

The Cold-Inducible CBF1 Factor–Dependent Signaling Pathway Modulates the Accumulation of the Growth-Repressing DELLA Proteins via Its Effect on Gibberellin Metabolism ^W

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Plants have evolved robust mechanisms to respond and adapt to unfavorable environmental conditions, such as low temperature. The C-repeat/drought-responsive element binding factor *CBF1/DREB1b* gene encodes a transcriptional activator transiently induced by cold that controls the expression of a set of genes responding to low temperature (the CBF regulon). Constitutive expression of *CBF1* confers freezing tolerance but also slows growth. Here, we propose that low temperature–induced *CBF1* expression restrains growth at least in part by allowing the accumulation of DELLAs, a family of nuclear growth-repressing proteins, the degradation of which is stimulated by gibberellin (GA). We show that cold/*CBF1* enhances the accumulation of a green fluorescent protein (GFP)–tagged DELLA protein (GFP-RGA) by reducing GA content through stimulating expression of GA-inactivating *GA 2-oxidase* genes. Accordingly, transgenic plants that constitutively express *CBF1* accumulate less bioactive GA and as a consequence exhibit dwarfism and late flowering. Both phenotypes are suppressed when *CBF1* is expressed in a line lacking two DELLA proteins, GA-INSENSITIVE and REPRESSOR OF GA1-3. In addition, we show that DELLAs contribute significantly to *CBF1*-induced cold acclimation and freezing tolerance by a mechanism that is distinct from the CBF regulon. We conclude that DELLAs are components of the *CBF1*-mediated cold stress response.

INTRODUCTION

Plants are sessile organisms and hence cannot escape unfavorable environmental conditions, such as cold. To overcome these limitations, plants have the potential to acclimate to low temperature by triggering a cascade of events leading to changes in gene expression and subsequently to biochemical and physiological modifications that enhance their freezing tolerance.

Transcript profiling experiments have shown that multiple regulatory pathways are activated during cold acclimation, and one such important pathway involves the CBF/DREB1 regulon (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Fowler and Thomashow, 2002; Rabbani et al., 2003; Lee et al., 2005). The CBF/DREB1 proteins (CRT binding factor or DRE binding protein) are transcriptional regulators that bind specifically to the cold and dehydration-responsive *cis*-element, designated the CRT (C-repeat)/DRE (dehydration response element), present in the promoter of *COR* (cold-regulated) and a multitude of other stress-responsive genes known as the CBF

regulon (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). CBF/DREB1 factors are members of the AP2/EREBP (Apeta1a2/ethylene-responsive element binding protein) family of transcription factors (Riechmann and Meyerowitz, 1998). In *Arabidopsis thaliana*, there are three genes encoding CBF/DREB1 proteins, known as CBF1, CBF2, and CBF3 (also referred to as DREB1b, DREB1c, and DREB1a, respectively) (Gilmour et al., 2004). Following cold exposure, these genes are rapidly and transiently induced via an abscisic acid–independent pathway, and their products activate the CBF regulon to enhance freezing tolerance (Agarwal et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2006). Multiple mechanisms appear to contribute to this increase in freezing tolerance, including the synthesis of cryoprotective polypeptides (such as COR15a) and the accumulation of solutes, such as soluble sugars and Pro (Thomashow, 1998; Gilmour et al., 2004).

Expression of any one of the *CBF* proteins from a cDNA under control of the 35S cauliflower mosaic virus (CaMV) promoter in transgenic plants gives rise to strong constitutive expression of the CBF regulon and hence increased freezing tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000, 2004). *Arabidopsis* CBF proteins are also effective in several other plant species; thus, a large number of transgenic plants overexpressing CBF proteins have been engineered to enhance freezing tolerance (Agarwal et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2006). However, overexpression of these genes also

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resulted in severe growth retardation under normal growth condition (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2004).

The phytohormone gibberellin (GA) promotes important processes of plant growth and development, such as seed germination, growth through elongation, and floral transition (Richards et al., 2001). Key components of the GA-signaling pathway are the nuclear-localized growth repressing DELLA proteins (DELLAs) (Peng et al., 1997; Silverstone et al., 1998; Dill and Sun, 2001; King et al., 2001), a subset of the GRAS family of transcriptional regulators (Bolle, 2004; Zentella et al., 2007; De Lucas et al., 2008; Feng et al., 2008). The *Arabidopsis* genome encodes five DELLAs (GA-INSENSITIVE [GAI], REPRESSOR OF GA1-3 [RGA], RGA-LIKE1 [RGL1], RGL2, and RGL3) that all share the DELLA motif in their N-terminal domain (Peng et al., 1997; Dill and Sun, 2001; Silverstone et al., 2001; Lee et al., 2002; Wen and Chang, 2002). Recent genetic analyses have revealed both distinct but also overlapping functions for individual DELLAs in the regulation of plant development (Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004; Achard et al., 2006). According to the relief of restraint model, DELLAs restrain plant growth, whereas GA promotes growth by overcoming DELLA-mediated growth restraint (Harberd et al., 1998; Dill and Sun, 2001; King et al., 2001; Silverstone et al., 2001; Harberd, 2003). The GA signal is perceived by a GA receptor with homology to human hormone-sensitive lipase, GA-INSENSITIVE DWARF1 (GID1) (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006). The binding of bioactive GAs to GID1 promotes an interaction between GID1 and the DELLA domain of DELLAs (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2007; Willige et al., 2007). Subsequently, this interaction enhances the affinity between DELLAs and a specific SCF E3 ubiquitin-ligase complex (involving the F-box protein called SLY1 [in *Arabidopsis*] or GID2 in rice [*Oryza sativa*]) (Griffiths et al., 2006; Willige et al., 2007), thus promoting the eventual destruction of DELLAs by the 26S proteasome (McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004). Thus, according to the current model, DELLAs restrain plant growth, whereas GA promotes growth by targeting the DELLAs for destruction.

Recent advances have revealed the role of DELLAs in many aspects of plant growth, including those that are influenced by environmental conditions (Lee et al., 2002; Cheng et al., 2004; Cao et al., 2005; Achard et al., 2006, 2007a, 2007b, 2008; Penfield et al., 2006). Interestingly, the growth of mutant plants lacking four of the five DELLAs (GAI, RGA, RGL1, and RGL2) is less inhibited by salt stress than is the growth of wild-type plants. By contrast, growth restraint conferred by DELLAs promotes survival (Achard et al., 2006, 2008). Thus, DELLA-dependent growth restraint is advantageous to plants and permits flexible and appropriate modulation of growth in response to changes in natural environments.

