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Fitness Benefits and Costs of Cold Acclimation in *Arabidopsis thaliana*

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ABSTRACT: When resources are limited, there is a trade-off between growth/reproduction and stress defense in plants. Most temperate plant species, including *Arabidopsis thaliana*, can enhance freezing tolerance through cold acclimation at low but nonfreezing temperatures. Induction of the cold acclimation pathway should be beneficial in environments where plants frequently encounter freezing stress, but it might represent a cost in environments where freezing events are rare. In *A. thaliana*, induction of the cold acclimation pathway critically involves a small subfamily of genes known as the *CBF*s. Here we test for a cost of cold acclimation by utilizing (1) natural accessions of *A. thaliana* that originate from different regions of the species' native range and that have experienced different patterns of historical selection on their *CBF* genes and (2) transgenic *CBF* overexpression and T-DNA insertion (knockdown/knockout) lines. While benefits of cold acclimation in the presence of freezing stress were confirmed, no cost of cold acclimation was detected in the absence of freezing stress. These findings suggest that cold acclimation is unlikely to be selected against in warmer environments and that naturally occurring mutations disrupting *CBF* function in the southern part of the species range are likely to be selectively neutral. An unanticipated finding was that cold acclimation in the absence of a subsequent freezing stress resulted in increased fruit production, that is, fitness.

Keywords: fitness, costs, cold acclimation, freezing tolerance, *CBF* genes, *Arabidopsis thaliana.*

Introduction

Phenotypes involved in tolerance or defense against environmental stress can be inducibly or constitutively expressed (Maleck and Dietrich 1999; Wittstock and Gershenzon 2002). Inducible phenotypes are expressed only in response to specific cues indicating that a defense is needed. The most common explanation for the evolution of inducible defenses is that a constitutive defense is too costly to maintain in the absence of an environmental stress. This is because of an associated allocation cost (Strauss et al. 2002; Walters and Heil 2007) whereby energy and resources allocated to stress defense cannot be utilized for growth or reproduction (Bazzaz et al. 1987; Herms and Mattson 1992). Allocation costs can be documented when, by experimental manipulation, a defense phenotype is expressed in the absence of an environmental stress (Heil 2001, 2002; Heil and Baldwin 2002; Cipollini et al. 2003).

Freezing temperatures represent an important environmental stress for plants and limit their growth, productivity, and geographic distribution. Most temperate plant species, including the model species *Arabidopsis thaliana*, can significantly increase freezing tolerance through preexposure to low but nonfreezing temperatures, a phenomenon known as cold acclimation (Gilmour et al. 1988; Xin and Browse 2000). This process involves extensive physiological and biochemical changes that may be metabolically costly, including distinct changes in membrane lipid composition and abundance, global gene expression patterns, and intracellular compatible osmolytes (Welti et al. 2002; Cook et al. 2004; Hannah et al. 2005). Although the genetics and physiology of plant cold acclimation have been studied intensively in recent years, the ecological and evolutionary consequences of cold acclimation as an inducible response has received far less attention, especially as it relates to population-level variation in freezing tolerance across diverse temperature environments.

We previously documented a steep latitudinal cline in freezing tolerance in *A. thaliana* that follows a gradient of temperature variation across the species' native range (Zhen and Ungerer 2008*a*). This pattern of clinal variation was shown to be attributable, at least in part, to relaxed purifying selection on the *CBF* (C-repeat binding factor) subfamily of transcription activators in the southern, warmer part of the species' range. The *CBF* genes are critical components of a genetic network involved in initiating the cold acclimation pathway in *A. thaliana* and numerous other temperate plant taxa (Jaglo et al. 2001;

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Vogel et al. 2005; Van Buskirk and Thomashow 2006; Xiong and Fei 2006). The *A. thaliana* genome harbors three *CBF* genes known alternatively as *CBF1*, *CBF2*, and *CBF3* or *DREB1b*, *DREB1c*, and *DREB1a*. These genes are induced rapidly and transitorily at low, nonfreezing temperatures, and they encode transcriptional activators that regulate the expression of approximately 100 downstream cold-responsive (*COR*) genes (Fowler and Thomashow 2002; Vogel et al. 2005). The inference of relaxed selection on this subfamily was based on observations that *A. thaliana* accessions from the southern part of the species' range were found to have an approximate threefold increase in nonsynonymous nucleotide substitution rates in their *CBF* genes as well as to possess a number of regulatory mutations leading to abrogated expression of certain *CBF* subfamily members in particular southern accessions (Zhen and Ungerer 2008*b*).

