Role of CBF transcription factors during long-term acclimation to high light and low temperature in two ecotypes of Arabidopsis thaliana

christopher baker[1], Jared Stewart[2], Cynthia Amstutz[1], Jeffrey Johnson[1], Lindsey Ching[1], Krishna Niyogi[1], William W. Adams[3], and Barbara Demmig-Adams[3]

[1]University of California Berkeley
[2]USDA-ARS Plains Area
[3]University of Colorado Boulder

September 24, 2021

Abstract

When grown under cool temperature, winter annuals upregulate photosynthetic capacity as well as freezing tolerance. Here, the role of three cold-induced C-repeat-Binding Factor (CBF1–3) transcription factors in photosynthetic upregulation and freezing tolerance was examined in two Arabidopsis thaliana ecotypes originating from Italy (IT) or Sweden (SW), and their corresponding CBF1–3-deficient mutant lines it:cbf123 and sw:cbf123. Photosynthetic, morphological, and freezing-tolerance phenotypes as well as gene expression profiles were characterized in plants grown from seedling stage under different combinations of light level and temperature. Under high light and cool growth temperature (HLC), a greater role of CBF1–3 in IT versus SW was evident from both phenotypic and transcriptomic data, especially with respect to photosynthetic upregulation and freezing tolerance of whole plants. Overall, features of SW were consistent with a different approach to HLC acclimation than seen in IT, and an ability of SW to reach the new homeostasis through involvement of transcriptional controls other than CBF1–3. These results provide tools and direction for further mechanistic analysis of the transcriptional control of approaches to cold acclimation suitable for either persistence through brief cold spells or for maximization of productivity in environments with continuous low temperatures.

Title

Role of CBF transcription factors during long-term acclimation to high light and low temperature in two ecotypes of *Arabidopsis thaliana*

Running head

Gene regulation in cold-grown *Arabidopsis* ecotypes

Authors

Christopher R. Baker[1*] , Jared J. Stewart[2], Cynthia L. Amstutz[1], Lindsey G. Ching[1], Jeffrey D. Johnson[1], Krishna K. Niyogi[1,3], William W. Adams III[2], Barbara Demmig-Adams[2]

[1]Howard Hughes Medical Institute, Dept. of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA

[2]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309-0334, USA

[3]Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

*Corresponding author: cbaker@berkeley.edu

Abstract

When grown under cool temperature, winter annuals upregulate photosynthetic capacity as well as freezing tolerance. Here, the role of three cold-induced C-repeat-Binding Factor (CBF1–3) transcription factors in photosynthetic upregulation and freezing tolerance was examined in two *Arabidopsis thaliana* ecotypes originating from Italy (IT) or Sweden (SW), and their corresponding CBF1–3-deficient mutant lines it:*cbf123* and sw:*cbf123* . Photosynthetic, morphological, and freezing-tolerance phenotypes as well as gene expression profiles were characterized in plants grown from seedling stage under different combinations of light level and temperature. Under high light and cool growth temperature (HLC), a greater role of CBF1–3 in IT versus SW was evident from both phenotypic and transcriptomic data, especially with respect to photosynthetic upregulation and freezing tolerance of whole plants. Overall, features of SW were consistent with a different approach to HLC acclimation than seen in IT, and an ability of SW to reach the new homeostasis through involvement of transcriptional controls other than CBF1–3. These results provide tools and direction for further mechanistic analysis of the transcriptional control of approaches to cold acclimation suitable for either persistence through brief cold spells or for maximization of productivity in environments with continuous low temperatures.

KEYWORDS

*Arabidopsis thaliana***,** cold tolerance, local adaptation, photosynthetic acclimation, regulation of leaf morphology

Accessions numbers: Gene expression omnibus (https://www.ncbi.nlm.nih.gov/geo/) GSE154349

1 [?] INTRODUCTION

Functional acclimation to cool temperatures in winter annuals has two essential components. These are activation of traits that (i) permit survival during periods of subfreezing temperatures (e.g., enhanced freezing tolerance; Thomashow 1999; Zhen & Ungerer 2008; Kang *et al.* 2013; Oakley, Ågren, Atchison & Schemske 2014) and (ii) support for continued productivity on cool days via upregulation of photosynthetic capacity, which compensates for cold-dependent inhibition of enzymatic activity (Berry & Björkman 1980; Hüner, Öquist & Sarhan 1998; Savitch *et al.* 2002; Cohu, Muller, Adams & Demmig-Adams 2014). Photosynthetic capacity is enhanced by synthesis of greater numbers of photosynthetic proteins (Hüner *et al.* 1993; Strand*et al.* 1999; Stitt & Hurry 2002; Demmig-Adams, Stewart & Adams 2017; Adams, Stewart & Demmig-Adams 2018a) as well as augmentation of related features, such as the infrastructure for photosynthate export from leaves (Leonardos, Savitch, Huner, Öquist & Grodzinski 2003; Dumlao *et al.* 2012; Adams, Cohu, Muller & Demmig-Adams 2013; Adams, Stewart, Cohu, Muller & Demmig-Adams 2016). Leaves of winter annuals grown in cool versus warm temperatures are also thicker and contain more chloroplast-rich mesophyll cells per unit area (Gorsuch, Pandey & Atkin 2010; Cohu *et al.* 2014; Adams *et al.*2016). By virtue of upregulation of photosynthetic capacity in leaves that develop under cool temperatures (Cohu, Muller, Stewart, Demmig-Adams & Adams 2013b), plants are able to maintain sugar production and transport for underground storage while limiting above-ground growth and exposure of leaves to freezing temperature (Eremina, Rozhon & Poppenberger 2016). This enhancement of photosynthesis-related traits illustrates how acclimatory adjustment leads to a new homeostasis that minimizes internal stress despite a challenging environment (Anderson, Chow & Park 1995). Notably, a similar upregulation of photosynthesis-related features takes place during acclimation to high growth-light intensity (Gauhl 1976; Boardman 1977; Munekage, Inoue, Yoneda & Yokota 2015) in many species, including*Arabidopsis thaliana* (Stewart *et al.* 2017b; Stewart, Polutchko, Adams & Demmig-Adams 2017a; Hoshino, Yoshida & Tsukaya 2019). Common regulatory networks may thus be involved in both cold and high light acclimation, such as signaling networks that respond to the level of excitation pressure in the chloroplast (Anderson *et al.*1995; Hüner *et al.* 2012; Hüner, Dahal, Bode, Kurepin & Ivanov 2016).

The transcription factor family of C-repeat-Binding Factors (CBFs) has been proposed as a regulatory net-

work that may orchestrate photosynthetic upregulation and enhance freezing tolerance in response to growth under cool temperatures and/or high light intensities (Savitch*et al.* 2005; Hüner *et al.* 2016). *Arabidopsis thaliana* contains three tandemly duplicated *CBF* paralogs (*CBF1* , *CBF2* , and *CBF3* ; abbreviated to*CBF1–3* in this text) that are strongly induced by cold temperature and orchestrate transcriptional and physiological changes necessary for enhanced freezing tolerance (Thomashow 1999; Knight & Knight 2012; Shi, Ding & Yang 2018). Laboratory studies revealed largely overlapping functions for the CBF1–3 transcription factors as well as a requirement for combined loss-of-function mutations in all three genes to strongly reduce induction of freezing-tolerance genes and freezing tolerance itself (Gilmour, Fowler & Thomashow 2004; Zhao*et al.* 2016; Jia *et al.* 2016). CBF over-expressing lines exhibited higher freezing tolerance as well as greater leaf thickness, chlorophyll levels, and photosynthetic rates per unit area even when grown under low light and warm temperature (Gilmour *et al.* 2004; Savitch *et al.* 2005). Thus, CBF overexpression induced both the survival trait of enhanced freezing tolerance and the productivity-maintenance trait of photosynthetic upregulation.

Following a five-year, reciprocal transplant investigation of two*A. thaliana* ecotypes (Ågren & Schemske 2012), Rodasen-47 from Sweden (SW) and Castelnuovo-12 from Italy (IT), numerous studies provided insight into the ecophysiology and genetics underlying local adaptation in this model organism. Anatomical and physiological studies revealed that SW exhibited considerably greater foliar phenotypic plasticity in response to both growth light intensity and temperature compared to IT (Cohu *et al.* 2013b; Adams, Cohu, Amiard & Demmig-Adams 2014; Adams, Stewart, Polutchko & Demmig-Adams 2018b; Stewart *et al.* 2015, 2016, 2017b). While possessing a similar constitutive freezing tolerance, in warm-grown plants, SW also induced greater freezing tolerance relative to IT when grown under controlled cold conditions (Gehan *et al.* 2015; Park, Gilmour, Grumet & Thomashow 2018; Sanderson *et al.* 2020). Under field growth conditions, the *CBF1–3* region was identified as a QTL for fitness (Ågren, Oakley, McKay, Lovell & Schemske 2013) as well as freezing tolerance (Oakley *et al.* 2014). In fact, IT possesses a naturally occurring 8-bp deletion in its *CBF2* gene that renders the CBF2 transcription factor nonfunctional (Gehan *et al.* 2015). Nevertheless, CBF2-deficient lines of SW still maintained greater cold-induced freezing tolerance than IT (Park *et al.* 2018; Sanderson *et al.* 2020). Likewise, a CBF1–3- deficient line created in SW maintained greater cold induced freezing tolerance than a CBF1–3- deficient line created in IT (Park *et al.* 2018).

In the present study, IT and SW were grown under a factorial design of different light intensity and temperature regimes. Transcriptome data from fully expanded leaves were generated to compare expression patterns of genes associated with the functional traits of freezing tolerance and photosynthesis, and chloroplast redox state (reduction state of the primary electron acceptor of photosystem II, Q_A) was assessed to address the relationship between chloroplast excitation pressure and *CBF1–3* expression levels. Under the two most different growth conditions, the wild-type ecotypes, IT and SW, were grown alongside the corresponding CBF1–3-deficient mutant lines it:*cbf123* and sw:*cbf123* (Park *et al.* 2018). Fully expanded leaves of these plants that had developed under the respective growth conditions were assayed for freezing tolerance, morphological and photosynthetic characteristics, and expression of genes associated with the latter phenotypic traits.

2 [?] MATERIALS AND METHODS

2.1 [?] Plant material and growth conditions

Arabidopsis thaliana ecotypes IT (Castelnuovo-12 [ABRC stock number: CS98761], sub-line 24) and SW (Rodasen-47 [ABRC stock number: CS98762], sub-line 29) were grown from seed in Conviron E15 (Controlled Environments Ltd., Manitoba, Canada) and then in E36-HID (Percival Scientific, Perry, IA, USA) growth chambers alongside the corresponding CBF1–3-deficient lines it:*cbf123* and sw:*cbf123* that had been generated by Park, Gilmour, Grumet, & Thomashow (2018) via CRISPR/Cas9 (for more information on the ecotypes, see Agren & Schemske 2012; Adams *et al.* 2016). For selected experiments, sw:*cbf2* , a CBF2-deficient line created in SW by the same group (Park, Gilmour, Grumet, & Thomashow 2018), was included as well. *CBF1–3* genotypes of it:*cbf123* , sw:*cbf123* , and sw:*cbf2* plants used in this study were confirmed by Sanger sequencing.