Although it is clear that both the cold-induced CBF and GA-signaling pathways regulate plant growth and stress tolerance, it is unclear whether and how these pathways interact. Recent experiments have shown that overexpression of *DWARF AND DELAYED-FLOWERING1* (*DDF1*), an AP2 transcription factor closely related to the CBF/DREB1 family, conferred dwarfism, late flowering, and increased salt stress tolerance in *Arabidopsis*

(Magome et al., 2004, 2008). Interestingly, GA treatment restored the wild-type phenotype. Similarly, constitutive expression of heterologous *Arabidopsis CBF1* in tomato (*Solanum lycopersicum*) also conferred dwarfism, a phenotype that was prevented by application of GA (Hsieh et al., 2002). However, GA-treated transgenic tomato plants still exhibited a greater degree of dehydration and chilling tolerance compared with wild-type plants, which is not seen in *Arabidopsis* expressing *DDF1* constitutively (Magome et al., 2004). Furthermore, the dwarf phenotype of transgenic *Arabidopsis* and tobacco (*Nicotiana tabacum*) plants overexpressing *Arabidopsis CBF3* cannot be reversed by GA treatment (Agarwal et al., 2006). Thus, at present, it is unclear whether the CBF/DREB1 response pathway interacts with the GA pathway. Here, we undertook a molecular and genetic approach to evaluate the role of GA metabolism and signaling in the CBF1-dependent cold acclimation process in *Arabidopsis*. Essentially, we show that seedling roots of DELLA-deficient mutants have a reduced capacity for cold-induced root growth inhibition. We also show that cold-induced or constitutive expression of *CBF1* promotes the accumulation of the green fluorescent protein (GFP)-tagged DELLA RGA and reduces plant growth. Accordingly, GA treatment or lack of functional *GAI* and *RGA* genes suppresses the growth defect of *CBF1-ox* plants. Thus, cold-induced CBF1 restrains growth at least in part via a DELLA-dependent mechanism. Moreover, we show that CBF1 reduces bioactive GA levels by increasing the abundance of transcripts encoding GA 2-oxidases, which may explain the CBF1-induced DELLA accumulation. Finally, we show that DELLA accumulation contributes significantly to the CBF1-induced cold acclimation and freezing tolerance by a mechanism that is independent of the well-known CBF regulon. We conclude that DELLA restraint is a component of the CBF1-mediated cold stress response.

RESULTS

Cold-Mediated Root Growth Inhibition Is Associated with DELLA Accumulation

Although well characterized as growth inhibitors during adverse conditions, it is unknown whether the GA-signaling nuclear DELLA proteins inhibit growth during cold stress. We therefore investigated the involvement of DELLAs in the inhibition of primary root growth in response to cold using double-DELLA (*gai-t6 rga-24*) and quadruple-DELLA (*gai-t6 rga-t2 rgl1-1 rgl2-1*) mutant *Arabidopsis* lines that lack the corresponding DELLAs (King et al., 2001; Dill and Sun, 2001; Achard et al., 2006). In nature, plants are exposed to alternating periods of high and low temperatures that often correspond to day and night cycles (Guy, 1990). We found that DELLAs inhibit primary root growth to a degree proportionate to cold period length. Whereas the growth of *gai-t6 rga-24* and quadruple-DELLA mutant roots was less affected by a daily 8-h cold exposure (4°C) than wild-type roots, the differences in root growth among these three lines decreased progressively with increase in cold period length (Figure 1). Thus, DELLA function restrains primary root growth during cold exposure but becomes less critical as the cold period length increases. Therefore, it is likely that additional (DELLA-independent) factors

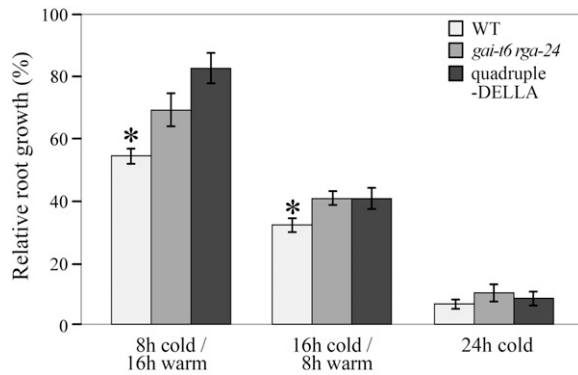


Figure 1. Low Temperature Slows Root Growth via a DELLA-Dependent Mechanism.

Mean (\pm SE) relative growth of primary roots of 8-day-old wild-type (white), *gai-t6 rga-24* (light gray), and the quadruple-DELLA mutant (*gai-t6 rga-t2 rgl1-1 rgl2-1*; dark gray) seedlings were grown for 5 d in alternating cold (4°C)/warm (22°C) temperatures as indicated (expressed as percentage of non-cold-treated control for each genotype). An asterisk indicates a significant difference between the wild type and *gai-t6 rga24*/quadruple-DELLA mutant ($P < 0.05$).

also restrict growth at low temperatures, particularly under long periods of exposure to cold.

Next, we investigated whether cold-induced inhibition of root growth is associated with DELLA protein accumulation. For this purpose, we used *pRGA:GFP-RGA* transgenic seedlings expressing a GFP-RGA fusion protein that is detectable by protein gel blotting using an anti-GFP antibody (see Supplemental Figure 1 online; Silverstone et al., 2001). We found a twofold increase in GFP-RGA protein level within 4 h of transfer of *pRGA:GFP-RGA* seedlings from warm (22°C) to low temperature (4°C) (Figure 2A). This increase in GFP-RGA levels could be the consequence of an increase in *GFP-RGA* transcript levels or to a posttranscriptional effect, as both mechanisms are known to regulate DELLA protein accumulation (McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004; Oh et al., 2007). To discriminate between these possibilities, we performed an RNA gel blot experiment to compare *GFP-RGA* and *RGA* transcript levels in cold-treated and control seedlings. As shown in Figure 2B, exposure to cold had no detectable effect on either *GFP-RGA* or *RGA* transcript levels. Thus, cold enhances GFP-RGA accumulation (and probably that of other DELLAs) through a posttranslational mechanism, thereby inhibiting root growth.

To investigate whether cold affects the expression of other *DELLA* genes, we performed quantitative RT-PCR experiments for all five *DELLAs*. Interestingly, we found that *RGL3* transcript levels were transiently increased by cold (Figure 2C), indicating that cold also specifically enhances *RGL3* accumulation via its effect on gene expression.

CBF1 Restrains Plant Growth via the DELLA-Dependent Signaling Pathway

At present, the best-understood genetic system with a role in cold acclimation is the CBF cold response pathway. Upon

exposure to low temperature, the CBF genes are rapidly induced and their products activate the expression of downstream CBF-regulated genes (Figure 2B; Jaglo-Ottosen et al., 1998; Gilmour et al., 2004). We found that GFP-RGA levels increase after 4 h of cold treatment, thus following *CBF1* induction (Figure 2B). Furthermore, it was previously reported that transgenic *Arabidopsis* plants overexpressing *CBF1* were dwarfed and exhibited a late-flowering phenotype compared with wild-type plants (Gilmour et al., 2004), a phenotype that is usually observed with GA-deficient plants (Richards et al., 2001). Altogether, these observations strongly suggest that low temperature enhances DELLA accumulation via a CBF-dependent activation.

To examine the involvement of the GA/DELLA components in the CBF cold response pathway, we created transgenic *Arabidopsis* plants that constitutively express *CBF1* under the control of the 35S CaMV promoter. Several T3 lines were identified, and a transgenic line that moderately expresses the corresponding *CBF1* transgene (*CBF1-ox*) was selected for further studies (see Supplemental Figure 2 online). RNA gel blot analysis confirmed that *CBF1-ox* plants accumulated *CBF1* transcripts in the absence of cold stress in contrast with wild-type plants (Figure 3A).

