in the region encoding a portion of the AP2 DNA binding domain, and the second set of primers are in the 3' region of the transcript. The primer binding sites for both *COR14B* and *DHN5* occur at the 3' end of the transcribed region of the gene and these sites are 100 % conserved across multiple cultivars including 'Aurea' (AJ512944), 'Georgie' (M60732), and 'Haruna nijo' (AK359508) in the instance of *COR14B*; and 'Dicktoo' (AF043096), 'Haruna nijo' (AK248391), 'Himalaya' (M95810), and 'Morex' (AF181455) in the instance of *DHN5*. The primer binding sites for both sets of *DHN8* primers are 100 % conserved across multiple cultivars including 'Dicktoo' (AF043093), 'Georgie' (X84056), 'Harun nijo' (AK252668), and 'Morex' (AF181458). The *DHN8* primer binding sites occur in the middle of the transcribed region of the gene and span the single intron. For the later generation of plants, qRT-PCRs were carried out in a final volume of 20 µl using 10 ng of cDNA, 10 nM each primer, and 10 µl of "Power Master Mix". Cycling was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, www.appliedbiosystems.com).

The specificity of the PCR was confirmed by sequencing and product melt curves were analyzed using standard procedures. Data was analyzed using Sequence Detection Software (v 1.4) (Applied Biosystems, www.appliedbiosystems.com) and Microsoft Excel (Microsoft, www.microsoft.com). Standard curves indicated linear amplification of the products between 1 pg and 1 ng of template and all qPCR reactions were performed with template concentrations within this range.

Samples of PCR products were sequenced to confirm products consisted only of that expected. A melt curve was obtained from the product following cycling by heating from 70 to 99 °C, which was also used to detect the presence of any non-specific sequences. Data was analyzed using Sequence Detection Software (v 1.4) (Applied Biosystems, www.appliedbiosystems.com) and Microsoft Excel (Microsoft, www.microsoft.com). A tenfold dilution series replicated three times indicated that *COR14B* amplification was linear ($R = 0.9967$) using between 1 pg and 10 ng of cDNA template per reaction. Amplification products of *CBF2* increased linearly using 1 pg to 1 ng template, and began to plateau at the higher concentration range. All qPCR reactions were carried out using this concentration range of template.

Morphological analyses

T₃ and T₄ generation plants were grown alongside 'Golden Promise' plants in a glasshouse with supplementary fluorescent lighting over an Australian spring season. Maximum/minimum temperatures were 28 °C/15 °C during the day/night. Plants were grown in 15 cm-diameter pots with one plant per pot.

Plants were photographed using an IXUS 70 digital camera (Canon, www.canon.com). Reported plant height is the distance from the soil to the tip of the tallest leaf. The 1,000 grain weight was calculated by weighing 100 randomly selected seed that were threshed from multiple spikes and then multiplying by ten. For plant weight, each mature plant's aerial portion (3 cm above soil surface) was harvested. Prior to weighing, the plant aerial tissue was dried at room temperature for 4 weeks and the spikes were dried at 37 °C for 1 week. The reported total plant biomass is the combined dry weights from aerial tissue and spikes. Yield is the number of grain from each plant.

Statistical analysis

Analyses of variance (ANOVAs) were performed using the general linear model procedure implemented in SAS v9.1 (SAS Institute Inc, Cary, NC). Statistical significance of survival scores at a given time point were determined by two independent mean separation tests using SAS v9.1: Duncan's multiple-range test and least square means

separation after a False Discovery Rate (Benjamini and Hochberg 1995) multi-test adjustment. In all cases identical results were obtained with both methods. Means followed by the same letter are not significantly different at $P < 0.05$.

Results

Generation and characterization of the Hv-*CBF2A* overexpressor lines

A construct encompassing the *CBF2A* coding sequence and flanking untranslated regions from the facultative barley ‘Dicktoo’ was placed under control of the CaMV 35S promoter and introduced into the spring barley ‘Golden Promise’ (Fig. 1a). Eighteen independent transgenic lines were recovered that were both hygromycin-resistant and β -glucuronidase (GUS) positive. Of these, six lines were selected for further evaluation based on transgene expression and fertility relative to the other lines.

To verify the six transgenic lines resulted from independent T-DNA insertion events, *Xho*I-digested DNAs of the transgenic lines, non-transgenic ‘Golden Promise’ and ‘Dicktoo’ were hybridized to the *hygromycin phosphotransferase* (*hph*) and β -glucuronidase (*uidA*) gene probes (Fig. 1b, c). The molecular weights of the fragments cross-hybridizing to the *hph* and *uidA* probes are expected to be different for each independent transformation event depending on the distance between the *Xho*I site in the ‘Golden Promise’ genome and the *Xho*I sites in the insert (Fig. 1a). Distinct hybridization banding patterns across the six transgenic lines confirmed that all six lines originated from independent transformation events (Fig. 1b, c). Multiple cross hybridizing fragments were detected with both probes, suggesting multiple inserts (Fig. 1b, c).

The same DNA blots were also hybridized with the *CBF2* coding sequence (CDS), and the region immediately downstream of the CDS, the latter of which served as a gene specific probe (Fig. 1d, e). These hybridizations confirmed the presence of the *CBF2A* transgene in each line (Fig. 1d, e). The signal intensity of the band cross-hybridizing to the transgene was higher than that cross-hybridizing to the endogenous gene (Fig. 1d), consistent with multiple copies of the transgene in each line. Fragments of the expected size were observed in five of the six lines. The banding pattern obtained with line 2 using the *CBF2* probes differed from that predicted (Fig. 1d, e). This unexpected banding pattern appeared to be due to a rearrangement because hybridization to *Bam*HI restricted DNAs, which should produce a single fragment via *Bam*HI sites internal to the construct, also differed from that predicted (Fig. 1f).

To verify the transgene was expressed and that expression was stably maintained through generations, Hv-*CBF2A* transformants were assayed for *CBF2* expression in a subset of early generation plants (T_2 and T_3) using qRT-PCR (Supplemental Fig. S1), and in latter generation plants (T_4 and T_5) using RNA blot hybridization (Fig. 2). In both generational sets, expression was assayed at normal warm growth temperatures and at cold temperatures for different lengths of time following the temperature decreases. With each set of plants *CBF2* levels were elevated above levels in ‘Golden Promise’ and ‘Dicktoo’ at warm and cold temperatures (Fig. 2, Supplemental Fig. S1). In the latter generation plants sampling at 2 h intervals over a 12 h time course and again at 30 and 54 h indicated that *CBF2* transcript levels remained relatively constant throughout the time course (Fig. 2). In comparison, *CBF2* in both ‘Golden Promise’ and ‘Dicktoo’ exhibited an increase in transcript levels relatively early in the time course that was followed by a decrease at latter time points (Fig. 2).