The following four growth conditions—based on a factorial design of contrasting light intensities and leaf temperatures—were employed: low light, warm temperature (LLW; 9-h photoperiod of 100 μmol photons m^{-2} s^{-1} and leaf temperature 25°C/20°C [light/dark]), low light, cool temperature (LLC; 9-h photoperiod of 100 μmol photons $m^{-2}s^{-1}$ and leaf temperature maximum of 16°C/12.5°C [light/dark]), high light, warm temperature (HLW; 9-h photoperiod of 1000 μmol photons m^{-2} s^{-1} and leaf temperature 25°C/20°C [light/dark]), and high light, cool temperature (HLC; 9-h photoperiod of 1000 μmol photons m^{-2} s^{-1} and leaf temperature maximum of 16°C/12.5°C [light/dark]), the last of which was chosen as the key condition for the present study based on previously reported phenotypic differences between IT and SW (Cohu *et al.* 2013b; Cohu, Muller, Demmig-Adams & Adams 2013a). The controlled conditions used here do not represent the full extremes these plants experience in natural settings; high/low light (HL/LL) and cool/warm (C/W) temperature are used as descriptive terms to characterize the factorial growth regime. All plants were grown from seeds soaked in water at 4°C for 4 days and then germinated in six-pack seed-starting trays containing 50 mL of Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA, USA) under 9-h photoperiods of either 100 (LLW and LLC) or 1000 (HLW and HLC) μmol photons m^{-2} s^{-1} and a common air temperature of 25°C during the photoperiod and 20°C during the dark period. Following germination, individual seedlings were transplanted with 50-mL soil from their respective cells into larger (2.9-L) pots, and then transitioned to their final growth conditions (for details, see Fig. S1; see also (Cohu *et al.* 2013b)). Plants received water daily with nutrients added every other day as previously described (Stewart *et al.* 2017a).

2.2 [?] Leaf phenotypic traits

Leaf photosynthetic capacity was determined as light- and CO_2-saturated oxygen evolution with leaf disc oxygen electrodes (Hansatech Instruments Ltd., Norfolk, UK; Delieu & Walker, 1981) as previously described (Stewart *et al.* 2017a). The reduction state of the primary electron acceptor of photosystem II, Q_A, was assessed via measurements of chlorophyll fluorescence using a pulse-amplitude-modulated (PAM) chlorophyll fluorometer (FMS2; Hansatech Instruments Ltd., Norfolk, UK). Leaves were darkened for 20 min, exposed to a far-red light of 0.6 μmol photons m^{-2} s^{-1} for 5 min, and then subjected to 5-min exposures of increasing light intensities. At the end of each 5-min exposure, steady-state fluorescence (Strand *et al.*1999) were recorded, maximum fluorescence levels (F_m´) were obtained by applying a saturating pulse of light (0.8 s of 3000 μmol photons m^{-2} s^{-1}), and then minimum fluorescence levels (F_o´) were recorded by briefly darkening the leaf. Q_A reduction state was calculated as $1 - q_L = (1/F_s - 1/F_m')/(1/F_o' - 1/F_m')$. Measurements on LLW plants were conducted in the laboratory at ambient temperature (approximately 22degC) and measurements on HLC plants were conducted inside the growth chamber in which they were grown (with an air temperature of 8degC). Chlorophyll *a* and *b* content was determined via high-performance liquid chromatography as previously described (Stewart *et al.* 2015) or via spectrophotometry as previously described (Arnon 1949) from leaf discs (0.30 cm^2) collected at the end of the 15-h dark period.

Leaf dry mass was measured with an A-160 balance (Denver Instruments Company, Denver, CO, USA) from leaf discs that were dried at 70degC for 7 d. For leaf-thickness measurements, leaves were embedded in 7% (w/v) agarose and sectioned into 80–100 μm thick sections using a 752/M Vibroslice tissue cutter (Campden Instruments Ltd., Loughborough, UK). Sections were stained with 0.02% toluidine blue O for 30 s, and images were taken approximately 150 μm away from the mid-vein (where no major veins or trichomes were present) with an AxioImager (Zeiss, Oberkochen, Germany) coupled with a MicroPublisher color camera (QImaging, Surrey, Canada). Leaf thickness was quantified for 10 representative sections of each plant (i.e., 10 technical replicates for each biological replicate) using ImageJ (Schindelin *et al.* 2012).

2.3 [?] Freezing tolerance assays

Freezing tolerance of leaf tissue was determined via electrolyte leakage assays based on those described by Thalhammer, Hincha & Zuther 2014. Leaves (grown under LLW or HLC conditions) with fresh-cut petioles were placed in 300 ml of deionized H_2O (petioles submerged) and subjected to subfreezing temperatures using an Arctic A25 refrigerated water bath (Thermo Fisher Scientific, Waltham, MA, USA) and a cooling rate of 4°C h^{-1}. Electrical conductivity was measured using an Exstik II probe (Extech Instruments, Nashua, NH, USA). The data for each replicate were fitted to a four-parameter logistic model, and lethal freezing

temperatures (LT_{50}) values were determined as the inflection points from these models. Maximal intrinsic photosystem II efficiency in darkness was assessed in parallel with the electrolyte leakage assays after overnight incubation on ice (4degC) to thaw frozen leaves for measurements of chlorophyll fluorescence with an Imaging-PAM Maxi (Walz, Effeltrich, Germany). Minimal fluorescence levels (F_o) were recorded after a 20-min dark period at room temperature following the freezing treatments, and then maximal fluorescence levels (F_m) were recorded by applying a pulse of saturating light (2500 µmol photons m^{-2} s^{-1}). Maximal intrinsic photosystem II efficiency was calculated as $F_v/F_m = (F_m - F_o)/F_m$, and false-colored images of F_v/F_m were generated using ImageJ (Schindelin *et al.* 2012).

Freezing tolerance of whole plants was determined via survival assays based on previously described protocols (Xin & Browse 1998; Sanderson*et al.* 2020). Seeds were germinated and transferred to LLW or HLC growth conditions as described above with the exception that seedlings were not transferred to individual pots and were instead thinned to prevent overcrowding. After ten days under LLW or HLC growth conditions, plants with six to eight leaves were transferred to $\frac{1}{2}$ MS-agar plates, chilled to -1degC in the presence of ice chips for 8 h, and frozen overnight (16 h) at an average freezer temperature of -10degC. Plates were then transferred to 4degC for one day, and plant survival was assessed after another two days of recovery in LLW conditions. Surviving plants remained green and erect, whereas non-surviving plants were white and no longer erect.

2.4 [?] Gene expression analysis using real-time qPCR

RNA extraction, cDNA synthesis, and qPCR were performed as previously described (Wakao *et al.* 2014). All primer pairs were confirmed as having 90–105% amplification efficiency and linear amplification within their dynamic range in experimental samples using serial dilutions of cDNA prior to experiments. Relative transcript levels were calculated by the ΔΔCt method (Livak & Schmittgen 2001) using*PEX4* (AT5G25760) as the internal reference. *PEX4* , a peroxisomal ubiquitin-conjugating enzyme, is an established RT-qPCR internal reference (Dekkers *et al.* 2011) and was confirmed in the RNAseq dataset to have constant expression levels in all conditions and ecotypes. Primers were designed using Primer3 (Untergasser *et al.*2012) against the 3´-UTR of each gene to avoid binding to off-target paralogous genes. A single peak in melt-curve analysis with a unique melting temperature was observed for each amplicon, verifying that off-target amplification of paralogous genes was negligible.

2.5 [?] RNAseq library preparation and analysis

Two flash-frozen leaf discs of 0.73 cm^2 collected at the end of the 15-h dark period were homogenized in liquid nitrogen by bead beating, and RNA was extracted and DNase-treated using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Integrity of purified RNA was validated using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and concentration determined using a QuBit fluorometer (Thermo Fisher Scientific, Massachusetts, USA). Plant rRNA was depleted from 2 mg of purified RNA using the RiboZero rRNA removal kit for plants (Illumina, San Diego, CA, USA). Barcoded cDNA libraries were generated from our rRNA-depleted RNA samples using the TruSeq RNA library preparation kit (Illumina, San Diego, CA, USA). Sequencing of barcoded cDNA libraries was performed at the Vincent J. Coates Genomics Sequencing Laboratory (Berkeley, CA, USA) using a HiSeq2500 platform with 50 bp single-end reads (Illumina, San Diego, CA, USA).

2.6 [?] Statistical analyses

RNAseq analysis was performed using the genomic analysis tools available through Galaxy (Afgan et al., 2018). Quality of RNAseq runs was validated by FastQC and adapter sequences were clipped using FASTQ (Gordon & Hannon 2017). Reads were mapped to the *A. thaliana*reference genome (TAIR10) and preliminary differential expression analysis was conducted using HISAT and StringTie (Pertea *et al.*2015). Differential expression analysis was conducted using DESeq2 as well as the calculation of adjusted *P*- values, which limit high false positive discovery rates due to multiple testing (Love, Huber & Anders 2014). Data can be accessed on the Gene Expression Omnibus at GSE154349. Log_2 fold-changes were transformed with the regularized log function to minimize variance caused by low expression genes, then clustered and plotted using pheatmap. In pheatmap, each sample was clustered on the horizontal axis based on the similarity of

its transcriptome to the 23 other transcriptomes. On the vertical axis, individual genes were clustered based on the similarity of their expression profile across the 24 samples to the expression profile of other genes.

Comparisons of two means were evaluated via Student's t tests and comparisons of multiple means evaluated via one-way analysis of variance (ANOVA) coupled with post hoc Tukey–Kramer Honestly Significant Differences (HSD) tests. The effects of genotype (e.g., CBF1–3 deficiency) and growth conditions as well as genotype response to the growth conditions for the IT (IT & it:*cbf123*) and SW (SW & sw:*cbf123*) genetic backgrounds were each assessed via two-way ANOVA. Nonlinear curves were generated using 3-parameter exponential and 4-parameter logistic models. All statistical analyses, excluding those of RNAseq data, were conducted using JMP software (Pro 15.0.0; SAS Institute Inc., Cary, NC, USA).