Mutations arising and persisting in the *CBF* subfamily in southern *A. thaliana* accessions could be selectively neutral or possibly selectively beneficial depending on whether there is an allocation cost associated with cold acclimation. For example, if cold acclimation is metabolically costly and if the cold acclimation pathway is induced in the southern part of the species' range but temperatures rarely drop to where plants experience freezing-induced damage or death, then mutations that compromise proper functioning of the cold acclimation pathway might be favored by natural selection. In contrast, in the absence of such a cost, cold acclimation capacity is unlikely to be selected against, and thus mutations compromising *CBF* function are expected to be selectively neutral in the southern part of the species' range. In this article, we test these alternative hypotheses by quantifying the fitness benefits and the potential fitness costs of cold acclimation in natural *A. thaliana* accessions from both northern and southern regions of the species' native range as well as in *CBF* T-DNA insertion lines and *CBF* overexpression transgenic lines. We discuss our results as they relate to intraspecific variation in freezing tolerance in *A. thaliana* and in a more general context of detecting plasticity costs.

Material and Methods

Plant Materials

Natural Accessions. Seeds of 12 accessions of *Arabidopsis thaliana* (L.) Heynh were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at the Ohio State University. These accessions represent wild populations originally collected from the species' native range. Accessions were categorized as being from the northern part of the range (N1–N6) or the southern part of the range (S1–S6; table A1 in the online edition of the *American Naturalist*) on the basis of differences in both geographic origin and maximum freezing tolerance (Zhen and Ungerer 2008*a*, 2008*b*). Northern accessions are derived from latitudes at or above 49.4°N, where mean January temperatures are below 0°C. These northern accessions have higher cold acclimation capacity and possess *CBF* genes with normal expression levels and low levels of nonsynonymous nucleotide polymorphism. In contrast, southern accessions are derived from latitudes at or below 42°N, where mean January temperatures equal or exceed 7.7°C. These accessions possess multiple coding and/or regulatory mutations in their *CBF* genes resulting from relaxed purifying selection on these genes in warmer climates (Zhen and Ungerer 2008*b*).

Overexpression Lines. Seeds of four *A. thaliana* transgenic lines overexpressing individual *CBF* genes and of a single null vector control line (B6) were graciously provided by the laboratory of M. Thomashow at Michigan State University (table A2 in the online edition of the *American Naturalist*). Lines used in this study include two *CBF1* overexpression lines (G5, G6) and single *CBF2* and *CBF3* overexpression lines (E24 and A40, respectively). These transgenic lines have a single inserted *CBF* copy driven by the CaMV 35S promoter in the background of *A. thaliana* accession Ws-2 (Gilmour et al. 2000, 2004). The Ws-2 accession originates from Russia and exhibits high cold acclimation capacity and freezing tolerance. All overexpression lines were confirmed via real-time polymerase chain reaction (RT-PCR) in non-cold-acclimated plants before experiments were initiated.

T-DNA Insertion Lines. Seeds of individual T-DNA insertion lines with insertions in or near the three *CBF* genes were obtained through the ABRC. Confirmation of insertions and determination of homozygosity was accomplished using the "iSct Primers" tool available at the Salk Institute Genomic Analysis Laboratory Web site (http://signal.salk.edu/tdnaprimers.2.html). Individuals homozygous for T-DNA insertions within or near the three *CBF* genes were tested via RT-PCR for expression of the relevant *CBF* copy during cold acclimation. Lines lacking (or with greatly reduced) expression of the *CBF* target were retained. These individuals were allowed to undergo self-fertilization, and the resulting seeds were utilized in our study (table A3 in the online edition of the *American Naturalist*). Three *CBF2* and two *CBF3* T-DNA insertion lines were identified by these methods; no *CBF1* T-DNA insertion line was identified by our screen.

Experimental Design, Cold Acclimation, and Freezing Treatments

Plants were subjected to four different combinations of cold acclimation and freezing stress (table 1). Comparison of fitness differences among genotypes (i.e., natural accessions and/or transgenic lines) between treatments 1 and 2 allows quantification of the benefit of cold acclimation when plants experience freezing stress. Comparison of fitness differences among genotypes between treatments 3 and 4 enables quantification of the cost of cold acclimation in the absence of freezing stress.