Taken together these data indicate that the six transgenic lines result from independent transformation events. Each line constitutively expresses the *CBF2A* transgene at levels higher than the non-transgenic ‘Golden Promise’ and ‘Dicktoo’. Additionally, transgene expression was stably maintained through generations.

Hv-*CBF2A* overexpression negatively affects plant development

Overexpression of *CBF* genes is often associated with detrimental side effects (Liu et al. 1998; Gilmour et al. 2000, 2004; Jaglo et al. 2001; Ito et al. 2006). This was also the case with many of the primary ‘Golden Promise’ transformants. Numerous T_0 lines were infertile while many of those that were fertile produced non-viable seed. To more thoroughly characterize the effect of *CBF2A* overexpression on several phenotypic characters and yield-associated traits, six of the Hv-*CBF2A* overexpressing lines were grown to maturity alongside ‘Golden Promise’, and a set of phenotypic characters were measured during their life cycle. Figure 3a shows the expression levels of the Hv-*CBF2A* transgene in seedlings of the same group of plants phenotyped (Fig. 3b). Relative to ‘Golden Promise’ the Hv-*CBF2A* overexpressors showed varying degrees of stunted growth (Fig. 3b, c). The final plant height of lines 2, 3, 6, and 15 was significantly less than ‘Golden Promise’ ($P < 0.0001$) (Fig. 3b, c). Visually, lines 10 and 13 also exhibited shorter stature compared to ‘Golden Promise’, although this difference was not significant (Fig. 3b, c). Total biomass and total grain weight produced by each Hv-*CBF2A* overexpressor line was significantly less than that of ‘Golden Promise’ ($P < 0.0001$) (Fig. 3d, e). Many of

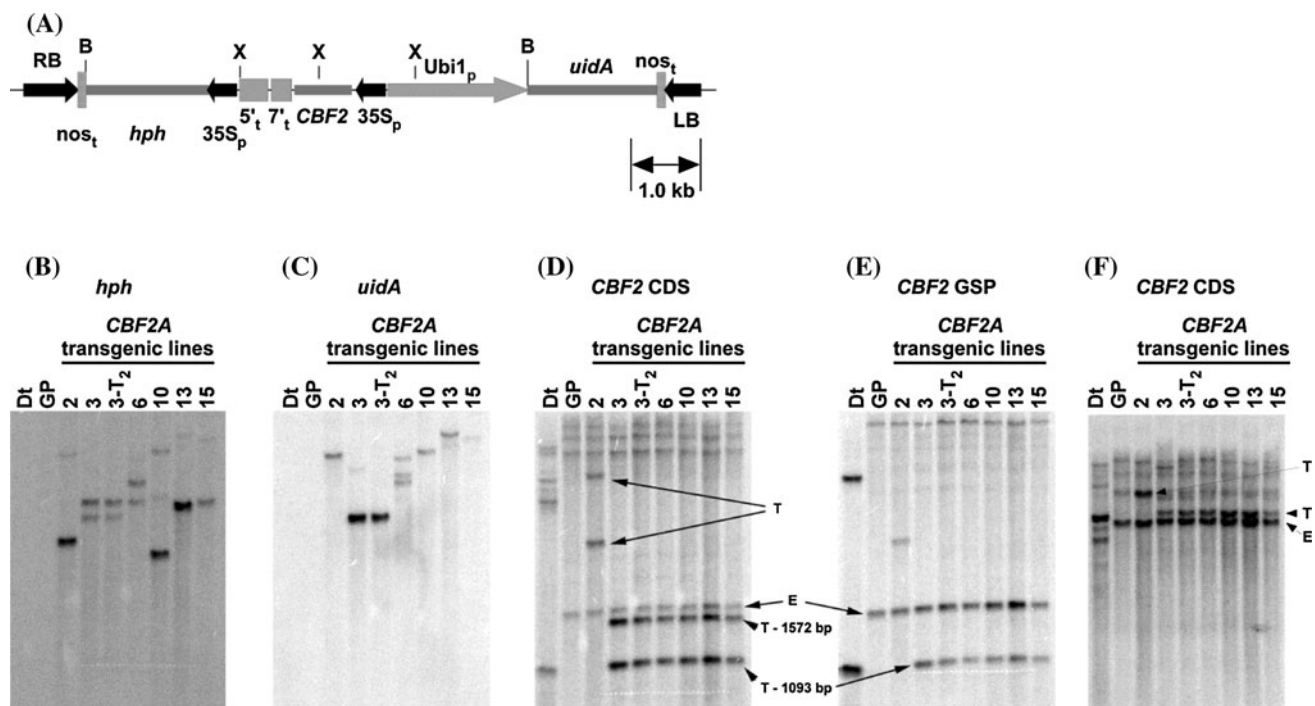


Fig. 1 DNA blot hybridization indicates *CBF2A* overexpressing lines result from independent transformation events. **a** Map of the construct used for transformation shows the region between the *right* and *left* borders of pWBVec10a binary vector. *Bam*HI, *B*, and *Xho*I sites, *X*, are identified above the map. **b** *hph* (*hygromycin phosphotransferase*), **c** β -*glucuronidase* (*GUS*), **d**, **f** *CBF2A* coding sequence (CDS), and **e** *CBF2A* 3' gene-specific probe (GSP) radiolabeled DNA probes were hybridized to *Xho*I (**a–e**) and *Bam*HI (**f**)-digested DNAs of 'Dicktoo'

(Dt), 'Golden Promise' (GP), and *CBF2A*-transformed lines in the 'Golden Promise' background. Arrows marked by *E* identify the endogenous *CBF2* in 'Golden Promise', arrowheads marked by *T* identify the predicted *CBF2A* transgene cross-hybridizing fragments, and arrows marked by *T* identify the unexpected MW *CBF2A* transgene cross-hybridizing fragments in line 2. Transgenic lines 2, 3, 6, 10, and 13 were T₄ generation plants, Line 15 was T₃, and Line 3-T₂ was T₂

the spikes produced on the Hv-*CBF2A* overexpressors were shorter with fewer grains, resulting in lower seed yield (Fig. 3f). Taken together this data indicates that Hv-*CBF2A* overexpression has a negative effect on growth and developmental processes at normal temperature. While it is possible that these aberrant phenotypes are the result of the transformation and regeneration processes; that similar aberrant phenotypes occur in other plant species transformed with *CBF* genes suggests this is probably not the case.