3 [?] Results

3.1 [?] Interaction of growth environment with ecotype in shaping photosynthesis and related features, expression of *CBF1–3* genes, and leaf transcriptome

3.1.1 [?] Photosynthesis and related features

For both IT and SW, the highest levels of photosynthetic capacity (Fig. 1a), leaf dry mass (Fig. 1b), and chlorophyll $a + b$ (Fig. 1c) per leaf area were seen in plants grown under high light and cool temperature (HLC). Whereas photosynthetic capacity and leaf dry mass per area were higher in plants of both ecotypes grown under high light and warm temperature (HLW) compared to low light and warm temperature (LLW), chlorophyll $a + b$ levels were similar. Chlorophyll a / b ratios were higher in both ecotypes in high compared to low growth light irrespective of growth temperature (Fig. 1d). Significant ecotype-specific differences were also observed, with higher photosynthetic capacity per area in SW compared to IT under HLC and HLW (Fig. 1a), higher leaf dry mass per area in SW compared to IT under HLC (Fig. 1b), higher chlorophyll $a + b$ in SW compared to IT under all conditions tested (Fig. 1c) and higher chlorophyll a / b ratios in IT compared to SW under HLW and a similar, albeit not significant, trend under HLC (Fig. 1d).

Excitation pressure in the chloroplast was ascertained as photosystem II (Q_A) reduction state after short experimental exposure to a range of light intensities in leaves of plants grown in HLC and LLW. Q_A reduction state was similar in the two LLW-grown ecotypes grown across a range of light intensities (Fig. 2a), with both ecotypes exhibiting a relatively steep increase to high Q_A reduction states with increasing light intensities. In contrast, plants of both ecotypes grown under HLC compared to LLW exhibited considerably lower Q_A reduction states (Fig. 2a,b). Furthermore, the light response of Q_A reduction state differed between IT and SW plants grown under HLC, with a significantly lower Q_A reduction state (more oxidized Q_A) in SW compared to IT under experimental exposure to higher light intensities (Fig. 2b).

3.1.2 [?] Expression of CBF1–3 genes and leaf transcriptome

In both ecotypes, the strongest *CBF1–3* transcript expression was also seen in plants grown under HLC (Fig. 3). As was the case for photosynthetic capacity and leaf dry mass per area, *CBF1* and *CBF3* expression were also greater in SW compared to IT plants grown under HLC. In plants grown under LLC on the other hand, only IT moderately induced *CBF1–3* but SW did not (Fig. 3).

Growth under HLC compared to LLW also resulted in sweeping differences in the leaf transcriptome in both ecotypes (Fig. 4). 1912 and 1415 genes were induced in HLC-grown IT and SW, respectively, with an adjusted P -value of less than 0.01 and a minimum fold-change of 2 (Table S1, S2). Similar numbers of genes were downregulated under HLC compared to LLW, i.e., 1671 and 1531 genes, for IT and SW, respectively (Tables S3, S4). For both ecotypes grown under each of the four conditions, all three biological replicates co-clustered (after hierarchical clustering) and the transcriptomic response of IT and SW in HLC conditions co-branched (Fig. 4a). This co-branching of HLC transcriptomes of the two ecotypes was due at least in part to large blocks of co-clustering genes either induced specifically or downregulated under HLC in both ecotypes (Fig. 4a, Tables S5–S7). Genes specifically induced under HLC were strongly enriched for a number of gene ontology (GO) categories, of which the most enriched unique categories were starch

catabolism/starch metabolism (GO:0005983/GO:0005982), sucrose biosynthesis (GO:0005986), and cold acclimation (GO:0009631) (Table S8). GO analysis also revealed pathways repressed specifically in HLC; the three categories most strongly downregulated in HLC were water transport (GO:0006833), brassinosteroid metabolism (GO:0016131), and auxin polar transport (GO:0009926) (Table S9).

In addition to these shared responses, there were also substantial differences between IT and SW in how gene expression responded to HLC (Table S10-16). In SW compared to IT, growth under HLC conditions enriched more strongly for induction of genes in the overlapping GO categories of cold acclimation (GO:0009631), response to water deprivation (GO:0009414), and response to abscisic acid (GO:0009737) (Table S11). Conversely, the three most enriched unique GO categories among genes with significantly higher induction in IT compared to SW in HLC were protein refolding (GO:0042026), glucosinolate biosynthesis (GO:0019761), and phosphate starvation response (GO:0016036) (Table S14). Moreover, genes exhibiting greater down-regulation in HLC in IT compared to SW were enriched for cytokinin-activated signaling pathway (GO:0009736), auxin polar transport (GO:0009926), and response to auxin (GO:0009733) (Table S16).

Genes in the photosynthesis GO category (GO:0015979) also exhibited unique expression patterns in each ecotype under HLC (Fig. S2). With the exception of the light-stress-induced light-harvesting complex *LHCB4.3* (AT2G40100), genes involved in light harvesting were downregulated in both ecotypes under HLC, but more so in IT relative to SW. Conversely, genes more strongly induced in IT compared to SW (Fig. S2) had in common that they are typically induced under abiotic and/or oxidative stress (see discussion), such as chloroplast glucose-6 phosphate/phosphate translocator *GPT2* (AT1G61800), chloroplast envelope K^+/H^+ antiporter *KEA2* (AT4G00630), phosphofructokinase (AT1G76550), cytosolic fumarase (AT5G76550), ferritin (AT2G30400/AT3G56090), and pyridoxal phosphate synthase protein (AT5G01410).

For both ecotypes, genes preferentially induced under HLC were enriched for those that had also been induced by *CBF1–3* overexpression in the absence of either high light or cool temperatures (Park *et al.* 2018) (P -values of 10^{-19} and 10^{-18} for IT and SW, respectively; Fig. 4b, Table S17). Moreover, these genes preferentially induced under HLC were also enriched for genes expressed at lower levels in it:*cbf123* and sw:*cbf123* following sudden transfer from warm growth conditions to 4degC for 24 h (Park *et al.* 2018) (P -values of 10^{-28} and 10^{-38}, for IT and SW, respectively; Table S18). Overall, while CBF1–3 target genes (Jia *et al.* 2016; Park *et al.* 2018) were strongly induced in both ecotypes in HLC, these genes tended to be more strongly induced in SW compared to the IT ecotype in this condition. Examples for genes in this previously defined CBF1–3-regulated group that were more strongly induced in SW included the cold-acclimation-regulating protein kinase *CIPK25* (AT5G25110) and a suite of cold-induced dehydrin proteins, *COR47* (AT1G20440), *LTI30* (AT3G50970), and *LTI29* (AT1G20450) (Fig. 4b). In contrast, CBF1–3-regulated protein phosphatase *EGR2* (AT5G27930), a repressor of growth, was more strongly induced in IT compared to SW under HLC (Fig. 4b).

3.2 [?] CBF1–3 deficiency, photosynthesis, morphology, freezing tolerance, and gene expression

3.2.1 [?] *Photosynthesis and morphology*

CBF1–3- deficiency significantly lessened the extent of upregulation of photosynthetic capacity and leaf dry mass under HLC relative to LLW and abolished upregulation of chlorophyll $a + b$ content in it:*cbf123* compared to IT but, remarkably, did not impede upregulation of these traits in HLC compared to LLW in sw:*cbf123* compared to SW (Fig. 5a–c, Table 1). Despite the difference in chlorophyll $a + b$ content, IT and it:*cbf123* did not differ in chlorophyll a/b under either LLW or HLC (Fig. 5d).

Similar trends were observed for leaf morphology in that IT and it:*cbf123* grown under HLC exhibited significant differences, whereas SW and sw:*cbf123* did not (Figs. 6,7). Specifically, leaves were thinner (Fig. 6a–c) and rosettes were larger (had a larger diameter) in it:*cbf123* compared to IT (Fig. 7a–c) in plants grown in HLC. In contrast, leaf thickness was the same (Fig. 6a,d,e) and rosette diameter was similar in HLC-grown plants of SW and sw:*cbf123* (Fig. 7a,d,e).

3.2.2 [?] *Freezing tolerance*

An initial assessment of leaf freezing tolerance was made using electrolyte leakage and chlorophyll fluorescence, where a sharp increase in leakage and/or decrease in intrinsic photosystem II indicates freezing damage to membranes (Fig. 8). While LLW-grown plants of all genotypes exhibited the same high susceptibility to freezing damage by these criteria (Fig. 8a), HLC-grown plants were shifted to greater tolerance that was also more pronounced in SW compared to IT and was substantially impaired by CBF1–3 deficiency in both backgrounds (Fig. 8b, Table 2). Figure 8c shows these same data transformed to mean lethal temperature (LT_{50}) upon exposure to stress; onset of significant electrolyte leakage occurred with an LT_{50}near -5.6degC for all genotypes grown in LLW but was shifted to lower sub-freezing temperatures in leaves grown in HLC compared to LLW to varying degrees depending on genotype. LT_{50} of freezing tolerance in sw:*cbf123* was 3.5degC warmer than that of SW (Fig. 8b,c). Similarly, LT_{50} of it:*cbf123* was 3.4degC warmer than that of IT (Fig. 8b,c). This greater electrolyte leakage in sw:*cbf123* compared to SW and it:*cbf123* compared to IT was accompanied by more pronounced freezing-induced depression of intrinsic PSII efficiency F_v/F_m (Fig. 8d). At the same time, the lesser electrolyte leakage in both it:*cbf123* and sw:*cbf123* lines grown under HLC compared to LLW indicated contributions from CBF1–3-independent freezing-tolerance mechanisms.

The results from excised leaves (Fig. 8) were complemented by tests of whole plant survival upon exposure to freezing temperatures (Fig. 9). Whole-plant survival was extremely low in LLW-grown plants of all genotypes and was much enhanced by growth under HLC (Fig. 9). The whole plants (Fig. 9) were even more sensitive to freezing stress than the leaves shown in Figure 8. The impairment of freezing tolerance of whole plants by CBF1–3-deficiency was much more pronounced than that of leaves in the IT background, i.e., in it:*cbf123* compared to IT. In contrast, there was much less impairment of freezing tolerance in whole plants by CBF1–3- deficiency in sw:*cbf123* compared to SW (Fig. 9).

3.2.3 [**?**] *CBF1–3-dependent gene expression*

This section focuses on selected genes that exhibited response patterns reminiscent of the trends exhibited by photosynthesis and leaf/plant morphology (Fig. 10, Table 1) as well as selected genes known to be cold regulated (Fig. 11, Table 2). From among 31 genes that were identified as CBF1–3-target genes in prior work (Park *et al.* 2018) and showed considerable induction under HLC in IT (Fig. 4), nine were selected for validation by RT-qPCR with priority given to genes encoding proteins that can be linked to a role in photosynthetic or leaf-morphological acclimation phenotypes based on either previous studies on these proteins or the presence of a protein domain with an established role in acclimation phenotypes. Expression level of five of these nine genes (Fig. 10; Table 1) exhibited an impact of CBF1–3 deficiency mirroring that on leaf photosynthetic and morphological traits in the two ecotypes. Specifically, these five genes exhibited a strong reduction in the extent of upregulation under HLC compared to LLW in it:*cbf123* compared to IT but no to little difference in sw:*cbf123* compared to SW. These genes included cold- and salt-responsive protein *RCI2A* (AT3G05880; Fig. 10a), transmembrane protein AT5G44565 (Fig. 10b), sucrose synthase *SUS1*(AT5G20830; Fig. 10c), cysteine-rich, defensin-like protein *LCR69*(AT2G02100; Fig. 10d), and oleosin-B3-like stress protein AT1G13930 (Fig. 10e).