Twenty replicate individuals per genotype were evaluated for all treatments. All seeds were sowed in planting media (two parts Sun Gro MetroMix 350 planting media to one part sand) in 72-well plastic flats (54 cm × 27 cm) and subirrigated throughout the experiment. Each flat comprised two complete replicates of all genotypes, with all plant positions randomized. After sowing, flats were kept at 4°C in the dark for 3 days for stratification and to promote uniform germination. Following this initial treatment, plants were transferred to a 21°C growth room and grown under short days (10L : 14D). Twenty-five days after sowing, plants at the rosette stage were subjected to cold acclimation and/or freezing treatments, depending on the treatment group.

Cold acclimation was conducted in a 4°C walk-in cold chamber, where plants experienced the same photoperiod as in the growth room. This treatment consisted of subjecting plants to three consecutive rounds of 3 days at 4°C, with an intervening day between rounds 1 and 2, and between rounds 2 and 3, in the 21°C growth room. The rationale for multiple rounds of cold acclimation was to increase the likelihood of detecting a cost (if present) by multiple inductions of the *CBF* pathway. After the third round of cold acclimation, plants were either returned to the growth room (treatment 4) or subjected to freezing stress immediately (treatment 2).

Freezing treatments were conducted in an ESPEC ESU-3CA Platinous series environmental test chamber (Hudsonville, MI). Plants were exposed to two nights of freezing stress (−10°C for the first night and −14°C for the second night), with the intervening day at 4°C. During periods of cooling and warming within the chamber, rates of temperature change were set to 2°C h^{-1}, and minimum temperatures (−10° or −14°C) were maintained for 2.5 h. During cooling periods, ice chips were added to flats when the chamber temperature reached −1°C, to induce ice nucleation and prevent plant tissue supercooling. Following freezing treatments, plants were transferred back to the 21°C growth room.

Table 1: Experimental treatments utilized in the current study

Treatment	Cold acclimation	Freezing
1	−	+
2	+	+
3	−	−
4	+	−

Note: Each treatment consists of a different combination of cold acclimation and freezing stress. Plus and minus symbols indicate presence and absence, respectively.

Phenotypic Measurements

Survivorship of plants exposed to freezing stress (treatments 1 and 2) was recorded as the proportion of replicates per accession/transgenic line that were still alive after 3 weeks posttreatment. For plants not experiencing freezing stress (treatments 3 and 4), reproductive output was recorded as fruit number on the main inflorescence and the axillary stems and basal shoots. Total fruit number was determined by summing the above. Mean seed number per fruit was averaged by counting the seeds of five normal-looking fruits per plant. Total seed number was estimated by multiplying total fruit number and mean seed number per fruit. Several additional fitness-related morphological traits were also measured, including bolting time, rosette leaf number at bolting, early flower number (the cumulative number of flowers produced 10 d after bolting), maximum rosette diameter (measured at the end of experiment), and final plant height.

Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence data were collected as a potential physiological component of fitness variation in response to cold acclimation. Dark-adapted chlorophyll fluorescence, F_v/F_m, measures the potential quantum efficiency of photosystem II (F_v is the total amount of variable fluorescence; F_m is the maximum fluorescence yield). This physiological measure is sensitive to environmental stress–induced photoinhibition of photosystem II reaction centers (Maxwell and Johnson 2000; Ehlert and Hincha 2008). The more stressful the physiological condition, the lower the F_v/F_m ratio. If there is a measurable cost of cold acclimation in natural accessions, then we can expect a greater reduction in the F_v/F_m ratio in northern accessions compared with southern accessions during cold acclimation and/or a slower recovery in days following acclimation.

Predawn dark-adapted F_v/F_m ratio was measured on northern and southern accessions in treatment 4, using a photosynthesis yield analyzer MINI-PAM (Heinz Walz).

Measurements were taken on four to six individuals per accession at the following time points: (1) immediately before the cold acclimation treatment (CA), (2) on the first day of the first CA, (3) on the third day of the first CA, (4) on the intervening day at room temperature following the first CA, (5) on the third day of the second CA, (6) on the intervening day at room temperature following the second CA, (7) on the first day of the third CA, (8) on the third day of the third CA, and (9) every day for 7 d following the third CA (app. E, which is available online in a zip file).

Data Analysis

Mean survivorship of different plant categories (i.e., northern accessions, southern accessions, overexpression lines, and T-DNA insertion lines) in treatment 2 were compared, using one-way ANOVA and Tukey's HSD test. Phenotypic data of northern and southern accessions from treatments 3 and 4 were analyzed using ANOVA according to the model

$$y = \mu + \text{Line (Origin)} + \text{Origin} + \text{Acclimation} + \text{Line (Origin)} \times \text{Acclimation} + \text{Acclimation} \times \text{Origin} + E, \quad (1)$$

where Line is a random effect and Origin represents either the northern or the southern accession designation. Repeated-measures ANOVA was used to compare chlorophyll florescence data from the northern and the southern accessions in treatment 4.