Overexpression of Hv-*CBF2A* enhances freezing tolerance upon cold acclimation

Given the role of CBFs in affecting freezing tolerance, a central question was whether the Hv-*CBF2A* overexpressors were altered in the capacity to survive freezing temperatures. To address this question we assayed the freezing tolerance levels of several Hv-*CBF2A* overexpressor lines alongside non-transformed 'Golden Promise' and 'Dicktoo'. Freezing tolerance levels were assayed by quantifying survival percentages of both whole plants and excised crowns subjected to -3 and -6 °C, and by measuring LT₅₀

values of crowns excised from cold acclimated and non-acclimated plants.

Using the whole plant freezing assays, none of the plants regrew following freezing to -6 °C. Freezing to -3 °C produced differences in regrowth; the percentages of plants resuming growth after freezing to -3 °C were 14 % for 'Golden Promise', 95 % for 'Dicktoo', and 50–75 % for the four Hv-*CBF2A*-overexpressing lines 2, 10, 13, and 15 (Fig. 4).

In separate experiments using excised crowns, survival of lines 2 and 10 alongside 'Golden Promise' and 'Dicktoo' was assayed following freezing to -3 °C (Fig. 5a) and -6 °C (Fig. 5b). In these experiments none of the crowns excised from any of the non-acclimated plants (0 h time point) resumed growth following freezing to -3 and -6 °C (Fig. 5a, b). In comparison, crowns excised from the cold acclimated plants resumed growth, and the regrowth percentage increased with increasing lengths of cold acclimation (Fig. 5a, b). Following freezing to -3 °C, the regrowth percentage of excised crowns from plants cold-acclimated for 4 days was about 65 % for 'Golden Promise' and 85–100 % for the two Hv-*CBF2*-overexpressing lines and 'Dicktoo' (Fig. 5a). Regrowth

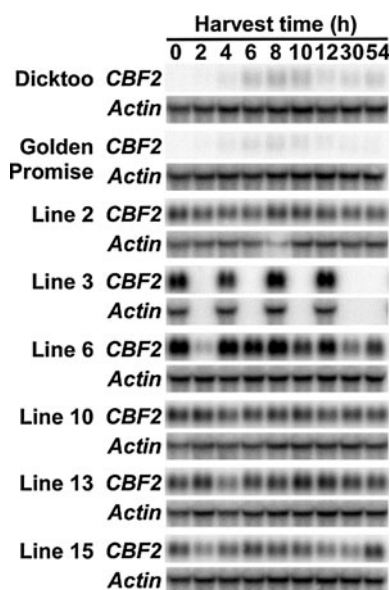


Fig. 2 *CBF2* and *Actin* expression profiles assayed using RNA blot hybridization of ‘Dicktoo’, ‘Golden Promise’, and the Hv-*CBF2A* transformants following a temperature decrease. Plants were grown for 3 weeks under short day (8 h light/16 h dark) at 18 °C. After 3 weeks growth the growth chamber temperature was decreased to 6 °C at subjective daybreak. Following the temperature decrease plants were harvested at 2 h intervals over the first 12 h, and again at 30 and 54 h (6 h into the subjective day each day). The 0 h time point was made at 18 °C, immediately preceding the subjective daybreak and temperature decrease. *CBF2* and *Actin* were hybridized in succession to the same filter. (limited seed numbers of line 3 precluded assaying at every time point)

percentages of excised crowns from all genotypes including ‘Golden Promise’ approached 100 % when the plants had been cold acclimated for 1 week (Fig. 5a). Following freezing to −6 °C the regrowth percentage of excised crowns from plants cold-acclimated for 1 week was approximately 80 % for lines 2 and 10, and ‘Dicktoo’, whereas excised crowns from ‘Golden Promise’ did not resume growth (Fig. 5b). After 2 weeks of cold acclimation these percentages increased to approximately 100 % for the two Hv-*CBF2A* overexpressors and ‘Dicktoo’, while ‘Golden Promise’ increased to approximately 30 % (Fig. 5b). After 4 weeks cold acclimation 100 % of ‘Golden Promise’ excised crowns also exhibited regrowth (Fig. 5b).

Determining the temperature at which 50 % of the excised crowns failed to regrow (LT_{50}) indicated that at 1 week of cold acclimation the LT_{50} of lines 2 and 10, and ‘Dicktoo’ were about −7 °C, and the LT_{50} of ‘Golden Promise’ was about −4 °C (Fig. 5c). The LT_{50} values remained relatively unchanged for lines 2 and 10, and ‘Dicktoo’ after 2 weeks of cold acclimation while ‘Golden Promise’ increased to about −5 °C (Fig. 5c). After 4 weeks of cold acclimation the LT_{50} of line 2 was −7 °C while that of line 10, ‘Golden Promise’, and ‘Dicktoo’ were all approximately −8 °C (Fig. 5c).

These experiments indicate overexpression of Hv-*CBF2A* in the spring barley ‘Golden Promise’ increases the ability of isolated ‘Golden Promise’ crowns to regrow following exposure to freezing temperatures to levels similar to those of the facultative barley ‘Dicktoo’. These data also indicate that a period of cold acclimation is necessary to impart this ability for regrowth.