Moreover, expression of nine selected cold acclimation genes was affected by CBF1–3 activity in both IT and SW grown in HLC (Fig. 11). Under HLC, expression of galactinol synthase *GolS3* (AT1G09350), the protein kinases *CIPK25* (AT5G25110) and *KIN2*(AT5G15970), and the protein phosphatase *EGR2* (AT5G27930) were higher in SW compared to IT and were also higher in both wildtype genotypes compared to their corresponding CBF1–3-deficient mutants (Fig. 11a–d). In contrast, expression of cold-regulated genes*COR78* (AT5G52310) and *COR15A* (AT2G42540) was higher in IT compared to SW (Fig. 11e,f), and expression of the dehydrin *LTI30*(AT3G50970), the cold-regulated gene (necessary for chloroplast membrane integrity in freezing) *COR15B* (AT2G42530), and lipid-sensing-domain-containing AT1G21790 was similar in SW and IT (Fig. 11g–i). Induction of the latter genes (expressed either more strongly, or similarly, in IT compared to SW) under HLC versus LLW was associated to some extent with CBF1–3 since it was partially inhibited in sw:*cbf123* compared to SW (Fig. 11, Table 2) and partially (Fig. 11a–c,e–h) or completely (Fig. 11d,i) inhibited in it:*cbf123*compared to IT (Table 2).

While the focus of this work was the effect of complete CBF1–3 deficiency, it should be noted that there was

no difference, or only a relatively small effect, for expression of selected CBF1–3-regulated genes in plants deficient only in CBF2 in the SW background (sw:*cbf2*) relative to SW in HLC (Table S19). Similarly, no difference was observed between HLC-grown sw:*cbf2* and SW in leaf photosynthetic or morphological phenotypic traits (Figs. 5–7, Table S19) or in freezing tolerance of excised leaves (via electrolyte leakage or F_v/F_m; Fig. 8, Table S19). A small decrease in freezing tolerance of whole plants was observed for sw:*cbf2* relative to SW seedlings grown in HLC conditions, but its survivorship was still higher than that of IT plants (Fig. 9, Table S19).

4 [?] Discussion

4.1 [?] Response of plant function and gene expression to growth under HLC conditions

Neither cool temperature alone (maximum daytime leaf temperature of 16degC) nor high light alone strongly induced expression of*CBF1–3,* but combined high light and cool temperature (HLC) acted synergistically to induce *CBF1–3* . Increased excitation pressure in the chloroplast serves as one of the signals that induce elevated*CBF1–3* expression under HLC conditions (Huner *et al.*2012, 2016) and is integrated with additional photosynthetic retrograde signals (Lee & Thomashow 2012; Noren *et al.* 2016). Overwintering herbaceous plants, experiencing cold temperatures and the associated high excitation pressure in the chloroplast, enact the suite of acclimatory responses demonstrated here, including upregulation of photosynthetic capacity and leaf thickness (with more mass and chlorophyll per area), reduced leaf expansion, and enhanced freezing tolerance (see also Cohu *et al.* 2013b, 2014; Muller *et al.*2014; Sanderson *et al.* 2020). These changes can allow overwintering species to achieve full acclimation, defined as a new homeostasis where internal stress (with signs of oxidative stress) is minimized or absent.

The pronounced acclimation of plant form and function in SW and IT plants grown in HLC conditions was associated with sweeping changes in gene expression, with approximately 5.2% of total leaf transcriptome upregulated and 4.9% downregulated in HLC relative to growth in low light and warm temperature (LLW). The most strongly enriched gene ontology categories in HLC were related to starch metabolism, sucrose biosynthesis, and cold acclimation, which is consistent with the upregulation of photosynthetic capacity and of freezing tolerance. Continued photosynthetic productivity under cool temperatures in the absence of significant growth generates carbohydrate that can be stored in sink tissues (Demmig-Adams *et al.* 2017; Adams *et al.*2018a) and also contribute to the accumulation of compatible solutes and freezing point depression (Wanner & Junttila 1999; Reyes-Diaz *et al.* 2006; Cao, Song & Su 2007).

Pathways repressed in HLC in both SW and IT included those associated with growth hormones. Reduction of rosette expansion under winter conditions, involving decreased rates of cell elongation during leaf development (Yano & Terashima 2004; Hoshino *et al.* 2019) helps to minimize foliar freezing damage. Pathways repressed in HLC in both SW and IT included not only those associated with growth hormones (e.g., brassinosteroids and auxin polar transport) but also with water transport. In fact, vascular tissue is one of the targets of growth hormones (Fabregas *et al.* 2015; Etchells, Peter Etchells & Turner 2017) and acclimation to cool temperature is associated with adjustments of vascular anatomy (Cohu *et al.* 2013a; Adams*et al.* 2016; Stewart *et al.* 2016). Thus, effects of freezing on the plant vasculature may be involved in the pronounced vulnerability of it:*cbf123* plants. Further research is warranted into the possibility that the much greater impairment of freezing tolerance of whole plants by CBF1–3- deficiency in the IT compared to the SW background may be due to the well-characterized phenomenon of freeze-thaw-induced water conduit embolism and its dependence on xylem anatomy (Lens *et al.* 2013). Further research should also address plant ontology and age given that the electrolyte leakage assays in this study were performed on excised leaves of plants acclimated for multiple weeks, whereas, to achieve the necessary sample size, whole plants of a younger age/smaller size were used for survival assays.

4.2 [?] Differences between SW and IT in the extent of response to HLC

4.2.1 [?] Stronger enrichment in SW versus IT under HLC

Growth under HLC conditions prompted stronger enrichment in SW compared to IT in genes of the ontology

categories of acclimation to cold and drought (responses to water deprivation and to abscisic acid) that have overlapping features (Heino, Sandman, Lang, Nordin & Palva 1990). This pattern is consistent with the greater freezing tolerance and upregulation of photosynthetic capacity in SW compared to IT (see also Cohu *et al.* 2013b; Stewart *et al.* 2016) as well as the lesser excitation pressure in the chloroplast (more oxidized Q_A reduction state) of HLC-grown SW compared to IT under experimental high-light exposure. The stronger downregulation of genes involved in light-harvesting in HLC-grown IT suggests that IT limits excitation pressure by lowering light-collection capacity, which is consistent with the lower Q_A reduction state under very low light (when thermal dissipation is not triggered) in HLC-grown IT compared to SW as well as IT's lower chlorophyll $a + b$content and higher chlorophyll a/b ratio that are indicative of a smaller antenna size (due to preferential degradation of the outer, chlorophyll b -containing light-harvesting complexes). This is consistent with previous studies in which SW increased, rather than decreased, light absorption during cold acclimation and apparently limited excitation pressure by greater utilization of excitation energy in photosynthetic electron transport (Cohu et al. 2013b), as well as greater photoprotective thermal dissipation (Oakley *et al.* 2018). Our present findings in HLC growth conditions indicate that the acclimatory adjustments in SW are more conducive to productivity maintenance, while adjustments in IT still mitigate oxidative stress.

Two examples of genes with expression patterns that match those of the greater photosynthetic acclimation in SW compared to IT are *SUS1* (AT5G20830) and *EGR2* (AT5G27930). *SUS1* is a sucrose synthase strongly induced under abiotic stress but not required for sucrose accumulation under conditions favorable for growth (Kilian*et al.* 2007; Barratt *et al.* 2009). High foliar sucrose levels are, furthermore, linked to increased palisade cell height in leaves grown under high light (Katagiri *et al.* 2016; Hoshino*et al.* 2019). *EGR2* is a negative regulator of growth (Bhaskara, Wen, Nguyen & Verslues 2017). Over-expression of *EGR2*caused a reduction of cell elongation and rosette size, whereas*egr2* null mutation enhanced both processes (Bhaskara *et al.* 2017).

4.2.2 [?] Stronger enrichment in IT compared to SW under HLC

The well-characterized phenotypic features of cold acclimation do occur in IT, but to a lesser extent than in SW. It is noteworthy that the genes more strongly induced under HLC in IT compared to SW have been implicated in abiotic stress responses, as was reported for chloroplast glucose-6 phosphate/phosphate translocator *GPT2* (Dyson et al. 2015), chloroplast envelope K^+/H^+antiporter *KEA2* (Kunz et al. 2014), light-harvesting complex*LHCB4.3* (Klimmek, Sjodin, Noutsos, Leister & Jansson 2006), cytosolic phosphofructokinase (Kant *et al.* 2008), cytosolic fumarase (Pracharoenwattana *et al.* 2010), ferritins (Petit, Briat & Lobreaux 2001), and pyridoxal phosphate synthase (Denslow, Rueschhoff & Daub 2007). Future research should further test the hypothesis that both SW and IT make acclimatory adjustments that limit oxidative stress under HLC conditions, but that changes in SW focus more on enhancement of productivity (which also lowers excitation pressure more effectively), while IT undergoes alternative evasive changes that are somewhat less effective in controlling excitation pressure.

Moreover, genes exhibiting greater down-regulation in HLC in IT compared to SW were those involved in growth hormone transport and signaling (cytokinin-activated, auxin polar transport, and response to auxin). As stated above, such effects could be related to the hormonal control of vascular acclimation.

4.3 [?] CBF1–3 involvement in acclimation to HLC conditions

4.3.1 [?] Extent of CBF1–3 involvement in SW relative to IT

The present finding that CBF1–3 are necessary for full induction of freezing tolerance in SW and IT demonstrates their involvement in*A. thaliana* grown from seedling stage in HLC conditions as done here. Previous studies had shown that CBF1–3 are required for full induction of freezing tolerance in mature plants grown under warm conditions and transferred in one step to chilling conditions (Zhao*et al.* 2016; Jia *et al.* 2016; Park *et al.* 2018). However, as was also concluded from studies on warm-grown CBF1–3-deficient mutants abruptly transferred to cold conditions (Zhao*et al.* 2016; Jia *et al.* 2016; Park *et al.* 2018), both CBF1–3-dependent and CBF1–3-independent pathways contribute to freezing tolerance in plants grown from seedling stage under HLC – as illustrated here by the fact that freezing tolerance of both it:*cbf123* and sw:*cbf123*

was greater in HLC compared to LLW and that the induction of genes previously defined as CBF1–3-target genes was reduced to varying degrees, but was not fully blocked in CBF1–3-deficient lines grown under HLC.