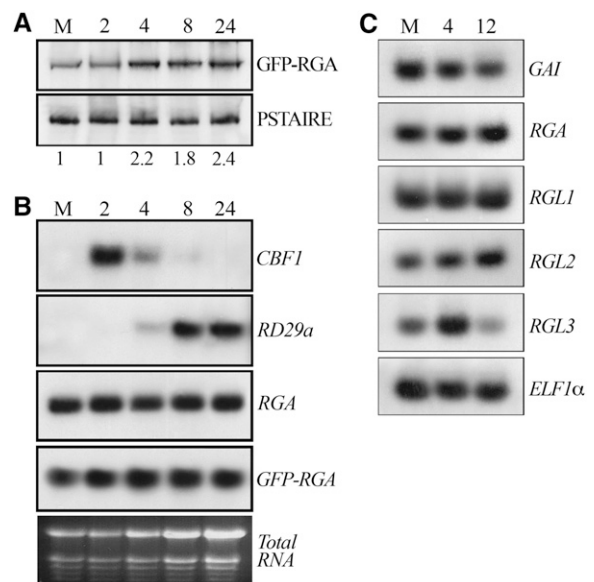


Figure 2. Cold Enhances *CBF1* Transcript and GFP-RGA Protein Levels.

(A) Immunodetection of GFP-RGA (with an antibody to GFP) in 7-d-old *pRGA:GFP-RGA* seedlings that had been subjected to cold (4°C) for the time (2, 4, 8, and 24 h) indicated (and controls, M). PSTAIRE serves as sample loading control. Numbers below the gels represent the fold increase in GFP-RGA protein levels relative to PSTAIRE levels.

(B) Expression of *CBF1*, *RD29a*, and *RGA* genes and the *GFP-RGA* transgene upon cold for the time as in **(A)** determined by RNA gel blot analysis. Each lane was loaded with 15 μ g of total RNA prepared from 7-d-old *pRGA:GFP-RGA* seedlings.

(C) Levels of *GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3* gene transcripts determined by a quantitative RT-PCR experiment in 7-d-old wild-type seedlings that had been subjected to cold for 4 and 12 h as indicated (and controls, M). *ELF1 α* transcripts provide loading control.

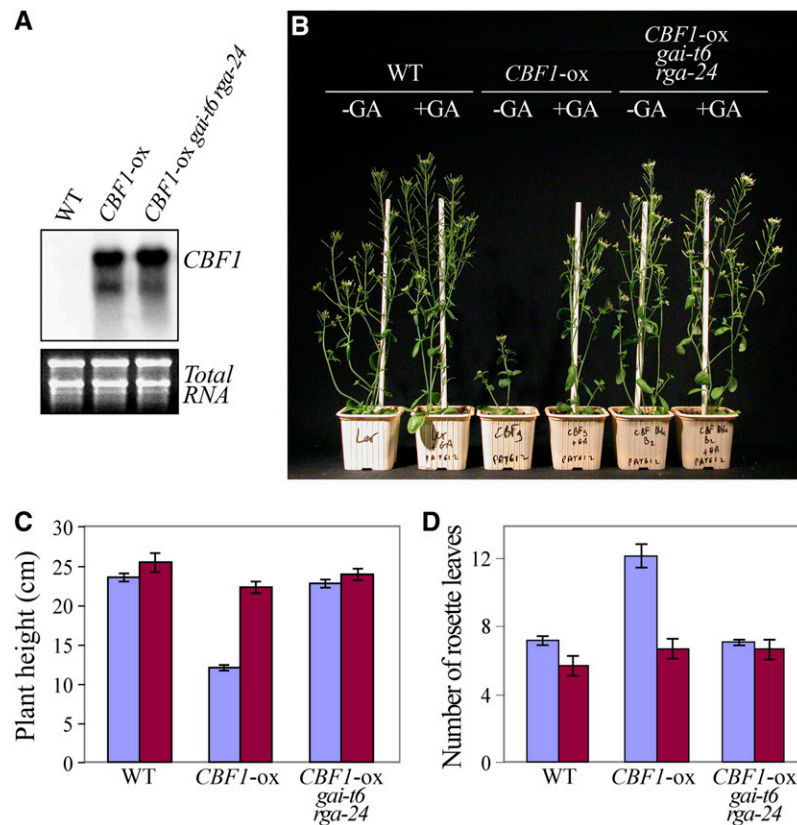


Figure 3. CBF1 Represses Growth and Delays Floral Transition through the GA-Signaling Pathway.

(A) Expression of *CBF1* gene in wild-type, *CBF1-ox*, and *CBF1-ox gai-t6 rga-24* plants. Each lane was loaded with 15 μ g of total RNA prepared from 2-week-old wild-type and transgenic plants. The RNA gel blot was hybridized with a specific probe for *CBF1*.

(B) Representative 4-week-old wild-type, *CBF1-ox*, and *CBF1-ox gai-t6 rga-24* plants treated with GA (+GA) or control (–GA).

(C) and **(D)** Mean (\pm SE) plant height (cm) **(C)**, and mean (\pm SE) rosette leaf number **(D)** of 4-week-old wild-type, *CBF1-ox*, and *CBF1-ox gai-t6 rga-24* plants GA-treated (violet) or control (blue).

CBF1-ox plants exhibited similar phenotypic alterations as previously described (Gilmour et al., 2004); the plants were shorter, flowered later, and their leaves were smaller and darker than those of wild-type plants. First, we investigated the responsiveness of *CBF1-ox* plants to exogenous GA. We found that GA treatment both rescued the normal growth and abolished the late-flowering phenotype of *CBF1-ox* plants (Figures 3B to 3D). Thus, as suggested above, CBF1 represses plant growth via a GA-dependent mechanism. The developmental effects of GA on plant growth are mediated by the destruction of DELLAs (Harberd, 2003; McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004). Because GA overcomes *CBF1-ox*-conferred dwarfism and late flowering, we next tested the hypothesis that CBF1 restrains growth via a DELLA-dependent mechanism. We found that lack of two DELLAs, GAI and RGA, in a *CBF1-ox gai-t6 rga-24* genetic background suppressed the growth inhibition and late flowering phenotype (Figures 3B to 3D). Thus, CBF1 does indeed restrain growth via a DELLA-dependent mechanism.

In a subsequent experiment, we determined the effect of CBF1 on DELLA protein accumulation. For this study, we crossed *CBF1-ox* with the *pRGA:GFP-RGA* line expressing a GFP-RGA fusion protein that is detectable in root cell nuclei (Silverstone et al., 2001). We found that the nuclear fluorescence attributable to GFP-RGA was brighter in cells of *CBF1-ox* root seedlings, particularly in the elongating zone of the root (Figure 4A). Consistently, the increase in GFP-RGA fluorescence observed in *CBF1-ox* plants was associated with an increase in GFP-RGA protein level (Figure 4B). Thus, CBF1 enhances DELLA protein accumulation and consequently restrains plant growth.

Finally, we investigated if CBF1-induced DELLA accumulation was attributable to an increase in *DELLA* transcript levels or to a posttranslational effect. Thus, we compared the levels of *DELLA* gene expression in wild-type and *CBF1-ox* plants. We found that expression of the *CBF1-ox* transgene, like cold treatment, had no detectable effect on either *DELLA* transcript levels, except for *RGL3*, which accumulated (Figure 4C). These results suggest that CBF1 enhances RGA accumulation (and most likely other

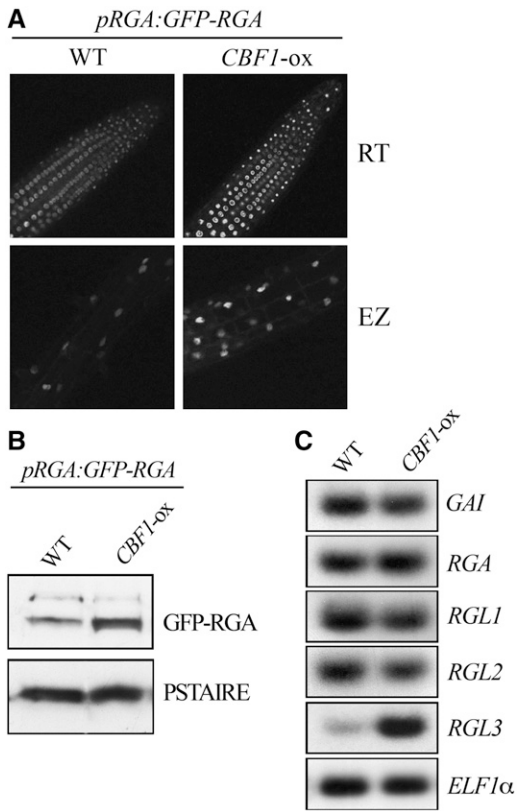


Figure 4. CBF1 Represses Plant Growth by Enhancing DELLA Accumulation.