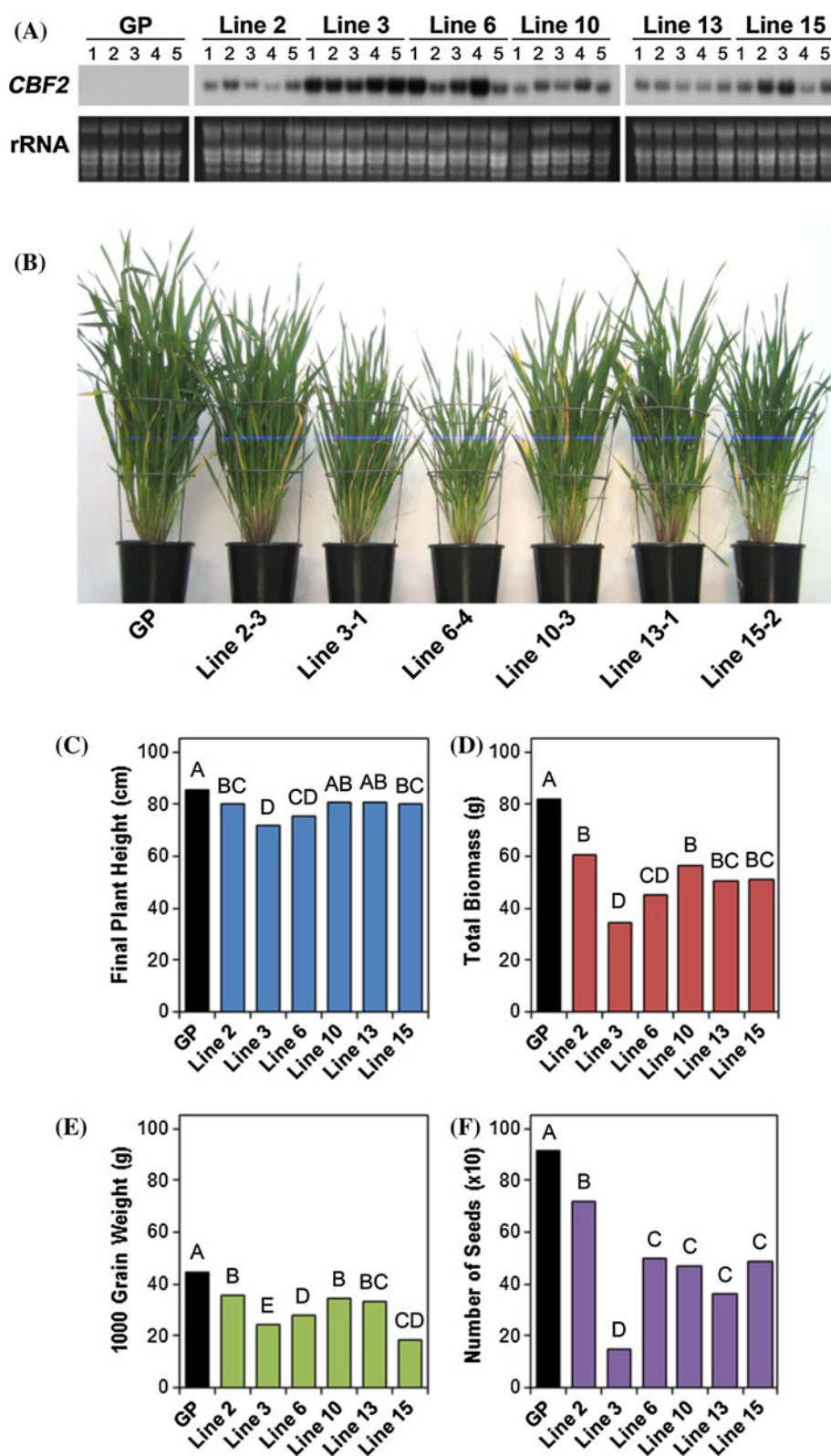
Hv-*CBF2A* overexpression accelerates *COR* and *DHN* transcript accumulation at low temperature

Increases in freezing tolerance during cold acclimation are typically associated with changes in the transcriptome, and mediating a significant portion of the transcriptome changes are the CBFs (van Buskirk and Thomashow 2006). Many of the changes that occur during cold acclimation can be induced at normal warm growth temperatures by constitutive overexpression of the CBFs (van Buskirk and Thomashow 2006). Given the requirement for cold acclimation to impart freezing tolerance to the Hv-*CBF2A* overexpressors we asked how genes predicted to be in the CBF pathway responded during cold acclimation. To do this we hybridized the same RNA filters used to assay the Hv-*CBF2A* overexpressors with *COR14B*, *DHN5*, and *DHN8* and carried out qRT-PCR for the same set of genes using the early generation plants.

Expression analyses indicated that there were increased levels of *COR14B*, *DHN5*, and *DHN8* in the Hv-*CBF2A* overexpressor lines at warm temperatures (Fig. 6b, c, Supplemental Fig. S1). However using RNA blot hybridization *COR14B* was not detectable in the Hv-*CBF2A* overexpressors at warm temperatures, and only a weak signal for *DHN5* was detected in the Hv-*CBF2A* overexpressor lines (Fig. 6a). Assaying this same RNA sample using RT-PCR indicated that in the Hv-*CBF2A* overexpressors *COR14B* transcript levels were about 100-fold higher than in ‘Golden Promise’ (C_T values 0.000292 vs. 0.000003, respectively) and *DHN5* transcript levels were about 20-fold higher (C_T values 0.0959297 vs. 0.005194, respectively, and Fig. 6c inset). *DHN8* transcript signals were detectable by RNA blot hybridization in both the Hv-*CBF2A* overexpressors and ‘Golden Promise’ and were 1.4-fold higher in the Hv-*CBF2A* overexpressors (Fig. 6a–c). This data indicates that at warm temperatures the *COR14B* and *DHN5* transcript levels in the Hv-*CBF2A* overexpressors and ‘Golden Promise’ were beyond the limit of detection using RNA blot hybridization.

As the time course proceeded *COR14B*, *DHN5*, and *DHN8* transcripts were detected at measurably higher levels in the Hv-*CBF2A* overexpressors relative to ‘Golden Promise’ (Fig. 6a–c). In the Hv-*CBF2A* overexpressors *COR14B*, *DHN5* and *DHN8* transcript levels were approximately 35-fold, fivefold, and 2.5-fold higher, respectively at the 54 h time point relative to their levels at the 6 h time

Fig. 3 *CBF2A* expression levels and trait comparison between ‘Golden Promise’ (GP) and the Hv-*CBF2A* overexpressing barley lines. Plants were grown at 28 °C day/15 °C night in a glasshouse with supplementary fluorescent lighting over an Australian spring season. **a** *CBF2A* transcript levels of five plants per line, as determined by RNA gel blot hybridization. **b** Side view showing a representative plant of each Hv-*CBF2A* overexpressing line nearing maturity (line numbers correspond to lines shown in part b); **c** average values of plant height (to tallest extended leaf). **d** Biomass (aerial portion including spikes). **e** 1,000 Grain weight (calculated from 100 grain weight). **f** Grain yield. T₄ generation plants were used for lines 2, 3, 6, 10, and 15, and T₅ generation plants for line 13. Bars with the same letter are not significantly different between genotypes at $P < 0.05$ by Duncan’s multiple range test



point. In comparison, in ‘Golden Promise’ *COR14B*, *DHN5* and *DHN8* transcript levels were about 14-fold, 2.3-fold, and 1.4-fold higher, respectively relative to their levels at the 6 h time point. This greater magnitude of increase at the 54 h

time point in the Hv-*CBF2A* overexpressors appeared to be due to continued increases in *COR14* and *DHN* levels combined with a reduction of their levels in ‘Golden Promise’; i.e., in ‘Golden Promise’ *COR14B*, *DHN5*, and *DHN8*