The striking difference in the extent to which CBF1–3- deficiency differentially impairs aspects of the acclimation process to HLC conditions in IT compared to SW is a key finding of the present study. While many genes previously defined as CBF1–3-responsive genes did exhibit strongly reduced expression in both it:*cbf123* compared to IT and sw:*cbf123* compared to SW, and may be associated with functions we did not characterize in this study, some genes instead exhibited trends matching those of photosynthetic acclimation and freezing tolerance of whole plants. For the latter genes, sw*:cbf123* compared to SW exhibited little or no difference as the result of CBF1–3- deficiency, whereas it:*cbf123* exhibited strongly reduced expression compared to IT. The central features of the acclimation of plant form and function to HLC, i.e., photosynthetic upregulation (and its associated morphological traits) as well as freezing tolerance, were only modestly impacted in sw:*cbf123* but were strongly impacted (especially in whole plants for the case of freezing tolerance) in it:*cbf123* compared to IT. These findings provide further indication for a role of CBF1–3-independent pathways in HLC acclimation of photosynthesis and freezing tolerance and suggest a greater contribution of such pathways in SW.

Growth is yet another trait exhibiting differential regulation between SW and IT in the context of CBF1–3-deficiency. The fact that rosettes of it:*cbf123* were larger relative to IT, but those of sw:*cbf123* were similarly large as those of SW under HLC is consistent with an obligatory role of CBF1–3 in growth depression under HLC conditions in IT but not in SW. Ding et al. (2019) reported a regulatory link between CBF1–3 induction by chilling stress, post-translational modification of EGR2, and whole-plant changes in rosette growth. As noted, under HLC conditions *EGR2* was induced in both ecotypes (more strongly so in SW) and preferentially attenuated in it:*cbf123* . Given *EGR2* 's role in repressing leaf elongation (Bhaskara *et al.* 2017), this gene may contribute to the larger rosette size of it:*cbf123* relative to IT in HLC growth conditions. Future research should further clarify the role of CBF1–3 (in IT) and/other regulators (in SW) in inducing EGR2-dependent growth depression under HLC.

4.3.2 [?] Role of paralog compensation

Paralog compensation is unlikely to explain the observed significant induction of genes defined as CBF1–3-target genes in CBF1–3-deficient mutants in HLC. CBF1–3 belong to the ERF/AP2 A-1 subfamily that includes three additional members located outside the *CBF1–3*gene locus in *A. thaliana* (Mizoi, Shinozaki & Yamaguchi-Shinozaki 2012); these three other ERF/AP2 A-1 subfamily members (*DDF1,* AT1G12610; *DDF2* , AT1G63030; *CBF4* , AT5G51990) were not expressed at detectable levels in leaf tissue of IT or SW under any of the four growth regimes in either the present study or a previous study (Park *et al.* 2018). On the other hand, while not a core topic for this work, the findings of the present study are fully consistent with the conclusion that paralog compensation significantly attenuates the effect of the sw:*cbf2* mutation alone on gene expression and freezing tolerance, as assessed by electrolyte leakage and F_v/F_m post-freezing (Gilmour*et al.* 2004; Zhao *et al.* 2016; Jia *et al.* 2016; Park *et al.* 2018). Several independent *A. thaliana*lineages have evolved loss-of-function mutations in individual*CBF1–3* genes without adverse effects on survival in regions with mild winters, suggesting a potential fitness advantage to attenuated CBF1–3 activity in southern ecotypes (Kang *et al.* 2013; Monroe*et al.* 2016). Likewise, our group, furthermore, reported that IT grow faster than SW in either low light or hot temperature under controlled conditions (Stewart *et al.* , 2016). The complete suite of CBF1–3 transcription factors may be required for tolerance to temperatures well below those used in this work (daytime air temperature of 8degC and maximum leaf temperature up to 16degC) as consistent with the results of the freezing survivorship assays performed by Sanderson*et al* . (2020).

4.3.3 [?] SW as a high-light adapted ecotype

It was previously shown that SW responds with stronger upregulation of photosynthetic capacity and associated leaf features than IT to growth in high light under warm temperature (Stewart *et al.* 2017a). Based on the latter response, SW was classified as having a high-light phenotype, presumably as an adaptation to the

extremely long summer days in its site of origin at high latitude (Adams *et al.* 2016). Photosynthetic upregulation is a developmental process involving changes at the organelle, cell, tissue, and whole plant levels (Yano & Terashima 2004; Hoshino *et al.* 2019), and involves integration of multiple regulatory pathways, including photoreceptors, photosynthesis-related sugar and redox signals, and phytohormone signals. For example, mutants in blue-light photoreceptor signaling and foliar sucrose (Lopez-Juez, Bowyer & Sakai 2007; Kozuka, Kong, Doi, Shimazaki & Nagatani 2011; Katagiri *et al.* 2016; Hoshino*et al.* 2019) have an effect of similar magnitude in increasing leaf thickness in HL-grown plants to those observed for CBF1–3-dependent leaf thickening in the it:*cbf123* mutant under HLC. The sucrose synthase *SUS1* may contribute to the differential leaf thickening phenotype of SW and IT in HLC conditions via sucrose-responsive leaf thickening (Katagiri *et al.* 2016) given that (a) it was induced in both ecotypes in HLC conditions, but more strongly in SW, and (b) its induction was unchanged in the sw:*cbf123* mutant but significantly attenuated in it:*cbf123*relative to each respective parental ecotype. Thus, its induction pattern in HLC closely mirrors the trends for leaf thickness reported here. In summary, the present findings suggest that light-responsive signaling pathways with overlapping functions compensate fully for CBF1–3 deficiency in sw:*cbf123* with respect to upregulation of photosynthetic capacity and associated leaf features, which were unaffected in sw:*cbf123* , but significantly (albeit modestly) reduced in it:*cbf123* . The particularly pronounced photosynthetic upregulation in SW is presumably demanded by the continuously low temperatures at its high-latitude site of origin, whereas the IT ecotype encounters intermittent cold spells (requiring oxidative-stress mitigation) and can quickly resume photosynthetic activity upon return to milder temperatures (for temperature profiles at the respective sites of origin, see Adams et al., 2016).

4.3.4 [?] CBF1–3 and the nature of acclimation

The more pronounced photosynthetic upregulation in SW plants grown from seedling stage under HLC suggest an acclimation directed at enhanced productivity in addition to mitigation of oxidative stress. Furthermore, the lesser excitation pressure (lower Q_A reduction state) in HLC-grown plants of SW compared to IT represents a lesser trigger for further acclimatory adjustment and evidence of more complete acclimation to HLC conditions in SW compared to IT plants (see also Cohu*et al.* 2013b a, 2014; Adams *et al.* 2013; Stewart *et al.* 2017b). Additionally, as described above, genes involved in plant response to oxidative stress were consistently more strongly induced in IT relative to SW under HLC growth conditions. Rather than maximizing excitation energy utilization for photosynthetic energy production and thereby minimizing oxidant production, IT thus apparently employs multiple mechanisms that mitigate oxidative stress. CBF1–3 may play a prominent role in the mitigation of oxidative stress in IT, and presumably also during the initial stages of cold acclimation (Fowler & Thomashow 2002) compared to completed acclimation in SW (see Park*et al.* 2018). This difference in transcriptional control in SW and IT may stem from evolution under the different environmental conditions at the sites of origin, where IT can presumably "wait out" infrequent, short-duration cold spells, while it is advantageous for SW to maintain productivity throughout long stretches of cool conditions. These contrasting strategies would be of interest for agriculture in locations with either short cold spells or continuously low temperatures.

In conclusion, several lines of evidence at the transcriptomic and physiological levels are consistent with the CBF1–3-dependent pathway playing a disproportionately greater role under HLC in IT but not in SW. It should be noted that this trend was already evident in young plants and not only in more mature plants. The system of IT and SW, and their CBF1–3-deficient mutants, can serve as a resource to further study CBF1–3-regulated genes that mitigate oxidative stress before, or in the absence of, fully regained productivity as well as genes that remain active after productivity has been fully restored. In addition, CBF1–3-independent pathways that contribute to full HLC acclimation can also be studied in the SW background. Tools for phenotyping and transcriptional profiling of Recombinant Inbred Line populations are available for these two populations (Agren *et al.* 2013; Oakley*et al.* 2018).

ACKNOWLEDGEMENTS

The lines sw:*cbf123* and it:*cbf123* were generously provided by Professor Michael Thomashow at Michigan State University. This work was supported by the Gordon and Betty Moore Foundation through Grant

GBMF 2550.03 to the Life Sciences Research Foundation [to C.R.B]. K.K.N. is an investigator of the Howard Hughes Medical Institute. This work was also supported by the National Science Foundation [DEB-1022236 to B.D.-A. and W.W.A., IOS-1907338 to J.J.S.] and the University of Colorado.

REFERENCES

Adams W.W. III, Cohu C.M., Muller O. & Demmig-Adams B. (2013) Foliar phloem infrastructure in support of photosynthesis. *Frontiers in Plant Science* **4** , 194.

Adams, W. W. III, Cohu C.M., Amiard V. & Demmig-Adams B. (2014) Associations between the acclimation of phloem-cell wall ingrowths in minor veins and maximal photosynthesis rate. *Frontiers in Plant Science* **5** , 24.

Adams, W. W. III, Stewart J.J., Cohu C.M., Muller O. & Demmig-Adams B. (2016) Habitat temperature and precipitation of *Arabidopsis thaliana* ecotypes determine the response of foliar vasculature, photosynthesis, and transpiration to growth temperature. *Frontiers in Plant Science* **7** , 1026.

Adams, W. W. III, Stewart J.J. & Demmig-Adams B. (2018a) Photosynthetic Modulation in Response to Plant Activity and Environment. In *The Leaf: A Platform for Performing Photosynthesis* . (eds W.W. Adams III & I. Terashima), pp. 493–563. Springer International Publishing, Cham.

Adams, W. W. III, Stewart J.J., Polutchko S.K. & Demmig-Adams B. (2018b) Leaf Vasculature and the Upper Limit of Photosynthesis. In*The Leaf: A Platform for Performing Photosynthesis* . (eds W.W. Adams III & I. Terashima), pp. 27–54. Springer International Publishing, Cham.

Agren J., Oakley C.G., McKay J.K., Lovell J.T. & Schemske D.W. (2013) Genetic mapping of adaptation reveals fitness tradeoffs in*Arabidopsis thaliana* . *Proceedings of the National Academy of Sciences of the United States of America* **110** , 21077–21082.