For both treatments 3 and 4, overexpression lines were compared with the control line B6 using one-way ANOVA and Dunnett's test. The T-DNA insertion line *CBF3*a was compared with its background line CS8846, using Student's *t*-test. All other T-DNA insertion lines were compared with their appropriate background line Col-0 using Dunnett's test. All statistical analyses were performed using JMP, version 7.0.1.

Results

Cold Acclimation Enhances Freezing Tolerance

The relatively severe stress imposed on plants subjected to freezing temperatures (one night at −10°C and the next night at −14°C) resulted in high mortality. In the absence of cold acclimation (treatment 1), mortality was 100% for all natural accessions and all *CBF* T-DNA insertion lines (fig. 1). The only plants to survive freezing stress under non-cold-acclimated conditions were *CBF* overexpression lines, albeit at low frequency (survivorship = 20.4%). This finding is consistent with previous reports demonstrating that transgenic *CBF* overexpression results in induction of the cold acclimation pathway even in the absence of low temperature (Gilmour et al. 2000, 2004).

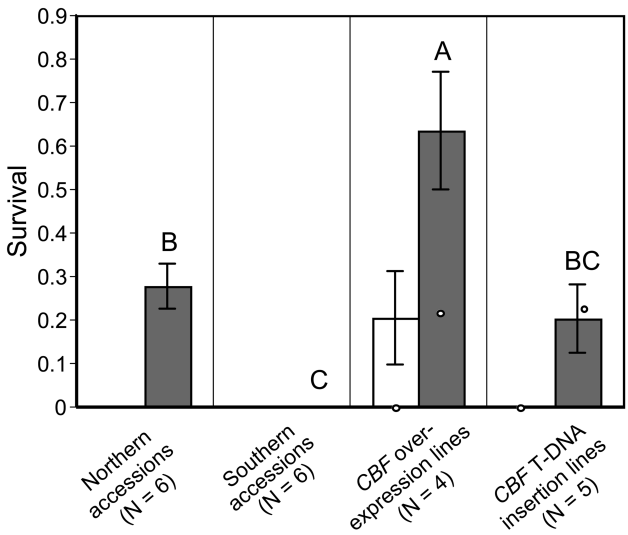

Figure 1: Survivorship following freezing stress (treatments 1 and 2). Gray bars indicate cold-acclimated plants; white bars indicate non-cold-acclimated plants. Missing bars indicate zero survivorship. Letters above gray bars indicate significant pairwise differences among different categories of cold-acclimated plants (Tukey-Kramer HSD test). Open circles indicate mean survivorship of the control line for *CBF* overexpression (null vector insertion line B6) or T-DNA insertion lines (genetic background lines Col-0 and cs8846). Error bars indicate ±1 SE.

Cold acclimation increased survivorship for all accessions/transgenic lines, with the exception of southern accessions, for which mortality remained 100% (fig. 1). This result also corroborates previous reports demonstrating that natural accessions of *A. thaliana* from the southern part of the species' range exhibit reduced cold acclimation capacity and maximum freezing tolerance relative to accessions from the northern part of the range (Cook et al. 2004; Zhen and Ungerer 2008*a*).

Under cold-acclimated conditions (treatment 2), the *CBF* overexpression lines exhibited the highest survivorship (63.4%), followed by the northern accessions (27.7%) and the *CBF* T-DNA insertion lines (20.2%; fig. 1). The higher survivorship of overexpression lines was likely attributable to their high constitutive *CBF* expression levels in addition to natural acclimation that took place under cold exposure. T-DNA insertion lines showed a trend of intermediate values relative to the northern and the southern accessions.

Evaluating the Cost of Cold Acclimation

Northern versus Southern Natural Accessions. For treatments not involving freezing stress (i.e., treatments 3 and 4), plant survivorship was 100% and thus fruit number was used as a measure of fitness. If there is a cost associated with cold acclimation, a decrease in fitness is predicted for cold-acclimated plants (treatment 4) compared with non-cold-acclimated plants (treatment 3), and the decrease is expected to be greater for the northern accessions because of their higher cold acclimation capacity (Zhen and Ungerer 2008*b*). Within this experimental framework, a cost of cold acclimation can be revealed by a significant Origin × Acclimation interaction in an ANOVA model (see eq. [1]) with the pattern of response described above. With total fruit number as the response variable, a significant effect of Acclimation was detected (app. B, which is available online in a zip file; $F = 21.4238$, $P = .0009$) but a significant Origin × Acclimation interaction was not revealed ($F = 1.0126$, $P = .3374$), indicating no difference among the northern and the southern accessions in their response to cold acclimation and thus no evidence of a higher cost of cold acclimation among the northern accessions. A significant effect was also detected for Line(Origin) ($F = 7.4306$, $P = .002$), but there was no significant Line(Origin) × Acclimation interaction ($F = 0.7917$, $P = .6369$).