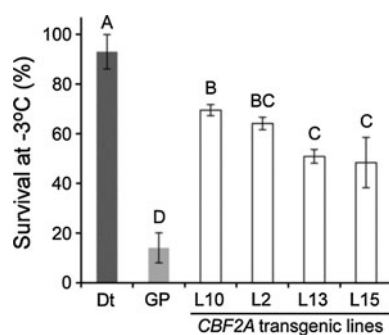


Fig. 4 Survival percentages of whole plants following freezing. Seedlings of ‘Dicktoo’ (Dt), ‘Golden Promise’ (GP) and four Hv-*CBF2A* overexpressing lines (L) in the ‘Golden Promise’ background were grown in wooden boxes at a density of approximately 180 plants per $\sim 1,000$ cm². The growth chamber was cooled from the normal growth temperature of 18 °C to the target freezing temperature of -3 °C at the rate of 0.5 °C h⁻¹. Target temperature was held for 24 h before allowing the growth chamber to return to normal temperatures. Error bars denote standard error. Bars with the same letter are not significantly different between genotypes at $P < 0.05$ by Duncan’s multiple range test

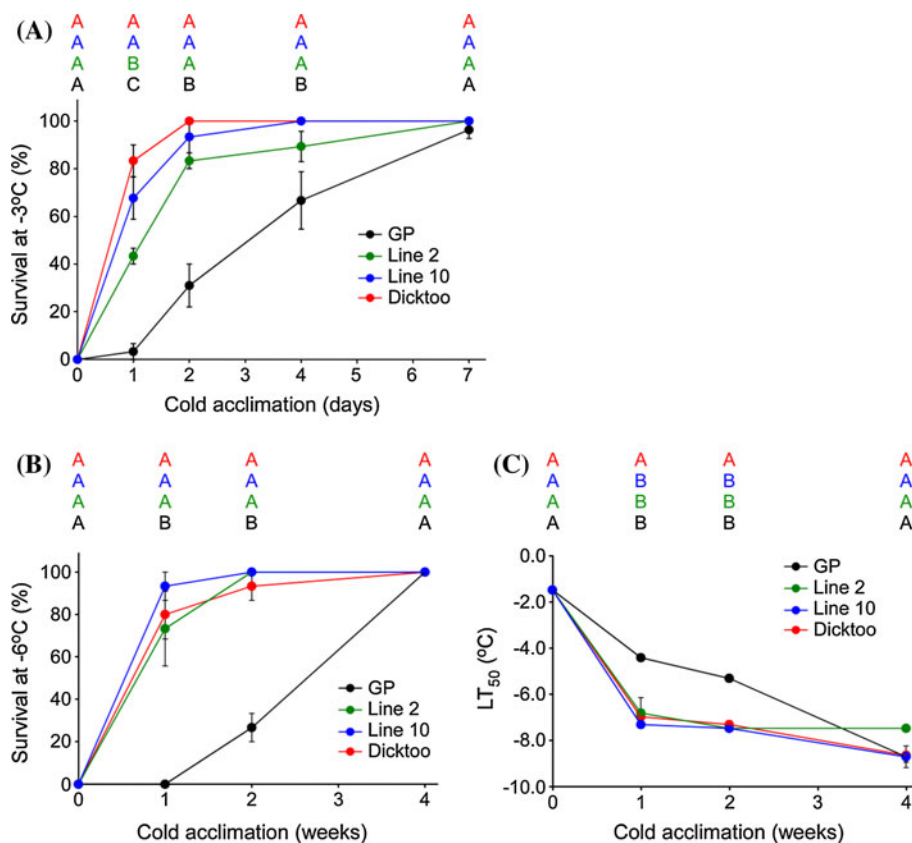
transcript levels relative to *actin* exhibited a peak at 30 h that tapered off at the 54 h time point whereas these transcript continued to increase in the Hv-*CBF2* overexpressors at the 54 h time point (Fig. 6c). *COR14B*, *DHN5*, and *DHN8* transcript levels were also higher in the Hv-*CBF2A*

overexpressing lines than in ‘Dicktoo’ (Fig. 6b). Taken together these data suggest Hv-*CBF2A* overexpression induces expression of *COR14B*, *DHN5*, and *DHN8* at normal growth temperatures and that cold temperature has a pronounced stabilizing effect upon their transcript levels.

Overexpression of Hv-*CBF2A* upregulates other *FR-H2* *CBF* genes

Sequencing the genomic regions encompassing the *CBF* genes at *FR-H2* revealed many of the *CBF* promoter regions have CRT/DRE motifs (Miller et al. 2006; Knox et al. 2010, Table 1). These data suggested *CBF* genes at *FR-H2* harboring CRT/DRE motifs might also be targets of the CBFs. To test for this possibility, relative levels of *CBF4*, *CBF9*, *CBF12*, *CBF14*, *CBF15*, and *CBF16* were also assayed in the Hv-*CBF2A* overexpressors. Carrying out RNA blot hybridizations with this set of probes indicated that in each Hv-*CBF2A* overexpressors line *CBF12*, *CBF15*, and *CBF16* transcripts were elevated above levels in ‘Golden Promise’ at all assayed time points (Fig. 6d, e). This was most pronounced for *CBF12*, followed by *CBF16* and *CBF15*. Over the time course *CBF12*, *CBF15*, and *CBF16* averaged 3.0-, 1.5-, and 2.2-fold higher, respectively, in the Hv-*CBF2A* overexpressors over ‘Golden Promise’ (Fig. 6e). The expression pattern of these three

Fig. 5 Survival percentages and LT₅₀ values of excised crowns following freezing in relation to different lengths of cold acclimation. **a** Survival after freezing to -3 °C for plants cold-acclimated 1–7 days. **b** Survival after freezing to -6 °C for plants cold-acclimated 1–4 weeks. **c** LT₅₀ values for plants cold-acclimated 1–4 weeks. Plants were grown under LD. Cold acclimation was at 3 °C (± 1 °C). Excised crowns from cold acclimated and non-cold-acclimated plants were frozen to the target temperatures at the rate of 2 °C h⁻¹. Values represent the mean \pm standard error from three independent experiments. (Standard error bars do not resolve from data points on several samples.) At a given day of cold acclimation, means marked with the same letter are not significantly different between genotypes at $P < 0.05$ by Duncan’s multiple range test



CBFs in the overexpressor lines was however similar to that in ‘Golden Promise’ in that *CBF* transcript levels rapidly increased following the temperature decrease, peaked at 8–12 h, and then decreased at the 30 h time point (Fig. 6d, e). Relative levels of *CBF9* were also slightly increased over that of ‘Golden Promise’, but this was less striking (Fig. 6d). Transcript levels of *CBF4* and *CBF14* over the time course were about the same in the Hv-*CBF2A* overexpressors as in ‘Golden Promise’. Among all the overexpressor lines, line 2 was the most consistent in having higher transcript levels of all *CBFs* at the different time points (Fig. 6d).