Agren J. & Schemske D.W. (2012) Reciprocal transplants demonstrate strong adaptive differentiation of the model organism *Arabidopsis thaliana* in its native range. *New Phytologist* **194** , 1112–1122.

Anderson J.M., Chow W.S. & Park Y.I. (1995) The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. *Photosynthesis Research* **46** , 129–139.

Arnon D.I. (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris* . *PlantPhysiology***24** , 1–15.

Barratt D.H.P., Derbyshire P., Findlay K., Pike M., Wellner N., Lunn J., ... Smith A.M. (2009) Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. *Proceedings of the National Academy of Sciences of the United States of America***106** , 13124–13129.

Berry J. & Bjorkman O. (1980) Photosynthetic response and adaptation to temperature in higher plants. *Annual Review of Plant Physiology***31** , 491–543.

Bhaskara G.B., Wen T.-N., Nguyen T.T. & Verslues P.E. (2017) Protein phosphatase 2Cs and microtubule-associated stress protein 1 control microtubule stability, plant growth, and drought response. *Plant Cell* **29** , 169–191.

Boardman N.K. (1977) Comparative photosynthesis of sun and shade plants.*Annual Review of Plant Physiology* **28** , 355–377.

Cao S.Q., Song Y.Q. & Su L. (2007) Freezing sensitivity in the gigantea mutant of *Arabidopsis* is associated with sugar deficiency.*Biologia Plantarum* **51** , 359–362.

Cohu C.M., Muller O., Adams, W. W. III & Demmig-Adams B. (2014) Leaf anatomical and photosynthetic acclimation to cool temperature and high light in two winter versus two summer annuals. *Physiologia Plantarum* **152** , 164–173.

Cohu C.M., Muller O., Demmig-Adams B. & Adams, W. W. III (2013a) Minor loading vein acclimation for three *Arabidopsis thaliana* ecotypes in response to growth under different temperature and light regimes.*Frontiers in Plant Science* **4** , 240.

Cohu C.M., Muller O., Stewart J.J., Demmig-Adams B. & Adams, W. W. III. (2013b) Association between minor loading vein architecture and light- and CO_2-saturated rates of photosynthetic oxygen evolution among *Arabidopsis thaliana* ecotypes from different latitudes. *Frontiers in Plant Science* **4** .

Dekkers B.J.W., Willems L., Bassel G.W., van Bolderen-Veldkamp R.P. (Marieke), Ligterink W., Hilhorst H.W.M. & Bentsink L. (2011) Identification of Reference Genes for RT–qPCR Expression Analysis in Arabidopsis and Tomato Seeds. *Plant & Cell Physiology***53** , 28–37.

Demmig-Adams B., Stewart J.J. & Adams, W. W. III (2017) Environmental regulation of intrinsic photosynthetic capacity: an integrated view.*Current Opinion in Plant Biology* **37** , 34–41.

Denslow S.A., Rueschhoff E.E. & Daub M.E. (2007) Regulation of the*Arabidopsis thaliana* vitamin B6 biosynthesis genes by abiotic stress. *Plant Physiology and Biochemistry: PPB / Societe Francaise de Physiologie Vegetale* **45** , 152–161.

Ding Y., Lv J., Shi Y., Gao J., Hua J., Song C., ... Yang S. (2019) EGR2 phosphatase regulates OST1 kinase activity and freezing tolerance in *Arabidopsis* . *EMBO* **38** .

Dumlao M.R., Darehshouri A., Cohu C.M., Muller O., Mathias J., Adams, W. W. III & Demmig-Adams B. (2012) Low temperature acclimation of photosynthetic capacity and leaf morphology in the context of phloem loading type. *Photosynthesis Research* **113** , 181–189.

Dyson B.C., Allwood J.W., Feil R., Xu Y.U.N., Miller M., Bowsher C.G., ... Johnson G.N. (2015) Acclimation of metabolism to light in*Arabidopsis thaliana* : the glucose 6-phosphate/phosphate translocator GPT2 directs metabolic acclimation. *Plant, Cell & Environment* **38** , 1404–1417.

Eremina M., Rozhon W. & Poppenberger B. (2016) Hormonal control of cold stress responses in plants. *Cellular and Molecular Life Sciences: CMLS* **73** , 797–810.

Etchells J.P., Peter Etchells J. & Turner S.R. (2017) Realizing pipe dreams – a detailed picture of vascular development. *Journal of Experimental Botany* **68** , 1–4.

Fabregas N., Formosa-Jordan P., Confraria A., Siligato R., Alonso J.M., Swarup R., ... Ibanes M. (2015) Auxin influx carriers control vascular patterning and xylem differentiation in *Arabidopsis thaliana* . *PLoS Genetics* **11** , e1005183.

Fowler S. & Thomashow M.F. (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14** , 1675–1690.

Gauhl E. (1976) Photosynthetic response to varying light intensity in ecotypes of Solanum dulcamara L. from shaded and exposed habitats.*Oecologia* **22** , 275–286.

Gehan M.A., Park S., Gilmour S.J., An C., Lee C.-M. & Thomashow M.F. (2015) Natural variation in the C-repeat binding factor cold response pathway correlates with local adaptation of *Arabidopsis* ecotypes.*Plant Journal* **84** , 682–693.

Gilmour S.J., Fowler S.G. & Thomashow M.F. (2004) *Arabidopsis*transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Molecular Biology* **54** , 767–781.

Gordon A. & Hannon G. (2017) Fastx-toolkit. FASTQ/A short-reads pre-processing tools. 2010. *Unpublished available online at: http://hannonlab. cshl. edu/fastx_toolkit* .

Gorsuch P.A., Pandey S. & Atkin O.K. (2010) Temporal heterogeneity of cold acclimation phenotypes in Arabidopsis leaves. *Plant, Cell & Environment* **33** , 244–258.

Heino P., Sandman G., Lang V., Nordin K. & Palva E.T. (1990) Abscisic acid deficiency prevents development of freezing tolerance in*Arabidopsis thaliana* (L.) Heynh. *TAG. Theoretical and Applied Genetics.* **79** , 801–806.

Hoshino R., Yoshida Y. & Tsukaya H. (2019) Multiple steps of leaf thickening during sun-leaf formation in Arabidopsis. *Plant Journal* **100** , 738–753.

Huner N., Dahal K., Hollis L., Bode R., Rosso D., Krol M. & Ivanov A. (2012) Chloroplast redox imbalance governs phenotypic plasticity: the "grand design of photosynthesis" revisited. *Frontiers in Plant Science* **3** , 255.

Huner N.P.A., Dahal K., Bode R., Kurepin L.V. & Ivanov A.G. (2016) Photosynthetic acclimation, vernalization, crop productivity and "the grand design of photosynthesis." *Journal of Plant Physiology***203** , 29–43.

Huner N.P.A., Oquist G. & Sarhan F. (1998) Energy balance and acclimation to light and cold. *Trends in Plant Science***3** , 224–230.

Huner N.P., Oquist G., Hurry V.M., Krol M., Falk S. & Griffith M. (1993) Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. *Photosynthesis Research* **37** , 19–39.

Jia Y., Ding Y., Shi Y., Zhang X., Gong Z. & Yang S. (2016) The cbfs triple mutants reveal the essential functions of CBFs in cold acclimation and allow the definition of CBF regulons in*Arabidopsis* . *New Phytologist* **212** , 345–353.

Kang J., Zhang H., Sun T., Shi Y., Wang J., Zhang B., ... Gu H. (2013) Natural variation of C-repeat-binding factor (CBFs) genes is a major cause of divergence in freezing tolerance among a group of*Arabidopsis thaliana* populations along the Yangtze River in China. *The New Phytologist* **199** , 1069–1080.

Kant P., Gordon M., Kant S., Zolla G., Davydov O., Heimer Y.M., ... Barak S. (2008) Functional-genomics-based identification of genes that regulate *Arabidopsis* responses to multiple abiotic stresses.*Plant, Cell & Environment* **31** , 697–714.

Katagiri Y., Hasegawa J., Fujikura U., Hoshino R., Matsunaga S. & Tsukaya H. (2016) The coordination of ploidy and cell size differs between cell layers in leaves. *Development* **143** , 1120–1125.

Kilian J., Whitehead D., Horak J., Wanke D., Weinl S., Batistic O., ... Harter K. (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant Journal* **50** , 347–363.

Klimmek F., Sjodin A., Noutsos C., Leister D. & Jansson S. (2006) Abundantly and rarely expressed Lhc protein genes exhibit distinct regulation patterns in plants. *Plant Physiology* **140** , 793–804.

Knight M.R. & Knight H. (2012) Low-temperature perception leading to gene expression and cold tolerance in higher plants. *New Phytologist* **195** , 737–751.

Kozuka T., Kong S.-G., Doi M., Shimazaki K.-I. & Nagatani A. (2011) Tissue-autonomous promotion of palisade cell development by phototropin 2 in Arabidopsis. *Plant Cell* **23** , 3684–3695.

Kunz H.-H., Gierth M., Herdean A., Satoh-Cruz M., Kramer D.M., Spetea C. & Schroeder J.I. (2014) Plastidial transporters KEA1, -2, and -3 are essential for chloroplast osmoregulation, integrity, and pH regulation in *Arabidopsis* . *Proceedings of the National Academy of Sciences of the United States of America* **111** , 7480–7485.

Lee C.-M. & Thomashow M.F. (2012) Photoperiodic regulation of the C-repeat binding factor (CBF) cold acclimation pathway and freezing tolerance in *Arabidopsis thaliana* . *Proceedings of the National Academy of Sciences of the United States of America***109** , 15054–15059.

Lens F., Tixier A., Cochard H., Sperry J.S., Jansen S. & Herbette S. (2013) Embolism resistance as a key mechanism to understand adaptive plant strategies. *Current Opinion in Plant Biology* **16** , 287–292.

Leonardos E.D., Savitch L.V., Huner N.P.A., Oquist G. & Grodzinski B. (2003) Daily photosynthetic and C-export patterns in winter wheat leaves during cold stress and acclimation. *Physiologia Plantarum***117** , 521–531.

Livak K.J. & Schmittgen T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method.*Methods* **25** , 402–408.

López-Juez E., Bowyer J.R. & Sakai T. (2007) Distinct leaf developmental and gene expression responses to light quantity depend on blue-photoreceptor or plastid-derived signals, and can occur in the absence of phototropins. *Planta* **227** , 113–123.

Love M.I., Huber W. & Anders S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15** , 550.

Maruyama K., Takeda M., Kidokoro S., Yamada K., Sakuma Y., Urano K., ... Yamaguchi-Shinozaki K. (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant Physiology***150** , 1972–1980.