(A) GFP fluorescence (viewed by fluorescence confocal microscopy) in nuclear cells of the root tip (RT) and elongating zone (EZ) of 7-d-old seedlings of the *pRGA::GFP-RGA* (wild-type) and *CBF1-ox pRGA::GFP-RGA* lines. Images were taken with identical parameters to allow for comparison of fluorescence intensities.

(B) Immunodetection of GFP-RGA (with an antibody to GFP) in 7-d-old seedlings of *pRGA::GFP-RGA* (wild-type) and *CBF1-ox pRGA::GFP-RGA* lines. PSTAIRE serves as sample loading control.

(C) Levels of *GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3* gene transcripts determined by quantitative RT-PCR experiment in 7-d-old wild-type and *CBF1-ox* seedlings. *ELF1α* transcripts provide loading control.

DELLA proteins) through a posttranslational mechanism but also specifically enhances *RGL3* transcript accumulation.

GA 2-Oxidase Gene Transcripts Are Upregulated upon Exposure to Cold and in *CBF1-ox* Plants

Reduced bioactive GA levels cause an increase in DELLA protein accumulation and consequently growth inhibition (Silverstone et al., 2001; Fu et al., 2004). Moreover, GA metabolism is regulated by both developmental and environmental stimuli (Hedden and Phillips, 2000). We next investigated the possibility that cold/CBF1-induced accumulation of DELLAs might be the result of CBF1-mediated transcriptional regulation of genes involved in GA metabolism. GA metabolism is tightly regulated through the modulation of the expression of members of small

gene families encoding GA 20-oxidases (*GA20ox*) and GA 3-oxidases (*GA3ox*), which catalyze successive steps in the synthesis of bioactive GAs (Phillips et al., 1995; Chiang et al., 1995), but also GA 2-oxidases (*GA2ox*), which deactivate bioactive GAs (Thomas et al., 1999). We speculated that cold-mediated CBF1 induction either reduces *GA20ox* and/or *GA3ox* transcript levels and/or enhances *GA2ox* transcript levels. To test this hypothesis, we first determined the effect of cold and *CBF1-ox* on the accumulation of selected *GA20ox* and *GA3ox* gene transcripts. Surprisingly, we found that 2-week-old wild-type plants, exposed to cold for 4 h, and *CBF1-ox* plants accumulate substantially higher levels of *GA20ox1*, *GA20ox3*, and *GA3ox2* transcripts than control plants (non-cold-treated wild-type plants) (Figures 5A and 5B). Interestingly, these genes were only transiently upregulated by cold, as their expression levels dropped to normal after 12 h of cold exposure. By contrast, the level of *GA20ox2* transcripts was unchanged and the level of *GA3ox1* transcripts was reduced upon cold treatment but increased in *CBF1-ox* plants. Thus, the cold/CBF1-induced accumulation of DELLAs is not due to a transcriptional repression of *GA20ox* or *GA3ox* genes. It is noteworthy that previous work has shown that increased DELLA activity (as seen in GA-deficient and GA-insensitive mutants) results in increased levels of *GA20ox1* and *GA3ox1* transcripts and in decreased levels of *GA2ox* transcripts due to a feedback mechanism (Hedden and Kamiya, 1997; Cowling et al., 1998; Xu et al., 1999; Thomas et al., 1999). Thus, it is likely that the increase in *GA20ox* and *GA3ox* transcript levels observed in 4 h cold-treated wild-type plants and in *CBF1-ox* plants is the result of such a mechanism (Figure 5A). Accordingly, *CBF1-ox gai-t6 rga-24* plants exhibited reduced levels of *GA20ox* and *GA3ox* transcripts in comparison to *CBF1-ox* and wild-type plants.

Next, we determined the level of *GA2ox* gene transcripts in cold-treated wild-type plants and *CBF1-ox* plants (Figures 5A and 5B). We found that 2-week-old 4 h cold-treated wild-type plants contain relatively higher amounts of *GA2ox1*, *GA2ox3*, and *GA2ox6* transcripts in comparison to non-cold-treated plants. By contrast, *CBF1-ox* plants exhibited increased levels of *GA2ox3* and *GA2ox6* transcripts but not of *GA2ox1* transcripts. Thus, it is likely that cold enhances *GA2ox1* transcript levels in a CBF1-independent manner. Moreover, the increase in *GA2ox3* and *GA2ox6* transcripts is not related to DELLA status (to the feedback mechanism), as *CBF1-ox gai-t6 rga-24* plants also exhibited higher *GA2ox* transcript levels. Thus, in contrast with *GA20ox* and *GA3ox*, the increase in *GA2ox* transcripts observed in cold-treated wild-type plants and in *CBF1-ox* plants is not due to a feedback mechanism.

The upregulation of *GA2ox* expression in cold-treated and *CBF1-ox* plants may lead to a decrease in bioactive GAs. To determine if this was the case, we compared in separate experiments the endogenous GA contents in cold-treated and untreated wild-type plants and in wild-type and *CBF1-ox* plants. As shown in Table 1, the level of the major biologically active GA, GA_4 , was significantly reduced in *CBF1-ox* plants compared with wild-type plants, while the content of its inactive 2 β -hydroxylated product, GA_{34} , was increased. GA_4 , as well as GA_1 , contents were also reduced after cold treatment by ~50 and 30%, respectively. While the content of GA_8 , the 2 β -hydroxylated product of GA_1 ,

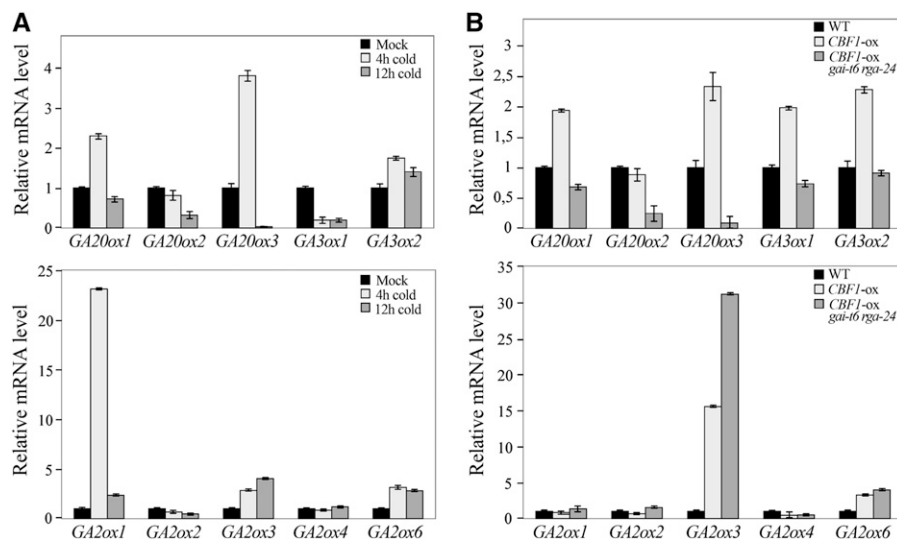


Figure 5. Cold and *CBF1*-ox Upregulate GA 2-oxidase Transcript Levels.