Discussion

An objective of this study was to further our mechanistic understanding of HvCBF4/CBFIV *CBFs* and the role they play in cold acclimation and freezing tolerance. Our data indicate that overexpression of Hv-*CBF2A* in spring barley ‘Golden Promise’ results in an accelerated acquisition of freezing tolerance and that the freezing tolerance of ‘Golden Promise’ increases to levels comparable to those of the winter hardy line ‘Dicktoo’. However, this increase in freezing tolerance occurs only after cold acclimation. After 1 day of cold acclimation the Hv-*CBF2A*

overexpressors and ‘Dicktoo’ exhibited about 50 % of their maximum freezing tolerance when plants were frozen to -3°C whereas ‘Golden Promise’ exhibited little to no increase in freezing tolerance at this same time point when frozen to -3°C . Similarly, when frozen to -6°C the Hv-*CBF2A* overexpressors and ‘Dicktoo’ exhibited near maximal freezing tolerance levels after 1 week of cold acclimation whereas ‘Golden Promise’ exhibited no freezing tolerance at the same time point. After 2 weeks of cold acclimation ‘Golden Promise’ was still only about 25 % of its maximal freezing tolerance. At 4 weeks ‘Golden Promise’ attained the same level of freezing tolerance. Measurements of the LT_{50} indicate the target freezing temperatures of -3°C and -6°C were probably not low enough to discriminate the Hv-*CBF2A* overexpressors and ‘Dicktoo’ from ‘Golden Promise’ at the latter time points in cold acclimation. Although most winter barleys exhibit greater freezing tolerance than -8°C , the LT_{50} of -8°C exhibited by ‘Dicktoo’ is about the maximum this genotype attains when grown and cold acclimated under the conditions we used in these experiments (Limin et al. 2007).

Cold temperatures also had a pronounced effect on *COR14B* and *DHN5* transcript levels in the Hv-*CBF2A* overexpressors. While *COR14B* and *DHN5* were induced at warm temperatures in the Hv-*CBF2A* overexpressors, cold temperatures led to substantially greater increases in their levels in the Hv-*CBF2A* overexpressors in comparison to their levels induced by cold temperatures in the non-transformed ‘Golden Promise’ plants. In comparison, *DHN8* was detected using RNA blot hybridization analysis in both the non-acclimated Hv-*CBF2A* overexpressors and non-acclimated ‘Golden Promise’ plants but its transcripts did not exhibit as dramatic a differential between the Hv-*CBF2A* overexpressors and the non-transformed ‘Golden Promise’ plants during the low temperature time course.

Transcript levels of other *CBF* genes at *FR-H2* were also increased in the Hv-*CBF2A* overexpressors. *CBF12* transcripts were about threefold higher in the Hv-*CBF2A* overexpressors compared to ‘Golden Promise’ and this differential increase remained nearly constant throughout the entire low temperature time course. The *CBFs* harboring fewer CRT/DRE motifs, including *CBF9*, *CBF15*, and *CBF16* exhibited more variable transcript levels, which were nonetheless elevated above those of ‘Golden Promise’. *CBF4* and *CBF14*, which do not harbor CRT/DRE motifs in their upstream regions, did not exhibit increased levels in the Hv-*CBF2A* overexpressors. Despite the increased levels of these other *FR-H2* *CBF* transcripts in the Hv-*CBF2A* overexpressors, these genes still exhibited a low-temperature-responsive expression profile that is characteristic of the *CBFs*; i.e., *CBF12*, *CBF16*, and *CBF15* transcript levels increased and then decreased over

Table 1 Number of CRT/DRE motifs in a 1,250 bp region upstream of the ATG in barley *CBF*, *COR*, and *DHN* genes

<i>CBF</i>	No. of CRT/DRE motifs
<i>CBF2A</i>	0
<i>CBF2B</i>	0
<i>CBF3</i>	0
<i>CBF4B</i>	0
<i>CBF6</i>	1
<i>CBF9</i>	1
<i>CBF10A</i>	1
<i>CBF10B</i>	7
<i>CBF12A</i>	5
<i>CBF12B</i>	5
<i>CBF13</i>	0
<i>CBF14</i>	0
<i>CBF15A</i>	1
<i>CBF15B</i>	1
<i>CBF16</i>	3
<i>COR14B</i>	3
<i>DHN5</i>	3
<i>DHN8</i>	10

Numbers are based on cultivar ‘Dicktoo’ (Choi et al. 1999; Knox et al. 2010) except *COR14B*, which is from barley cv. ‘Aurea’ (Dal Bosco et al. 2003)