Mizoi J., Shinozaki K. & Yamaguchi-Shinozaki K. (2012) AP2/ERF family transcription factors in plant abiotic stress responses.*Biochimica et Biophysica Acta* **1819** , 86–96.

Monroe J.G., McGovern C., Lasky J.R., Grogan K., Beck J. & McKay J.K. (2016) Adaptation to warmer climates by parallel functional evolution of CBF genes in *Arabidopsis thaliana* . *Molecular Ecology***25** , 3632–3644.

Muller O., Stewart J.J., Cohu C.M., Polutchko S.K., Demmig-Adams B. & Adams, W. W. III (2014) Leaf architectural, vascular and photosynthetic acclimation to temperature in two biennials. *Physiologia Plantarum* **152** , 763–772.

Munekage Y.N., Inoue S., Yoneda Y. & Yokota A. (2015) Distinct palisade tissue development processes promoted by leaf autonomous signalling and long-distance signalling in *Arabidopsis thaliana* . *Plant, Cell & Environment* **38** , 1116–1126.

Noren L., Kindgren P., Stachula P., Ruhl M., Eriksson M., Hurry V. & Strand A. (2016) HSP90, ZTL, PRR5 and HY5 integrate circadian and plastid signaling pathways to regulate CBF and COR expression.*Plant Physiology* , 00374.2016.

Oakley C.G., Ågren J., Atchison R.A. & Schemske D.W. (2014) QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs.*Molecular Ecology* **23** , 4304–4315.

Oakley C.G., Savage L., Lotz S., Rudd Larson G., Thomashow M.F., Kramer D.M. & Schemske D.W. (2018) Genetic basis of photosynthetic responses to cold in two locally adapted populations of *Arabidopsis thaliana* . *Journal of Experimental Botany* **69** , 699–709.

Park S., Gilmour S.J., Grumet R. & Thomashow M.F. (2018) CBF-dependent and CBF-independent regulatory pathways contribute to the differences in freezing tolerance and cold-regulated gene expression of two*Arabidopsis* ecotypes locally adapted to sites in Sweden and Italy. *PloS One* **13** , e0207723.

Pertea M., Pertea G.M., Antonescu C.M., Chang T.-C., Mendell J.T. & Salzberg S.L. (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature Biotechnology***33** , 290–295.

Petit J.M., Briat J.F. & Lobréaux S. (2001) Structure and differential expression of the four members of the Arabidopsis thaliana ferritin gene family. *Biochemical Journal* **359** , 575–582.

Pracharoenwattana I., Zhou W., Keech O., Francisco P.B., Udomchalothorn T., Tschoep H., ... Smith S.M. (2010) *Arabidopsis* has a cytosolic fumarase required for the massive allocation of photosynthate into fumaric

acid and for rapid plant growth on high nitrogen.*Plant Journal* **62** , 785–795.

Puhakainen T., Hess M.W., Mäkelä P., Svensson J., Heino P. & Palva E.T. (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis* . *Plant Molecular Biology***54** , 743–753.

Reyes-Diaz M., Ulloa N., Zuniga-Feest A., Gutierrez A., Gidekel M., Alberdi M., ... Bravo L.A. (2006) *Arabidopsis thaliana*avoids freezing by supercooling. *Journal of Experimental Botany***57** , 3687–3696.

Sanderson B.J., Park S., Jameel M.I., Kraft J.C., Thomashow M.F., Schemske D.W. & Oakley C.G. (2020) Genetic and physiological mechanisms of freezing tolerance in locally adapted populations of a winter annual.*American Journal of Botany* **107** , 250–261.

Savitch L.V., Allard G., Seki M., Robert L.S., Tinker N.A., Hüner N.P.A., ... Singh J. (2005) The effect of overexpression of two*Brassica* CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus* . *Plant and Cell Physiology* **46** , 1525–1539.

Savitch L.V., Leonardos E.D., Krol M., Jansson S., Grodzinski B., Hüner N.P.A. & Öquist G. (2002) Two different strategies for light utilization in photosynthesis in relation to growth and cold acclimation. *Plant, Cell & Environment* **25** , 761–771.

Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Pietzsch T., ... Cardona A. (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9** , 676–682.

Shi Y., Ding Y. & Yang S. (2018) Molecular regulation of CBF signaling in cold acclimation. *Trends in Plant Science* **23** , 623–637.

Stewart J.J., Adams, W. W. III, Cohu C.M., Polutchko S.K., Lombardi E.M. & Demmig-Adams B. (2015) Differences in light-harvesting, acclimation to growth-light environment, and leaf structural development between Swedish and Italian ecotypes of *Arabidopsis thaliana* .*Planta* **242** , 1277–1290.

Stewart J.J., Demmig-Adams B., Cohu C.M., Wenzl C.A., Muller O. & Adams, W. W. III (2016) Growth temperature impact on leaf form and function in *Arabidopsis thaliana* ecotypes from northern and southern Europe. *Plant, Cell & Environment* **39** , 1549–1558.

Stewart J.J., Polutchko S.K., Adams, W. W. III & Demmig-Adams B. (2017a) Acclimation of Swedish and Italian ecotypes of *Arabidopsis thaliana* to light intensity. *Photosynthesis Research***134** , 215–229.

Stewart J.J., Polutchko S.K., Adams, W. W. III, Cohu C.M., Wenzl C.A. & Demmig-Adams B. (2017b) Light, temperature and tocopherol status influence foliar vascular anatomy and leaf function in *Arabidopsis thaliana* . *Physiologia Plantarum* **160** , 98–110.

Stitt M. & Hurry V. (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in*Arabidopsis* . *Current Opinion in Plant Biology* **5** , 199–206.

Strand A., Hurry V., Henkes S., Huner N., Gustafsson P., Gardestrom P. & Stitt M. (1999) Acclimation of *Arabidopsis* leaves developing at low temperatures: increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. *Plant Physiology* **119** , 1387–1398.

Thalhammer A., Hincha D.K. & Zuther E. (2014) Measuring freezing tolerance: electrolyte leakage and chlorophyll fluorescence assays.*Methods in Molecular Biology* **1166** , 15–24.

Thalhammer A., Hundertmark M., Popova A.V., Seckler R. & Hincha D.K. (2010) Interaction of two intrinsically disordered plant stress proteins (COR15A and COR15B) with lipid membranes in the dry state.*Biochimica et Biophysica Acta* **1798** , 1812–1820.

Thomashow M.F. (1999) PLANT COLD ACCLIMATION: freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50** , 571–599.

Untergasser A., Cutcutache I., Koressaar T., Ye J., Faircloth B.C., Remm M. & Rozen S.G. (2012) Primer3—new capabilities and interfaces.*Nucleic Acids Research* **40** , e115–e115.

Wakao S., Chin B.L., Ledford H.K., Dent R.M., Casero D., Pellegrini M., ... Niyogi K.K. (2014) Phosphoprotein SAK1 is a regulator of acclimation to singlet oxygen in *Chlamydomonas reinhardtii* .*eLife* **3** , e02286.

Wanner L.A. & Junttila O. (1999) Cold-induced freezing tolerance in*Arabidopsis* . *Plant Physiology* **120** , 391–400.

Xin Z. & Browse J. (1998) eskimo1 mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proceedings of the National Academy of Sciences of the United States of America* **95** , 7799–7804.

Yano S. & Terashima I. (2004) Developmental process of sun and shade leaves in *Chenopodium album* L. *Plant, Cell and Environment***27** , 781–793.

Zhao C., Zhang Z., Xie S., Si T., Li Y. & Zhu J.-K. (2016) Mutational evidence for the critical role of CBF transcription factors in cold acclimation in *Arabidopsis* . *Plant Physiology* **171** , 2744–2759.

Zhen Y. & Ungerer M.C. (2008) Clinal variation in freezing tolerance among natural accessions of *Arabidopsis thaliana* . *New Phytologist* **177** , 419–427.

Tables

TABLE 1 Results of two-way ANOVAs for the effects of CBF1–3 deficiency (*cbf123*) and growth conditions as well as the interaction of these effects (*cbf123* x GC) on leaf photosynthetic capacity (Fig. 5a), leaf dry mass per area (Fig. 5b), and chlorophyll$a$ + b levels (Fig 5c) and expression of associated genes (Fig. 10) for the IT (i.e., IT and it:*cbf123*) and SW (i.e., SW and sw:*cbf123*) backgrounds.

Trait or gene	IT background *cbf123*	IT background Growth Condition	IT background *cbf123* × GC	SW background *cbf123*	SW background Growth Condition	S *c*
Photosynthetic capacity	***	***	***	*n.s.*	***	*n*
Leaf dry mass per area	***	***	***	*n.s.*	***	*n*
Chlorophyll a + b	*	**	*	*n.s.*	***	*n*
AT5G44565	***	***	***	*n.s.*	***	*n*
SUS1 (AT5G20830)	***	***	***	*n.s.*	***	*n*
LCR69 (AT2G02100)	***	***	***	*n.s.*	***	*n*
AT1G13930	***	***	***	*n.s.*	***	*n*
RCI2A (AT3G05880)	***	***	***	*	***	*n*

The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (*n.s.* = not significant).

TABLE 2 Results of two-way ANOVAs for the effects of CBF1–3 deficiency (*cbf123*) and growth conditions as well as the interaction of these effects (*cbf123* × GC) on freezing tolerance of discs from fully expanded leaves (LT_{50}; Fig. 8) and immature, whole plants of 6 to 8 leaves (% survival; Fig. 9) and expression of associated genes from mature leaves (Fig. 11) for the IT (i.e., IT and it:*cbf123*) and SW (i.e., SW and sw:*cbf123*) backgrounds.

Trait or gene	IT background *cbf123*	IT background Growth Condition	IT background *cbf123* × GC	SW background *cbf123*	SW background Growth Conditio
Freezing tolerance, LT_{50}	***	***	***	***	***
Freezing tolerance, % survival	***	***	***	*	***
GolS3 (AT1G09350)	***	***	***	***	***
CIPK25 (AT5G25110)	***	***	***	***	***

KIN2 (AT5G15970)	**	***	**	*	***
EGR2 (AT5G27930)	***	***	***	*n.s.*	***
COR78 (AT5G52310)	***	***	***	***	***
COR15A (AT2G42540)	***	***	***	***	***
LTI30 (AT3G50970)	***	***	***	***	***
COR15B (AT2G42530)	*	***	*	**	***
AT1G21790	*	***	*	*	***

The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (*n.s.* = not significant).

Figure Legends

FIGURE 1 (a) Photosynthetic capacity (i.e., maximal light- and CO_2-saturated rate of oxygen evolution) per leaf area, (b) leaf dry mass per area, (c) level of chlorophyll $a + b$per leaf area, and (d) chlorophyll a/b ratio in leaves of IT (red columns) and SW (blue columns) plants that were grown in low light/warm temperature growth conditions (LLW), low light/cool temperature growth conditions (LLC), high light/warm temperature growth conditions (HLW), or high light/cool temperature growth conditions (HLC). Mean values ± standard deviations ($n = 3$ or 4); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post hoc Tukey–Kramer HSD tests.