(A) Relative levels of GA biosynthesis *GA20ox1*, *GA20ox2*, *GA20ox3*, *GA3ox1*, and *GA3ox2* and GA deactivation of *GA2ox1*, *GA2ox2*, *GA2ox3*, *GA2ox4*, and *GA2ox6* gene transcripts (determined by real-time RT-PCR) in 2-week-old wild-type seedlings that had been subjected to cold for 4 h (light gray) and 12 h (dark gray) and control non-cold-treated (Mock, black). Data are means \pm SE.

(B) Relative levels of GA metabolism gene transcripts determined by real-time RT-PCR (as in **[A]**) in 2-week-old wild-type (black), *CBF1*-ox (light gray), and *CBF1*-ox *gai-t6 rga-24* (dark gray) plants.

increased after cold treatment, there was a slight reduction in GA_{34} content after this treatment. Some reduction in GA content in the cold-treated plants might be anticipated as a result of lower metabolic rates, but GA_4 content was reduced much more than that of GA_{34} , consistent with a higher rate of 2β -hydroxylation under these conditions. Taken together, the above results indicate that cold-mediated CBF1 induction enhances DELLA protein accumulation through a posttranslational mechanism mediated by a reduction of bioactive GA (via upregulation of *GA2ox* gene transcript levels). In turn, accumulation of DELLAs enhances *GA20ox* and *GA3ox* gene transcript levels by a feedback mechanism.

DELLAs Contribute to *CBF1*-Induced Freezing Tolerance and Cold Acclimation

It has recently been established that DELLAs promote salt tolerance (Achard et al., 2006, 2008). However, the contribution of DELLAs in promoting tolerance to other abiotic stresses, such as freezing, is unknown. To determine the role of DELLAs in freezing tolerance and cold acclimation, we examined the freezing tolerance of 2-week-old GA-signaling mutants before and after cold acclimation (4°C, 3 d). Plants were exposed for 24 h to -10°C , and survival was scored after 4 d of recovery under controlled growth conditions (see Supplemental Figure 3 online). Strikingly, we found that the GA response mutant *gai* (Koorneef et al., 1985; Peng et al., 1997) exhibited increased tolerance toward freezing, whereas *gai-t6 rga-24* mutant plants were less tolerant than wild-type plants (Figure 6). Thus, DELLA function is not restricted to salt stress and promotes tolerance to other

environmental stresses. Furthermore, *gai-t6 rga-24* mutant plants were significantly less freezing tolerant than wild-type plants after cold acclimation, despite the fact that *gai-t6 rga-24* plants were still responding to cold acclimation. Overall, these observations suggest that DELLAs contribute significantly in a cold adaptive response.

Next, we determined whether CBF1 promoted freezing tolerance in a DELLA-dependent fashion. Thus, we compared the freezing tolerance of *CBF1*-ox and *CBF1*-ox *gai-t6 rga-24* plants. Remarkably, we found that *CBF1*-ox *gai-t6 rga-24* plants

Table 1. Cold and *CBF1*-ox Reduce Bioactive GA Levels via Upregulation of GA 2-Oxidase Activity

ng/g Dry Weight	GA4	GA1	GA34	GA8
Wild type	11.37 \pm 0.26	0.68 \pm 0.03	19.41 \pm 0.13	1.82 \pm 0.02
Wild type cold 4 h	5.97 \pm 0.01	0.46 \pm 0.01	16.95 \pm 0.24	2.28 \pm 0.04
Wild type cold 12 h	6.02 \pm 0.02	0.44 \pm 0.01	17.21 \pm 0.14	2.30 \pm 0.08
ng/g Dry Weight	GA4	GA34		
Wild type	4.59 \pm 0.07	44.94 \pm 0.45		
<i>CBF1</i> -ox	2.74 \pm 0.03	66.58 \pm 0.16		

Concentrations of GAs (ng g dry weight $^{-1}$ \pm sd) in wild-type plants subjected to cold for 4 and 12 h (and controls, wild-type) and in separate experiments in wild-type and *CBF1*-ox plants. The values are means of three and two biological replicates, respectively.

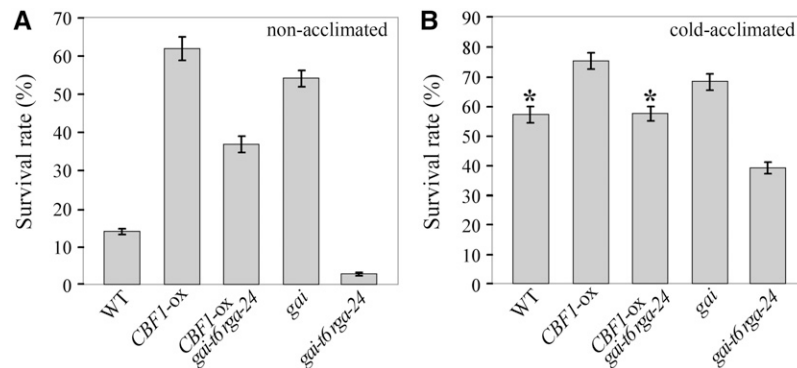


Figure 6. DELLAs Contribute to CBF1-Induced Freezing Tolerance and Cold Acclimation.

Freezing tolerance (expressed as rate of survival [%]; three replicates; $n = 24$) of 2-week-old wild-type, *CBF1-ox*, *CBF1-ox gai-t6 rga-24*, *gai*, and *gai-t6 rga-24* plants nonacclimated (**A**) or cold-acclimated (4°C , 3 d) (**B**). Plants were exposed for 24 h at -10°C , and survival was scored after 4 d of recovery under controlled growth conditions. The asterisks indicate no significant difference ($P < 0.05$) between wild-type and *CBF1-ox gai-t6 rga-24* cold-acclimated plants.

exhibited lower levels of freezing tolerance than *CBF1-ox* plants, suggesting that DELLAs contribute at least in part to CBF1-induced freezing tolerance. The fact that the survival rates of nonacclimated *CBF1-ox gai-t6 rga-24* mutant plants were higher (36.7%) than *gai-t6 rga-24* plants (2.8%) shows either that additional DELLAs (these plants retain RGL1, RGL2, and RGL3) mediate the effect of CBF1 on freezing tolerance or more likely that, in addition to the DELLA-dependent mechanism, CBF1 also promotes freezing tolerance via a DELLA-independent mechanism.