Interestingly, the cold acclimation treatment resulted in an actual increase in total fruit number (fig. 2*A*; Student's *t*-test, $P = .001$ for northern accessions, $P = .0154$ for southern accessions). Analyses of mean seed number per fruit and estimated total seed number showed similar patterns to those for total fruit number (apps. C and D, which are available online in a zip file). The increase in fruit number in response to cold acclimation was disproportionately attributable to elevated fruit production on axillary and basal shoots (fig. 2*B*, 2*C*).

Several additional fitness-related traits (i.e., plant height, bolting time, rosette leaf number at bolting, maximum rosette diameter, and early flower number) were also measured and analyzed. We failed to detect a significant origin × acclimation interaction for any of these additional traits (apps. C and D). However, a significant effect of line was detected for all traits; a significant effect of origin was detected for rosette leaf number at bolting and early flower number; a significant effect of acclimation was detected for rosette leaf number at bolting, maximum rosette diameter, and early flower number; and a significant line(origin) × acclimation interaction was detected for bolting time (app. D).

To explore a potential physiological component of fitness in natural accessions, we examined the dark-adapted F_v/F_m ratio, which is the maximum quantum efficiency of photosystem II. We observed declines of F_v/F_m ratio during each cold acclimation period for both the northern and the southern accessions, with the largest declines observed during the first cold acclimation treatment (app. E). Our data, however, failed to reveal a significant difference between the northern and the southern accessions in these response patterns (repeated-measures ANOVA: $F_{1,53} =$

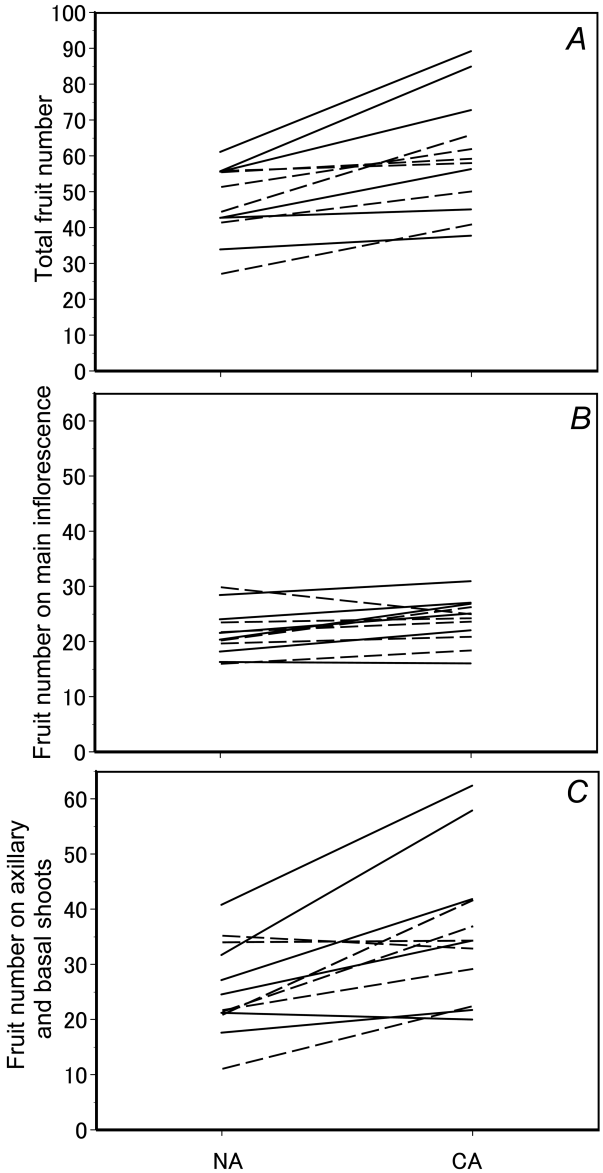

Figure 2: *A*, Total fruit number of northern (*solid lines*) and southern (*dashed lines*) natural accessions in the absence of freezing stress (treatments 3 and 4). *B*, Fruit number on the main inflorescence in the absence of freezing stress. *C*, Fruit number on axillary and basal shoots in the absence of freezing stress. *CA*, cold-acclimated plants; *NA*, non-cold-acclimated plants.