FIGURE 2 Light response of reduction state of the primary electron acceptor of photosystem II (Q_A, quantified by chlorophyll fluorescence using the equation 1 - q_L) of leaves from IT (red circles) and SW (blue squares) plants grown under (a) LLW and (b) HLC. Mean values +- standard deviations ($n = 3$); statistically significant differences between ecotypes based on Student's t -tests are indicated with *asterisks* (* =P < 0.05, ** = P < 0.01, *** =P < 0.001); *n.s* . = not significantly different.

FIGURE 3 Relative transcript abundance (via RT-qPCR) of (a)*CBF1* , (b) *CBF2* , and (c) *CBF3* in leaves of IT (red columns) and SW (blue columns) plants that were grown in LLW, LLC, HLW, or HLC conditions. Values are presented relative to the expression level for each respective gene in the IT ecotype grown under LLW. Mean values +- standard deviations ($n = 3$); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post hoc Tukey–Kramer HSD tests.

FIGURE 4 (a) Hierarchical clustering of the $\log_2$ expression data (via RNAseq) for 7,933 genes with an adjusted P- value below 0.01 in one of the pairwise comparisons for differential expression between ecotypes and growth conditions. The three biological replicates for each growth condition/ecotype set are shown as separate columns. (b) Log_2 expression data (via RNAseq) for IT and SW in HLC relative to LLW for genes previously characterized as regulated by CBF1–3 in Col-0 (Jia *et al.* 2016; Park *et al.* 2018). CIPK25 and KIN2 are protein kinases and EGR2 is a protein phosphatase, all participating in cold acclimation signaling (Thomashow 1999; Ding *et al.* 2019). COR47, LTI30, and LTI29 each are cold and drought-induced dehydrin proteins (Puhakainen*et al.* 2004). SUS1 and GOLS3 are stress-induced sucrose synthase and galactinol synthase enzymes, respectively (Maruyama *et al.*2009). COR15B is essential for chloroplast membrane integrity during freezing (Thalhammer, Hundertmark, Popova, Seckler & Hincha 2010).

FIGURE 5 (a) Photosynthetic capacity (light- and CO_2-saturated rate of oxygen evolution) per leaf area, (b) leaf dry mass per area, (c) level of chlorophyll $a + b$per leaf area, and (d) chlorophyll a/b ratio in leaves of IT (red columns), it:*cbf123* (light red columns), SW (blue columns), and sw:*cbf123* (light blue columns) plants that were grown in LLW or HLC. Mean values +- standard deviations ($n = 6$); Groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests.

FIGURE 6 (a) Leaf thickness of IT (red column), it:*cbf123* (light red column), SW (blue column), and

sw:*cbf123* (light blue column) plants that were grown in HLC, as well as representative images of leaf cross-sections for (b) IT, (c) it:*cbf123* , (d) SW, and (e) sw:*cbf123* . For (a), mean values +- standard deviations ($n = 3$); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests.

FIGURE 7 (a) Rosette diameter of IT (red column), it:*cbf123* (light red column), SW (blue column), and sw:*cbf123* (light blue column) after 40 days of growth in HLC, as well as images of representative (b) IT, (c) it:*cbf123* , (d) SW, and (e) sw:*cbf123* plants. For (a), mean values +- standard deviations ($n = 5$); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests.

FIGURE 8 (a–c) Cellular electrolyte leakage from mature leaves, and (d) images of mature leaves with photosystem II photochemical efficiency (visualized via false colors based on F_v/F_m values) from IT (red circles), it:*cbf123* (light red circles), SW (blue squares), and sw:*cbf123* (light blue squares) plants grown under LLW or HLC. For (a,b) mean values ($n = 3$). For (c), mean values +- standard deviations ($n = 3$); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests.

FIGURE 9 Percent survival after an overnight freezing treatment (16 h at -10degC) of IT (red columns), it:*cbf123* (light red columns), SW (blue columns), and sw:*cbf123* (light blue columns) plants grown under LLW or HLC. Mean values +- standard deviations ($n = 3$ plates, each of which contained 40 plants); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests.

FIGURE 10 Relative transcript abundance (via RT-qPCR) for (a) AT5G44565, (b) *SUS1,* (c) *LCR69,* (d) AT1G13930, and (e) *RCI2A* in leaves of IT (red columns), it:*cbf123* (light red columns), SW (blue columns), and sw:*cbf123* (light blue columns) plants grown in LLW or HLC. All values are normalized based on the expression levels of IT in LLW. Mean values +- standard deviations ($n = 3$); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests. SUS1 is a stress induced sucrose synthase (Barratt *et al.* 2009).

FIGURE 11 Relative transcript abundance (via RT-qPCR) for (a)*GolS3* , (b) *CIPK25,* (c) *KIN2* , (d) *EGR2* , (e)*COR78,* (f) *COR15A* , (g) *LTI30* , (h) *COR15B* , and (i) AT1G21790 in leaves of IT (red columns), it:*cbf123* (light red columns), SW (blue columns), and sw:*cbf123* (light blue columns) plants grown under LLW or HLC. CIPK25 and KIN2 are protein kinases and EGR2 is a protein phosphatase, all participating in cold acclimation signaling (Thomashow 1999; Ding *et al.* 2019). LTI30 is a drought-induced dehydrin protein (Puhakainen *et al.* 2004). GOLS3 is a galactinol synthase enzyme (Fowler & Thomashow 2002). COR15B is essential for chloroplast membrane integrity during freezing (Thalhammer *et al.* 2010). All values are normalized based on the expression levels of IT under LLW. Mean values +- standard deviations ($n = 3$); groups that share the same letters are not considered statistically different, and groups that do not share the same letters are considered statistically different based on one-way ANOVA and post-hoc Tukey–Kramer HSD tests.

Supplementary information

Supplemental Figure and Table legends

FIGURE S1 Timelines of set air temperatures and measured leaf temperatures for experiments under the LLW (low light & warm temperature), LLC (low light & cool temperature), HLW (high light & warm temperature), and HLC (high light & cool temperature) growth conditions.

FIGURE S2 Log_2 gene expression (via RNAseq) for genes from the heat map in Figure 4a that are a part of the photosynthesis GO category (GO:0015979). Genes that are more strongly induced in IT in response to HLC growth conditions relative to the response of SW to HLC conditions, such as the chloroplastic glucose-6P/phosphate transporter *GPT2* (AT1G61800), the cytosolic fumarase *FUM2* (AT5G76550), the chloroplastic, the phosphofructokinase AT1G76550, the ferritin *FER4 (* AT2G30400), the chloroplastic Na^+ efflux regulator *KEA2*(AT4G00630), and the high-light inducible LHC2-component *LHCB4.3*(AT2G40100), are located at the top of table. Whereas genes that are more strongly repressed in HLC conditions in the IT ecotype relative to the SW ecotype in HLC conditions can be found at the bottom of the table, such as LHC2-components *LHCB1.4* (AT2G34430), *LHCB3*(AT5G54270), *LHCB1.5* (AT2G34420), and *LHCB1.1*(AT1G29920), PSII component *PSBP-2* (AT2G30790), and triose phosphate transporter *TPT* (AT5G46110).

Table S1. The 1415 genes up-regulated in HLC-grown SW plants (minimum fold change of 2 and adjusted P -value of < 0.01).

Table S2. The 1992 genes up-regulated in HLC-grown IT plants (minimum fold change of 2 and adjusted P -value of < 0.01).

Table S3. The 1531 genes down-regulated in the HLC-grown SW plants (minimum fold change of 2 and adjusted P -value of < 0.01).

Table S4. The 1671 genes down-regulated in HLC-grown IT plants (minimum fold change of 2 and adjusted P -value of < 0.01).

Table S5 . The 1090 genes up-regulated in both HLC-grown IT and SW plants (minimum fold change of 2 and adjusted P -value of < 0.01)

Table S6. The 804 genes down-regulated in both HLC-grown IT and SW plants (minimum fold change of 2 and adjusted P -value of < 0.01).

Table S7. The 356 genes that co-clustered into the cluster labeled "HLC-Specific Group 1" and "HLC-Specific Group 2" in Fig. 4a.

Table S8. PANTHER Overrepresentation Test (Released 2019-07-11) on GO Ontology database (Released 2019-12-09) for genes up-regulated in both HLC-grown IT and SW plants.

Table S9. PANTHER Overrepresentation Test (Released 2019-07-11) on GO Ontology database (Released 2019-12-09) for genes down-regulated in both HLC-grown IT and SW plants.

Table S10. SW-HLC differentially regulated genes with induction in SW ecotype was at least two-folder greater than induction in the IT ecotype.

Table S11. PANTHER Overrepresentation Test (Released 2019-07-11) on GO Ontology database (Released 2019-12-09) for genes induced more strongly in SW ecotype.

Table S12. SW-HLC differentially regulated genes with down-regulation in SW ecotype was at least two-folder greater than down-regulation in the IT ecotype.

Table S13. IT-HLC differentially regulated genes with induction in IT ecotype was at least two-folder greater than induction in the SW ecotype.

Table S14. PANTHER Overrepresentation Test (Released 2019-07-11) on GO Ontology database (Released 2019-12-09) for genes induced more strongly in IT ecotype.

Table S15. IT-HLC differentially regulated genes with downregulation in IT ecotype was at least two-folder greater than downregulation in the SW ecotype.

Table S16. PANTHER Overrepresentation Test (Released 2019-07-11) on GO Ontology database (Released 2019-12-09) for genes induced more downregulated in IT ecotype.

Table S17. Genes previously reported to be CBF1–3-regulated—Comparison of transcriptomic data from four growth conditions for both ecotypes to genes identified as having diminished induction in it:*cbf123* and sw:*cbf123* mutants following a 24-hour 4degC treatment by Park, Gilmour, Grumet, & Thomashow (2018).

Table S18. Comparison of transcriptomic data from four growth conditions for both ecotypes to genes reported to be over-expressed under low light and warm temperatures by Park, Gilmour, Grumet, & Thomashow (2018).

Table S19. Data collected on sw:*cbf2* mutant (RT-qPCR, leaf thickness, photosynthetic capacity, electrolyte leakage, freezing survival, chlorophyll per unit area, rosette diameter).

Hosted file

`BakerCR_resubmission_Maintextfigures.pdf` available at https://authorea.com/users/343982/articles/538616-role-of-cbf-transcription-factors-during-long-term-acclimation-to-high-light-and-low-temperature-in-two-ecotypes-of-arabidopsis-thaliana