CBF1 Activates the CBF Regulon via a DELLA-Independent Mechanism

As a next step, we investigated whether DELLAs contribute to CBF1-induced freezing tolerance via the well-characterized CBF regulon. When plants are exposed to low temperature, CBF target genes are rapidly induced and act to increase freezing tolerance (see Supplemental Figure 4A online; Jaglo-Ottosen et al., 1998; Gilmour et al., 2004). As previously shown, we found that transcript levels of *RD29a* (*COR78*), *COR15a*, and *COR47*, three known CBF1-inducible *COR* genes (Gilmour et al., 2004), were upregulated in *CBF1-ox* plants in compared with wild-type plants (Figure 7A). Interestingly, there was no significant difference in transcript levels for these genes between the wild type and *gai* or *gai-t6 rga-24* (see Supplemental Figure 4A online) or between *CBF1-ox* and *CBF1-ox gai-t6 rga-24* plants (Figure 7A). The levels of Pro, an important protective osmolyte in higher plants, are also increased during cold acclimation or by *CBF1* overexpression in nonacclimated plants (Gilmour et al., 2004). It was previously shown that expression of the gene encoding Δ^1 -pyrroline-5-carboxylate synthase 2 (*P5CS2*), an enzyme involved in Pro biosynthesis, was upregulated in *CBF-ox* plants. Accordingly, we found that *P5CS2* transcript levels were elevated in *CBF1-ox* plants compared with wild-type plants (Figure 7A). However, lack of *GAI* and *RGA* in *CBF1-ox gai-t6 rga-24* plants

did not alter *P5CS2* transcript accumulation, which remains at the level of *CBF1-ox* plants. Thus, DELLAs do not modulate the expression levels of the *COR* and Pro biosynthesis genes tested here, suggesting that CBF1-induced DELLA accumulation enhances stress tolerance via a different mechanism.

The accumulation of simple sugars is another commonly observed biochemical change that occurs in cold-acclimated plants or in CBF-overexpressing plants (Gilmour et al., 2000, 2004). Soluble sugars, such as sucrose, glucose, or fructose, have cryoprotective properties (Gilmour et al., 2000; Rolland et al., 2002). Thus, it was of interest to determine whether *GAI/DELLA* contributes to CBF1-mediated stress tolerance by modulating sugar levels. We found that cold-acclimated wild-type plants and *CBF1-ox* plants accumulated soluble sugars (glucose, fructose, and sucrose) at a higher level (~ 1.5 -fold higher) than those found in control plants (non-cold-acclimated and wild-type plants, respectively) (see Supplemental Figure 4B online; Figure 7B). However, there was no difference between the wild type and *gai* or *gai-t6 rga-24* or between *CBF1-ox* and *CBF1-ox gai-t6 rga-24* plants. Thus, DELLAs do not contribute to CBF1-mediated increase in sugars levels.

Overall, these results strongly suggest that DELLAs contribute to CBF1-induced freezing tolerance by a mechanism that is distinct from the CBF regulon.

DISCUSSION

The process of cold acclimation involves numerous physiological and biochemical changes. The most notable changes include reduction or cessation of growth and accumulation of cryoprotective molecules, such as Pro and soluble sugars. The GA-regulated growth-repressing DELLA proteins integrate endogenous and environmental cues in the regulation of plant growth (Alvey and Harberd, 2005; Achard et al., 2006, 2007a, 2007b). It was proposed that DELLAs permit flexible and appropriate modulation of plant growth in response to changes in

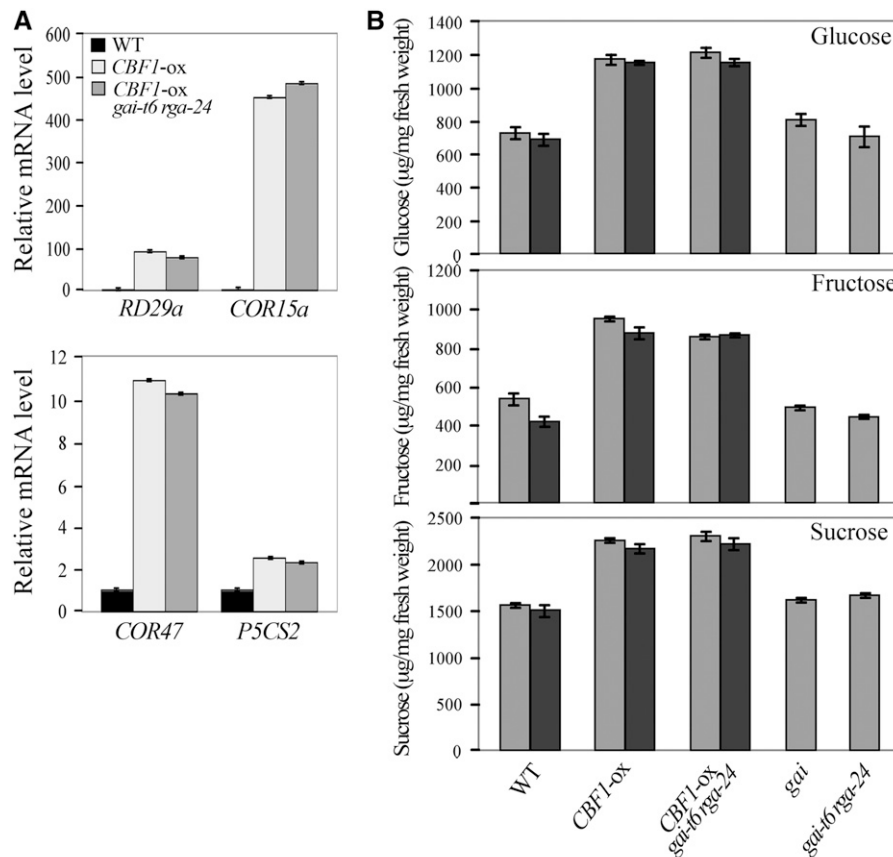


Figure 7. CBF1 Enhances Both CBF Regulon and Sugar Levels in a DELLA-Independent Fashion.

(A) Relative levels of *RD29a*, *COR15a*, *COR47*, and *P5CS2* gene transcripts (determined by real-time RT-PCR) in 2-week-old wild-type, *CBF1-ox*, and *CBF1-ox gai-t6 rga-24* plants. Data are means \pm SE.

(B) Mean (\pm SE) total soluble sugars (glucose, fructose, and sucrose) were determined from leaf tissue of 2-week-old wild-type, *CBF1-ox*, *CBF1-ox gai-t6 rga-24*, *gai*, and *gai-t6 rga-24* plants GA-treated (dark gray) or not (light gray). The levels of individual sugars were determined by enzymatic assays.

natural environments (Alvey and Harberd, 2005; Achard et al., 2006). The experiments described here investigate the relationship between the GA pathway and the CBF1-dependent cold signaling pathway.

Low temperature is one of the most important factors affecting plant performance and thus plant growth. All biochemical processes run more slowly during long exposure to cold (Stitt and Hurry, 2002). Low temperatures slow down enzyme-catalyzed reactions and modify the conformation of lipids and other macromolecules, with consequences for most biological processes. To minimize the impact of such processes, we initially looked at the effect of low temperatures in fast-growing tissue, such as seedling roots. We found that low temperature promoted the accumulation of a GFP-RGA fusion protein and that growth of *gai-t6 rga-24* and quadruple-DELLA mutant roots was less affected by a short exposure to cold than was growth of wild-type roots. The difference in root growth between these lines decreases progressively with increase in cold period length, prolonged exposure to cold mainly reducing growth by slowing

metabolism. Accordingly, cold treatment retarded at the same level the growth of wild-type and quadruple-DELLA mutant adult plants (see Supplemental Figure 5 online). Thus, low temperature represses growth in part via a DELLA-dependent pathway, the growth repression being more severe than that due simply to a slowing of metabolism at low temperature. This mechanism could have its importance particularly when plants are exposed to alternating periods of high and low temperatures.