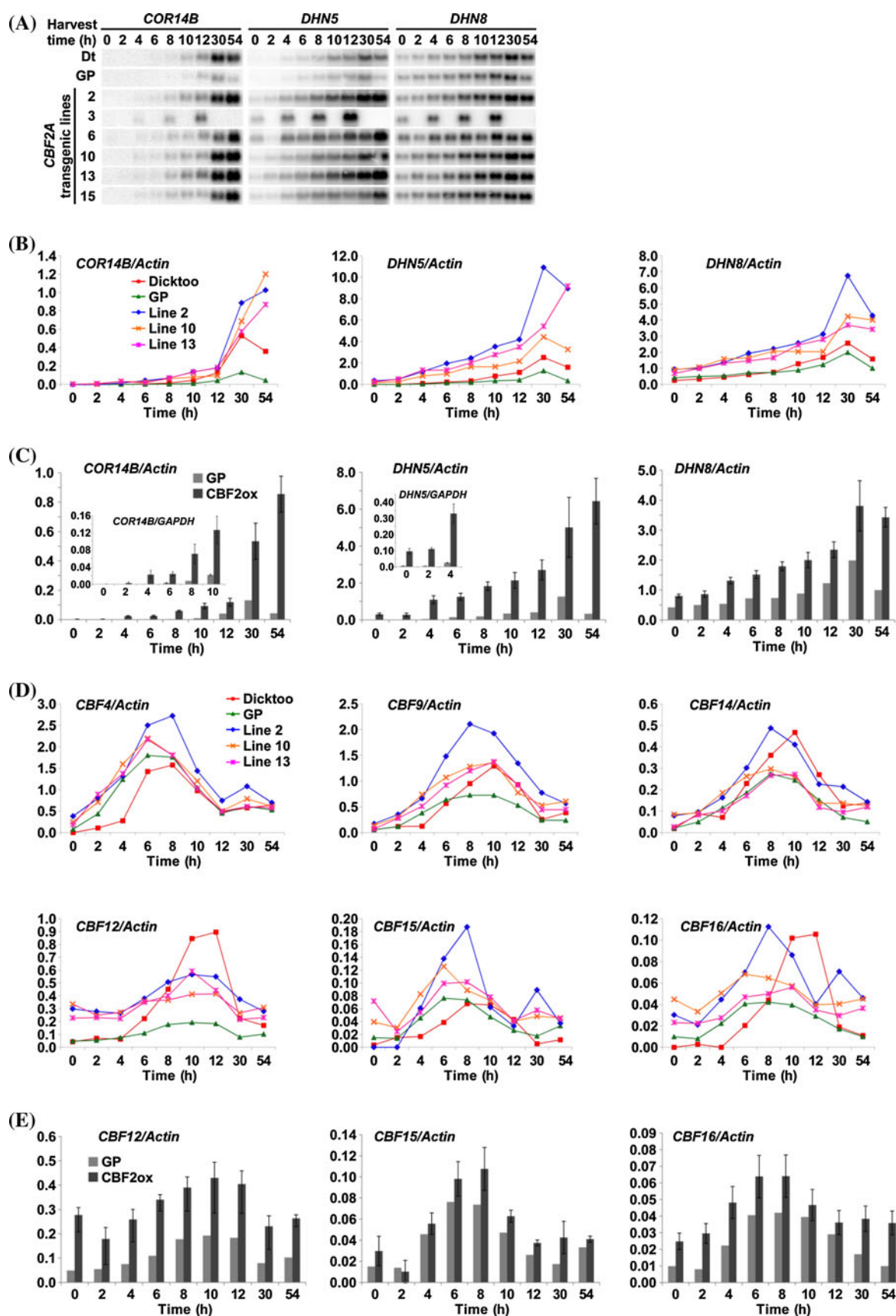


Fig. 6 *CBF* and *COR* expression profiles of ‘Dicktoo’, ‘Golden Promise’, and the Hv-*CBF2A* transformants following a temperature decrease. **a** RNA blot hybridization images. Probes were hybridized to the same RNA filters used to assay the Hv-*CBF2A* overexpressors and shown in Fig. 2. **b** *COR14B*, *DHN5*, and *DHN8* transcript signals normalized to *actin* (y-axis). **c** *COR14B*, *DHN5*, and *DHN8* in ‘Golden Promise’ relative to the mean of Hv-*CBF2A* overexpressor lines 2, 6, 10, 13, and 15 (y-axis). RT-qPCR data for *COR14B* and *DHN5* (insets) are threshold cycle (C_T) values normalized relative to *GAPDH* C_T values using the ΔC_T method. The same RNA samples used for RNA blot hybridization were used for generating the cDNA (1 μ g total RNA per cDNA synthesis reaction). **d** *CBF4*, *CBF9*, *CBF14*, *CBF12*, *CBF15*, and *CBF16* transcript signals normalized to *actin* (y-axis). **e** *CBF12*, *CBF15*, and *CBF16* in ‘Golden Promise’ relative to the mean of Hv-*CBF2A* overexpressor lines 2, 6, 10, 13, and 15 (y-axis)

the 12 h time course in both ‘Golden Promise’ and the Hv-*CBF2A* overexpressors. In the Hv-*CBF2A* overexpressors these *CBFs*—and *CBF12* in particular—were incrementally increased above levels in ‘Golden Promise’. Based on findings in *Arabidopsis* the cold response pathway appears to be affected by a cold-sensing mechanism responding to a temperature decrease that is then desensitized during continued exposure to a constant temperature (Zarka et al. 2003). Thus the finding that *CBF12* levels in the Hv-*CBF2A* overexpressors is additive onto levels in ‘Golden Promise’ throughout the time course, suggests the Hv-*CBF2A*-mediated increase in *CBF12* levels is independent of that of the normal endogenous cold response pathway.

The data from these experiments does not entirely resolve the question of whether the DNA binding and transcriptional activation activities of the CBF2 protein in barley plants are fully active, or whether these activities are increased by cold temperatures. The much greater increase in *COR14B* and *DHN5* transcripts during the low temperature time course in the Hv-*CBF2A* overexpressors than in ‘Golden Promise’ might be due to greater DNA binding or transcriptional activation activity, or both, of the overexpressed Hv-*CBF2A* protein under cold conditions. This would be consistent with the increase in DNA binding that occurs in vitro with recombinant HvCBF4/CBFIV subgroup proteins (Xue 2003; Skinner et al. 2005). However the parallel in *CBF12* transcript levels between the Hv-*CBF2A* overexpressors and ‘Golden Promise’ during the low temperature time course suggests the activity of the overexpressed CBF2 protein is not altered by cold temperatures. That the Hv-*CBF2A* overexpressing plants exhibited aberrant growth and development phenotypes at normal growth temperature is also consistent with activity arising from the overexpressed Hv-*CBF2A* construct at the normal growth temperatures. One possibility is that cold temperatures stabilize *COR14B* and *DHN5* transcripts but have no effect on *CBF12* transcripts. Directly addressing whether CBF2 protein binding to target sites in vivo is altered by temperature requires a means to assay bound and

unbound protein in the cell. Such experimentation may be possible using a robust anti-CBF2 antibody that recognizes epitopes specific to CBF2 or an epitope-tagged recombinant CBF2, and chromatin immunoprecipitation. If the activity of the CBF2 protein in the plant is not altered by cold temperatures, the increase in the activity of the *E. coli*-produced recombinant proteins detected in vitro (Xue 2003; Skinner et al. 2005) could be due to increased solubility, reduced misfolding, and reduced aggregation, all of which occur because the hydrophobic interactions contributing to these phenomena are weakened at colder temperatures (Baneyx and Mujacic 2004; Vera et al. 2007).

While *CBF2* transcript levels in the Hv-*CBF2A* overexpressors were very high there was disconnect between its levels and those of the presumed CBF target genes. Transcript levels of *CBF2* were more than 1,000-fold higher in the Hv-*CBF2* overexpressors than they were in either ‘Golden Promise’ or ‘Dicktoo’ (Supplemental Fig. S1). However *CBF12* exhibited only about threefold higher transcript levels in the Hv-*CBF2* overexpressors. The transcript levels of *COR* and *DHN* genes in the Hv-*CBF2A* overexpressors also did not parallel the 1,000-fold higher *CBF2* levels in these plants. This disconnect may be due to an upper limit on the amount of CBF protein in the cell controlled through post-transcriptional mechanisms, or the activity of CBF protein controlled through post-translational mechanisms, or a combination of both types of mechanisms. Again a robust anti-CBF2 antibody would aid addressing protein levels.