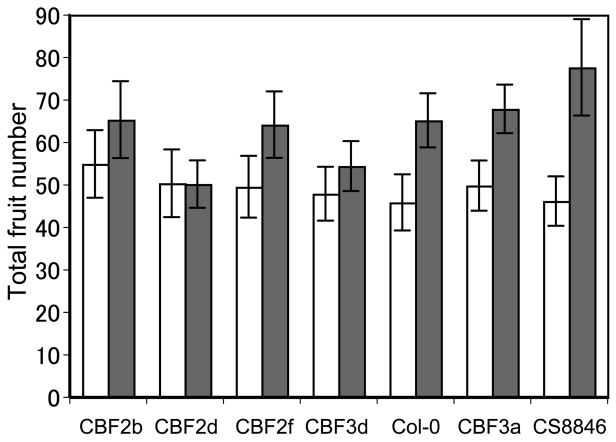

Figure 3: Total fruit number of *CBF* transfer DNA insertion lines and genetic background control lines. *Arabidopsis thaliana* accession CS8846 is the control line for *CBF3*a, whereas accession Col-0 is the control for all other insertion lines. White bars indicate non-cold-acclimated plants; gray bars indicate cold-acclimated plants. Error bars indicate ±1 SE.

0.0127, $P = .9107$). These findings were consistent with the results of our fruit number and other fitness-related morphological data indicating no detectable cost of cold acclimation in natural accessions. We noted that F_v/F_m ratios increased over the duration of the experiment. This result might be attributable to the fact that measurements were begun when plants were small and leaves were not fully expanded.

T-DNA Insertion Lines. To evaluate changes in the potential cost of cold acclimation arising from mutations in individual *CBF* copies, we compared the fitness of several *CBF* T-DNA insertion lines (for *CBF2* and *CBF3*) with their genetic background control lines under non-cold-acclimated and cold-acclimated conditions without a subsequent freezing stress (treatments 3 and 4). Total fruit number of T-DNA insertion line *CBF3*a was comparable to its genetic background control line CS8846 under both cold-acclimated (Student's *t*-test: $t = -0.7806$, $P = .44$) and non-cold-acclimated (Student's *t*-test: $t = 0.4398$, $P = .6626$) conditions (fig. 3). The T-DNA insertion lines *CBF2*b, *CBF2*d, *CBF2*f, and *CBF3*d also displayed similar values to their genetic background control line Col-0 under both cold-acclimated and non-cold-acclimated conditions (fig. 3; app. F, which is available online in a zip file). There were no *CBF1* T-DNA insertion lines recovered in our screen, and thus mutations in this *CBF* copy were not evaluated. Consistent with results for the natural accessions, a pattern of increased fruit production was observed in response to cold acclimation for all T-DNA insertion and control lines, with the exception of *CBF2*d.

Costs Associated with CBF *Overexpression*

The CBF transcription activators are thought to have largely redundant functions with regard to induction of the cold acclimation pathway (Gilmour et al. 2004). To explore potential variation among *CBF* copies to function in this capacity and to evaluate potential costs of this function, we utilized available *CBF1–3* overexpression lines and measured fitness variation under both non-cold-acclimated and cold-acclimated conditions without a subsequent freezing stress. Two *CBF1* overexpression lines, one *CBF2* overexpression line, and one *CBF3* overexpression line were evaluated. *CBF1* overexpression lines G5 and G6 displayed fitness values that were comparable to the null vector control line B6 both with and without cold acclimation treatment, indicating no cost of *CBF1* overexpression (fig. 4). In contrast, overexpression lines *CBF2* (E24) and *CBF3* (A40) exhibited significantly lower fitness compared with the null vector control both with and without cold acclimation treatment (fig. 4; app. F), indicating a clear cost of *CBF2* and *CBF3* overexpression.