The CBF cold response pathway plays a central role in cold acclimation (Cook et al., 2004). *CBF1*, *CBF2*, and *CBF3* genes have previously been shown to respond rapidly to low temperature and to induce the expression of various proteins (CBF regulon) that protect plants from freezing temperatures (Agarwal et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2006). Accordingly, overexpression of *CBF* genes in transgenic *Arabidopsis* plants conferred tolerance to freezing but also resulted in severe growth retardation under normal growth conditions (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Gilmour et al., 2000, 2004). Moreover, transgenic tomato plants overexpressing

Arabidopsis CBF1 exhibited dwarfism, which could be reversed by GA treatment (Hsieh et al., 2002). Dwarfism was also observed in transgenic *Arabidopsis* plants overexpressing *CBF4* and *DDF1* genes, close homologs of the *CBF/DREB1* gene family members, and it was reported that *DDF1* is involved in the regulation of GA metabolism (Haake et al., 2002; Magome et al., 2004, 2008). Here, we have shown that DELLA activity is substantially responsible for the dwarfism of *Arabidopsis CBF1-ox* plants, as lack of *GAI* and *RGA* (in *CBF1-ox gai-t6 rga-24*) suppressed this phenotype. Furthermore, we showed that a GFP-*RGA* protein accumulated to higher levels in *CBF1-ox* plants. Thus, we conclude that *CBF1* enhances DELLA accumulation and thereby inhibits plant growth. Because *DDF1* also modulates plant growth through a GA-dependent mechanism (Magome et al., 2004, 2008), it is likely that a large number of DREB factors regulate growth by interfering with the GA pathway.

Bioactive GA relieves DELLA restraint by promoting the degradation of DELLAs by the proteasome (Silverstone et al., 2001; Dill et al., 2004; Fu et al., 2004). We next determined the levels of gene transcripts encoding GA metabolic enzymes. This analysis revealed that cold and *CBF1* induction increase the level of *GA2ox3* and *GA2ox6* transcripts encoding an enzyme that deactivates bioactive GAs. Accordingly, we found that cold-treated wild-type plants and *CBF1-ox* plants contained reduced levels of bioactive GAs. We also found that exogenous GA rescued the growth of *CBF1-ox* plants. Thus, we conclude that cold-mediated *CBF1* induction modulates GA metabolism via upregulation of *GA2ox3* and *GA2ox6* gene transcript levels, contributing to the accumulation of DELLAs. Analyses of *GA2ox* gene promoter sequences have not revealed the presence of CRT/DRE-like *cis*-elements, suggesting that *CBF1* upregulates *GA2ox* gene expression indirectly. Interestingly and in contrast with *CBF1-ox* plants, we found that cold-treated wild-type plants also exhibited a transient increase in *GA2ox1* transcript levels, suggesting that cold enhances *GA2ox* levels via both *CBF1*-dependent and -independent manners.

It was recently suggested that DELLA-dependent growth restraint is advantageous in adverse environments (Achard et al., 2006). Thus, we speculated that DELLAs may contribute to the *CBF1*-mediated freezing tolerance. Indeed, we showed that lack of *GAI* and *RGA* (in *CBF1-ox gai-t6 rga-24*) substantially suppressed the freezing tolerance exhibited by *CBF1-ox* plants. Furthermore, cold-acclimated wild-type plants were more resistant to freezing than *gai-t6 rga-24* plants. Thus, cold-induced *CBF1* promotes freezing tolerance and cold acclimation at least in part by enhancing DELLA accumulation. However, because *CBF1-ox gai-t6 rga-24* plants were still more tolerant than *gai-t6 rga-24* plants, it is likely that *CBF1* also promotes freezing tolerance through DELLA-independent pathway(s).

Cold-induced or constitutive expression of *CBF1* results in upregulation of *COR* and Pro biosynthesis genes and the accumulation of soluble sugars, including sucrose, glucose, and fructose via modulation of the CBF regulon (Gilmour et al., 2004). There is evidence that *COR* polypeptides, Pro, and sugars contribute to freezing tolerance (Thomashow, 1998; Rolland et al., 2002; Gilmour et al., 2004). Our results suggest that *CBF1* modulates the CBF regulon via a DELLA-independent

pathway. Taken together, these findings lead us to propose that *CBF1* activates multiple mechanisms, DELLA-dependent and -independent, that work in concert to enhance freezing tolerance (Figure 8). The precise way by which DELLAs enhance freezing tolerance remains unclear. We have recently shown that DELLAs promote survival of adversity by reducing the accumulation of reactive oxygen species (ROS) levels, and it was reported that low temperature could lead to the production of ROS (Huner et al., 1998; Fowler and Thomashow, 2002; Achard et al., 2008). Perhaps DELLAs contribute to freezing tolerance through the regulation in ROS levels.

We propose a model in which the induction of *CBF1* expression by low temperature affects GA metabolism via upregulation of *GA2ox* gene transcripts (Figure 8). An increase in *GA2ox* enzyme levels results in a reduction in bioactive GAs, thus causing a higher accumulation of DELLAs (by increasing their stability). Our results also indicate that *CBF1* specifically regulates *RGL3* gene expression. As a consequence, increased DELLA accumulation restrains plant growth. Moreover, the *CBF1*-induced DELLA accumulation also contributes in a synergistic manner with the *CBF1*-induced *COR* pathway to promote cold adaptation. Finally, it is important to note that genetic studies suggest that the *CBF* cold response pathway is not the only system that contributes to cold acclimation. For example, *eskimo1* mutant plants are more compact in stature and are constitutively freezing tolerant, yet by a mechanism that is distinct from the *CBF* cold response pathway (Xin and Browse, 1998).

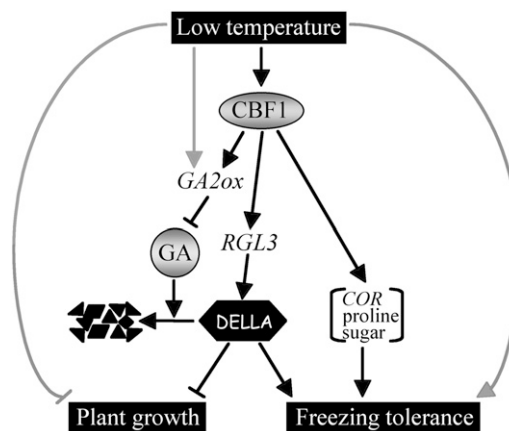


Figure 8. Scheme Showing Contribution of DELLAs to *CBF1*-Mediated Growth Regulation and Freezing Tolerance.

Cold-induced or constitutive expression of *CBF1* reduces bioactive GA levels (by increasing of *GA2ox3* and *GA2ox6* transcript abundance), thus promoting the accumulation in DELLAs by change in their protein stability. In addition, cold/*CBF1* specifically increases *RGL3* transcript levels. Low temperature also enhances *GA2ox1* expression levels via a *CBF1*-independent mechanism. DELLA accumulation in turn slows plant growth. *CBF1* also enhances freezing tolerance through the synergistic DELLA-dependent and *COR*-dependent pathways. Cold also restrains growth and promotes freezing tolerance by a DELLA-independent and *CBF*-independent cold response pathway.

METHODS

Plant Materials and Growth Conditions

All experiments used the Landsberg *erecta* laboratory strain of *Arabidopsis thaliana* as genetic background. The *gai*, *gai-t6 rga-24*, quadruple-DELLA mutant (*gai-t6 rga-t2 rgl1-1 rgl2-1*), and *pRGA:GFP-RGA* were as previously described (Achard et al., 2006). Plants were grown on soil or GM agar medium plates (Achard et al., 2007b) under long days (16 h light/8 h dark) at 22°C. Flowering time was expressed as the number of vegetative leaves produced before flowering. For root growth experiments, wild-type, *gai-t6 rga-24*, and quadruple-DELLA mutant seeds were surface sterilized and placed on GM plates at 4°C for 4 d to synchronize germination. Subsequently, plates were placed vertically in a controlled environment chamber (22°C; continuous light) for 3 d before transfer periodically (for 8 h or 16 h per day) or continuously to cold (4°C; continuous light). After 5 d, plates were scanned and root length was measured using ImageJ software (public domain; <http://rsb.info.nih.gov/ij>). For each experiment, roots from at least 30 seedlings were measured.