There were also growth and development problems resulting from Hv-*CBF2A* overexpression, as is typical when the *CBFs* are overexpressed to high levels (Liu et al. 1998; Gilmour et al. 2000). These phenotypes may in part be due to intersection of the CBF and Gibberellic acid pathways because overexpression of *CBF1* increases levels of DELLA proteins, which are involved in growth arrest (Achard et al. 2008). These phenotypes may also be the result of squelching, in which the CBF activation domain interacts with and sequesters other transcription factors, effectively pulling them away from their normal transcriptional activities (Gill and Ptashne 1988; Levine and Manley 1989). Overexpression of a fusion construct between the CBF1 COOH activation domain and the yeast GAL4 DNA binding domain also causes severe growth stunting (Wang et al. 2005). *COR* genes are not induced in the GAL4_{DBD}/CBF1_{AD} overexpressing plants, presumably because the GAL4_{DBD} does not tether the CBF1 activating region to the CRT/DRE (Wang et al. 2005). Because the growth stunting phenotype is alleviated when clusters of hydrophobic residues in the CBF activation domain are altered to alanine and the activating capacity of the fusion construct is abolished, the activating function appears to play a role in the growth stunting (Wang et al. 2005). To

circumvent the growth related defects resulting from constitutive high level *CBF* overexpression, the use of a stress-inducible promoter such as the *rd29A* promoter, which has been used in other systems (Kasuga et al. 1999; Pino et al. 2007), may be one means. Alternatively, the use of a weak promoter such as the NOS promoter (Sanders et al. 1987; Horstmann et al. 2004) may also provide a sufficient increase in transcript levels to effect an increase in regrowth following freezing without causing penalty.

During the revision of this manuscript Soltész and colleagues published findings from their work in which they overexpressed wheat *CBF14* and *CBF15* in ‘Golden Promise’ barley (Soltész et al. 2013). Ta-*CBF14*, like Hv-*CBF2*, is an HvCBF4/CBFIV CBF whereas Ta-*CBF15* is an HvCBF3/CBFIII CBF. In some aspects the findings of the two studies are similar, and in other aspects the findings seemingly are different. As was the case with our Hv-*CBF2A* overexpressing lines, most of the Ta-*CBF14* and Ta-*CBF15* overexpressing lines exhibited growth stunting and delayed development relative to ‘Golden Promise’ (Soltész et al. 2013). In plants that were grown in wooden boxes and had been cold acclimated, increases in plant survival following freezing were detected in independent lines overexpressing each of the two *CBFs* (Soltész et al. 2013). Under non-acclimating conditions two of eleven Ta-*CBF14* and Ta-*CBF15* overexpressing lines exhibited survival percentages that were significantly greater than that of ‘Golden Promise’ (Soltész et al. 2013). This result is similar to our results obtained in the freezing experiments carried out using whole plants grown in the wooden boxes, in which the non-acclimated Hv-*CBF2A* plants exhibited survival percentages greater than ‘Golden Promise’ when the temperature of the growth chamber was decreased by $0.5\text{ }^{\circ}\text{C h}^{-1}$. However our experiments with crowns isolated from non-acclimated plants showed no difference with ‘Golden Promise’. The contrast in results between the isolated crowns and whole plants in wooden boxes may be an indication that during the 42 h the plants in the wooden boxes were cooled from the $18\text{ }^{\circ}\text{C}$ growth temperature to the target freezing temperature of $-3\text{ }^{\circ}\text{C}$, they cold acclimated. These data are consistent with higher transcript levels of *CBFs* at warm temperatures being insufficient for increased freezing tolerance; rather higher levels in combination with cold temperatures are required.

Another area where the results of the two studies seemingly contrast is in the levels of *COR14B* transcripts detected. Whereas 1 day following cold temperature exposure both our Hv-*CBF2A* overexpressors and the Ta-*CBF14* and Ta-*CBF15* overexpressors had high levels of *COR14B*, in the non-acclimated plants the Ta-*CBF14* and Ta-*CBF15* overexpressing lines also exhibited high *COR14B* transcript levels (Soltész et al. 2013). The differences in *COR14B* transcript levels between the two sets of experiments in the

non-acclimated plants may be due more to differences in the experimental conditions used to assay gene expression rather than choice of which *CBF* gene is overexpressed. Our gene expression analyses were carried out using seedlings grown at a constant $18\text{ }^{\circ}\text{C}$ under an 8 h light/16 h dark photoperiod regimen. In comparison, the Ta-*CBF14* and Ta-*CBF15* overexpressing lines were grown using a $17/13\text{ }^{\circ}\text{C}$ day/night temperature differential under a 16 h light/8 h dark photoperiod regimen. Growth conditions very similar to the latter result in high *COR14B* transcript levels in freezing-tolerant lines and undetectable levels in freezing-sensitive lines, whereas no difference in *COR14B* accumulation occurs when plants are grown under a $25/20\text{ }^{\circ}\text{C}$ day/night temperature differential (Vágújfalvi et al. 2000, 2003). One possibility is that this temperature differential and the night time temperature in particular is having a large positive affect upon *COR14B* transcript accumulation in the Ta-*CBF14* and Ta-*CBF15* overexpressors whereas the constant $18\text{ }^{\circ}\text{C}$ used for gene expression analyses of the Hv-*CBF2A* overexpressing lines is not conducive for *COR14B* transcripts accumulation.

As relatively high levels of *CBF* transcripts are detected at warm temperatures in winter genotypes (Xue 2003; Kobayashi et al. 2005; Stockinger et al. 2007; Campoli et al. 2009), what the Hv-*CBF2A* overexpressing lines suggest is that these higher levels of *CBF* transcripts at the normal growth temperatures in winter genotypes may make possible for a more immediate and higher accumulation of *COR* gene transcripts upon exposure to cold temperatures. This suggests cold temperatures are having an effect downstream of *CBF* transcript production and that as long as *CBF* transcripts are produced plants can still cold acclimate. Drawing analogy to a field setting, plants should in essence still have the capacity to reacclimate when they deacclimate following a mid-winter thaw as long as *CBF* transcripts continue to be present. Identifying the factors that alter *CBF* expression during the growth phase transition that occurs in winter genotypes should enable greater precision in breeding for winter survival.

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Conflict of interest The authors declare that they have no conflict of interest.

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