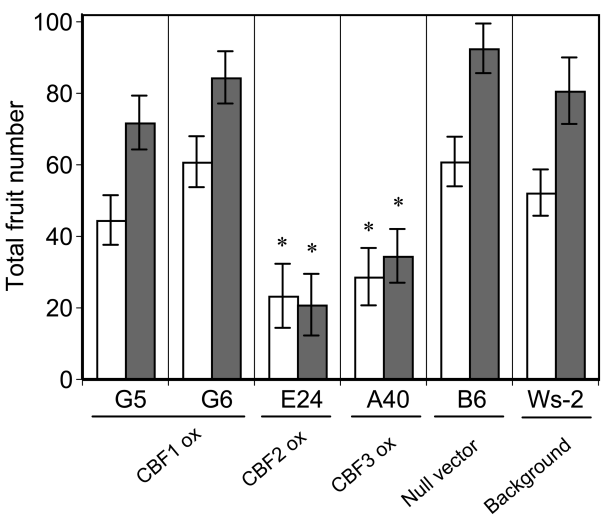

Figure 4: Total fruit number of *CBF1–3* overexpression lines and the null vector control line B6. Data for the genetic background line Ws-2 are shown but were not included in the statistical analysis. White bars indicate non-cold-acclimated plants; gray bars indicate cold-acclimated plants; an asterisk indicates a significant difference between overexpression lines and the null vector control line B6 (Dunnett's test). Error bars indicate ±1 SE.

Discussion

Benefits and Costs of Inducible Defenses

Inducible tolerance and defense phenotypes play a critical role in the ability of many organisms to respond to environmental stress factors. Investigating the benefits and potential costs of such phenotypes is important for a clearer understanding of organism-environment interactions and evolutionary population dynamics. Most studies that have explored the existence of such costs in plants have focused on inducible defenses against biotic stressors, such as pathogen or herbivore pressure. Costs have been revealed in many cases (Smedegaardpetersen and Stolen 1981; Baldwin 1998; Heil et al. 2000; Purrington 2000; Heil 2002; Heil and Walters 2009) but by no means are found to be universal (Bergelson and Purrington 1996).

Cold acclimation is an important inducible stress response in plants and represents an excellent phenotype for studying potential allocation costs in *Arabidopsis thaliana* because of the broad distribution of this species across diverse thermal environments and the detailed understanding of the underlying genetics of this phenotype. The CBF transcriptional activators are known to play a critical role in initiating the cold acclimation response (Thomashow 2001; Fowler and Thomashow 2002; Cook et al. 2004; Hannah et al. 2005; Van Buskirk and Thomashow 2006) and have been the focus of multiple studies investigating natural functional variation and historical patterns of selection on the cold acclimation pathway (Lin et al. 2008; McKhann et al. 2008; Zhen and Ungerer 2008*b*). Moreover, overexpression lines are available for all three *CBF* copies, and T-DNA insertion lines are available for *CBF2* and *CBF3*, thus providing additional useful resources for studying potential costs of the cold acclimation pathway governed by the *CBF*s.

The beneficial effects of cold acclimation on freezing tolerance have long been recognized, and the results presented in this study largely confirm previous observations. Cold acclimation increased freezing tolerance for all experimental groups examined herein, with the exception of southern accessions. The lack of increased freezing tolerance in this group was likely not attributable to a complete lack of cold acclimation capacity but rather to the severity of the freezing stress imposed (one night at −10°C and the next night at −14°C). Previous work (Hannah et al. 2005; Zhen and Ungerer 2008*a*) has indeed demonstrated that all natural *A. thaliana* accessions have at least some cold acclimation capacity, although in southern accessions this capacity appears to be diminished on account of relaxed selection in warmer climates (Zhen and Ungerer 2008*b*).

Also of note in this study is a trend (though not significant) of greater capacity for cold acclimation in the *CBF* T-DNA insertion lines relative to the southern accessions. This may reflect redundancy in *CBF1–3* function given that only a single *CBF* member is inactivated in each T-DNA insertion line. (T-DNA insertion lines were developed in the Col-0 or CS8846 genetic backgrounds, both of which are representative of northern accessions with *CBF* genes that remain under strong purifying selection.) In contrast, in southern accessions, the entire *CBF* subfamily has accumulated coding-region and regulatory mutations (Zhen and Ungerer 2008*b*), and thus the southern accessions might be predicted to exhibit lower capacity for cold acclimation as a consequence. We cannot rule out, however, that observed differences between T-DNA insertion lines and southern accessions also reflect additional variation in a pathway(s) unrelated to *CBF*.

Our study failed to identify (1) a fitness cost associated with cold acclimation and (2) differential costs in comparisons of the southern versus the northern accessions and comparisons of T-DNA insertion lines with their genetic background control lines. Indeed, an unanticipated finding in this study was that cold acclimation actually increased fitness as measured by total fruit number. This increase was observed, on average, across all analyzed natural accessions/transgenic lines and was found to be repeatable in a smaller follow-up experiment (app. G, which is available online in a zip file). This second experiment exposed plants to a control treatment (no cold acclimation), a 3-day cold acclimation treatment, a 7-day cold acclimation treatment, and three consecutive rounds of a 3-day cold acclimation treatment (as utilized in our larger study). The treatment of three consecutive rounds of cold acclimation resulted in the largest increase in total fruit number, although increases in total fruit number were detected across all treatments relative to the control.