Vector Construction, Transformation, and Crossing

The *CBF1* cDNA was amplified by RT-PCR from mRNA extracted from 2-week-old plants that had been cold treated (4°C) for 2 h and was cloned into the pGEM-T easy vector system (Promega). A *Bam*HI-*Eco*RI fragment containing the *CBF1* cDNA was inserted into pGreen0229 containing a 35S CaMV cassette (derived from the p35S-2 cassette) and Basta resistance marker (<http://www.pgreen.ac.uk>). The plasmid was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. *Arabidopsis* plants were transformed by floral dip. The *CBF1-ox pRGA:GFP-RGA* and *CBF1-ox gai-t6 rga-24* lines were isolated from F3 progeny of the appropriate crosses. Genomic PCR and kanamycin resistance were used to confirm the *gai-t6 rga-24* genotype and the presence of *pRGA:GFP-RGA* transgene, respectively (see also Achard et al., 2006).

Transcript Level Analyses

For expression analysis, total RNA was extracted using Trizol reagent (Molecular Research Center) from 7-d-old seedlings or 2-week-old rosette leaves as indicated. For RNA gel blot analysis of cold-treated seedlings, plants were placed at 4°C for 2, 4, 8, and 24 h. RNA gel blot transfers onto Hybond-N membrane (Amersham Biosciences) were performed in 10× SSC and hybridized at 42°C in SSPE containing 50% formamide with specific ³²P-random labeled probe (Promega). Blots were stripped at 80°C in 0.1× SSC/0.1% SDS. For quantitative real-time RT-PCR analysis, 2 μg of total RNA was treated first with 2 units of DNase I (Promega) and then reverse transcribed in a total volume of 40 μL with 2 μM oligo(dT)₂₀, 0.5 mM deoxynucleotide triphosphate, 5 mM DTT, and 200 units of Superscript III reverse transcriptase (Invitrogen). RT-PCR was performed using gene-specific primers in a total volume of 15 μL SYBR Green Master mix (Roche) on a Lightcycler LC480 apparatus (Roche) according to the manufacturer's instructions. The *GAPDH* and *At4g26410* (unknown function) genes were used as internal controls. The relative expression level of each gene in transgenic plants or in cold-treated plants was compared with that in non-cold-treated wild-type plants using GenEx Pro 4.3.5. software (MultiD Analyses) after normalization using the *GAPDH* cDNA level and averaging over three replicates. For quantitative RT-PCR experiments, after low-cycle PCR amplification (15 cycles) using specific primers, reaction products were blotted onto Hybond-N membrane (Amersham Biosciences) and probed at 65°C in 0.25 M sodium phosphate, pH 7.2/7% SDS with the equivalent PCR amplified ³²P-random-labeled fragment (Promega). Blots were exposed to film (within the linear range of detection). The images were scanned and

band intensities were quantified using ImageJ software. Two replicates were performed for each experiment. Primers for PCR amplification/probe preparation are listed in Supplemental Methods online.

Observation of GFP Fluorescence

Confocal microscopy images were obtained with a Zeiss LSM510 inverted confocal laser microscope with ×40 objectives. The excitation wavelength for GFP detection was 488 nm. All images were obtained with the same modifications and intensity parameters. GFP-RGA fluorescence was determined on 7-d-old *pRGA:GFP-RGA* and *CBF1-ox pRGA:GFP-RGA* root tip and elongating zone of the primary root.

Immunoblot Analyses

Tissues for protein analyses were ground in 2× SDS-PAGE sample buffer followed by boiling for 5 min. After centrifugation, the protein extracts were fractionated on a 10% SDS-PAGE gel and blotted onto membrane. Immunoblots were performed using a 2500-fold dilution of anti-GFP monoclonal antibodies from mouse (BD Biosciences) and a 5000-fold dilution of peroxidase-conjugated goat anti-mouse IgG (Molecular Probes). Signals were detected by film (within the linear range of detection) using the enhanced chemiluminescence protein gel blot analysis system (Amersham Biosciences). The blot was subsequently stripped with 0.2 N glycine, pH 2.5, and reprobed with anti-cdc2 (PSTAIRE) antibody (Santa Cruz Biotechnology) for loading control. Signals of GFP-RGA (Figure 2A) were scanned and then quantified using ImageJ software and normalized with the respective signals of PSTAIRE. Each immunoblot assay was repeated at least twice.

GA Determinations

GA determinations were performed on soil-grown 2-week-old vegetative rosettes. When indicated, the plants were subjected to cold for 4 and 12 h. Biological replicates (0.1 to 0.4 g dry weight) were analyzed by gas chromatography–mass spectrometry selected reaction monitoring with [²H₂]GA internal standards as described elsewhere (Rieu et al., 2008).

Sugar Analysis

Glucose, fructose, and sucrose were measured with a sequential enzymatic assay (production of NADH) using glucose (G2020), fructose (F2668), and sucrose assay (S1299) kits (Sigma-Aldrich). Production of NADH was monitored at 340 nm with a spectrophotometer ELx808 (Bio-Tek Instruments). The experiment was repeated three times (*n* = 10).

Freezing Tolerance Assays

Two-week-old plants grown in a growth room (22°C; 16 h photoperiod) were placed at –10°C for 24 h. Plants were then placed at 4°C for 6 h for recovery before being returned to the original growth room. Plant survival was scored after 4 d of recovery under normal growth conditions. Cold acclimation was performed at 4°C for 3 d. The experiment was repeated three times (*n* = 24).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CBF1/DREB1b*, AT4G25490; *GAI*, AT1G14920; *RGA*, AT2G01570; *RGL1*, AT1G66350; *RGL2*, AT3G03450; *RGL3*, AT5G17490; *GA20ox1*, AT4G25420; *GA20ox2*, AT5G51810; *GA20ox3*, AT5G07200; *GA3ox1*, AT1G15550; *GA3ox2*, AT1G80340; *GA2ox1*, AT1G78440; *GA2ox2*, AT1G30040; *GA2ox3*, AT2G34555; *GA2ox4*,

AT1G47990; *GA2ox6*, AT1G02400; *RD29a/COR78*, AT5G52310; *COR15a*, AT2G42540; *COR47*, AT1G20440; and *P5CS2*, AT3G55610.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The *pRGA:GFP-RGA* Transgenic Line Expresses a GFP-RGA Fusion Protein That Is Detectable by Protein Gel Blot Using an Anti-GFP Antibody.

Supplemental Figure 2. Expression of *CBF1* in T3 Transgenic *CBF1-ox* Lines.

Supplemental Figure 3. *Arabidopsis* DELLAs Contribute Significantly to Freezing Tolerance.

Supplemental Figure 4. Cold Enhances Both CBF Regulon and Sugar Levels via a DELLA-Independent Mechanism.

Supplemental Figure 5. Cold Treatment Slows at the Same Level the Vegetative Growth of Wild-Type and Quadruple-DELLA Mutant Plants.

Supplemental Methods. Primer List.

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