Across all experiments, the increases in fruit number were disproportionately attributable to fruit production on axillary and basal shoots rather than on the primary inflorescence (fig. 2*B*, 2*C*), which suggests that genes affecting architectural development may be cold responsive or that genes in the cold acclimation pathway may have pleiotropic effects on plant architecture. Transcriptome studies have shown that many genes involved in biosynthesis or signaling of plant hormones (i.e., abscisic acid, gibberellic acid, and auxin) and a number of transcription factors involved in development (e.g., ARF, GRAS, homeodomain, MADS, and NAC) are also cold responsive (Lee et al. 2005; Kilian et al. 2007). Although these studies did not specifically examine the effects of cold treatment on plant architecture, they suggest that growth and development may be influenced by low temperature. Our finding of increased fitness following cold acclimation is not unprecedented; there are reports in the agricultural literature demonstrating that low-temperature treatment

increases seed number in lettuce (Toledo et al. 1981), Chinese cabbage (Linwattana et al. 1997), and onion (Reghin et al. 2005).

Transgenic overexpression of individual *CBF* genes in *A. thaliana* induces the cold acclimation pathway in the absence of a low-temperature treatment (Jaglo-Ottosen et al. 1998; Gilmour et al. 2000), thus allowing evaluation of the potential costs of constitutive cold acclimation. A cost of overexpressing *CBF2* and *CBF3* (but not *CBF1*) was detected under both cold-acclimated and non-cold-acclimated conditions. These results are consistent with previous reports evaluating these same overexpression lines under similar experimental conditions (Jackson et al. 2004). While a cost associated with overexpression is consistent with the evolution of cold acclimation as an inducible response, the costs observed in this study are likely to be exaggerated given the unnaturally high *CBF* transgene expression driven by a strong promoter (CaMV 35S) and consequently must be viewed with caution. The finding of variation among different *CBF* overexpression lines is also noteworthy, as it may indicate potentially different contributions of these subfamily members to the process of cold acclimation, although we cannot definitively rule out the possibility that costs associated with *CBF2* and *CBF3* overexpression might be attributable to disruption of important genes via the actual transgene insertions themselves. However, it is interesting to note that natural induction levels of *CBF1* (for which no cost of overexpression was detected) are on average substantially lower than those for *CBF2* and *CBF3* (Zhen and Ungerer 2008*b*).

Implications for Clinal Variation in Freezing Tolerance in A. thaliana

In previous reports we documented extensive clinal variation in freezing tolerance in *A. thaliana* that is attributable to different patterns of historical selection on the *CBF* subfamily of transcriptional activators (Zhen and Ungerer 2008*a*, 2008*b*). This gene family was shown to be under strong purifying selection in the northern, colder regions of the species range but undergoing mutation accumulation in more southern, warmer regions (Zhen and Ungerer 2008*b*). Mutations that negatively impact *CBF* function could be positively selected if the cold acclimation pathway imposes a fitness cost in areas where freezing stress is rare. Evidence for such a scenario would manifest as the northern *A. thaliana* accessions (higher cold acclimation capacity) displaying greater fitness decrements in comparison to the southern accessions (lower cold acclimation capacity) when subjected to cold acclimation but in the absence of subsequent freezing stress. No such differential responses were observed. The absence of evidence for such an allocation cost supports the notion that cold acclimation capacity is not selected against in warmer environments and mutations in *CBF* genes in the southern accessions are likely to be selectively neutral.

General Conclusions and Implications

Strengths of the current study include (1) a system in which the inducible response is easily separable from the stress response, (2) clear ecological and genetic contexts in which to evaluate these responses, and (3) functional genetic tools (overexpression and T-DNA insertion lines) that enable specific *CBF* expression modifications to be evaluated alongside naturally occurring *CBF* genotypes. By utilizing these resources, we failed to detect a cost of cold acclimation, although the benefits of this response were clearly confirmed. The unanticipated finding of increased fruit number in response to cold acclimation across all treatment groups suggests that additional independent or semioverlapping pathways may need to be integrated into future studies investigating the dynamics of environmental temperature variation, plant thermal tolerance, and fitness.

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