Overexpression of the Arabidopsis *CBF3* Transcriptional Activator Mimics Multiple Biochemical Changes Associated with Cold Acclimation[1]

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We further investigated the role of the Arabidopsis *CBF* regulatory genes in cold acclimation, the process whereby certain plants increase in freezing tolerance upon exposure to low temperature. The *CBF* genes, which are rapidly induced in response to low temperature, encode transcriptional activators that control the expression of genes containing the C-repeat/ dehydration responsive element DNA regulatory element in their promoters. Constitutive expression of either *CBF1* or *CBF3* (also known as *DREB1b* and *DREB1a*, respectively) in transgenic Arabidopsis plants has been shown to induce the expression of target *COR* (cold-regulated) genes and to enhance freezing tolerance in nonacclimated plants. Here we demonstrate that overexpression of *CBF3* in Arabidopsis also increases the freezing tolerance of cold-acclimated plants. Moreover, we show that it results in multiple biochemical changes associated with cold acclimation: *CBF3*-expressing plants had elevated levels of proline (Pro) and total soluble sugars, including sucrose, raffinose, glucose, and fructose. Plants overexpressing *CBF3* also had elevated *P5CS* transcript levels suggesting that the increase in Pro levels resulted, at least in part, from increased expression of the key Pro biosynthetic enzyme Δ^1-pyrroline-5-carboxylate synthase. These results lead us to propose that *CBF3* integrates the activation of multiple components of the cold acclimation response.

Understanding the mechanisms that plants have evolved to survive freezing is of basic scientific interest and has the potential to offer new strategies to improve the freezing tolerance of agronomic plants. Toward this end, many investigators have focused on studying the phenomenon of cold acclimation, the process whereby certain plants increase in freezing tolerance in response to low temperatures (Hughes and Dunn, 1996; Thomashow, 1999). One line of investigation has been to identify genes that are induced at low temperature and to determine whether they have roles in freezing tolerance. This has led to the identification of scores of cold-induced genes, many of which encode either LEA (late embryogenesis abundant) or novel polypeptides (Thomashow, 1998). Recent results obtained with Arabidopsis indicate that at least one *COR* (cold-regulated) gene has a role in freezing tolerance. Overexpression of *COR15a*, which encodes a novel polypeptide that is targeted to the chloroplasts, has been shown to increase the freezing tolerance of chloroplasts in vivo and protoplasts in vitro (Artus et al., 1996). This increase in freezing tolerance results from the *COR15a*-encoded protein stabilizing membranes against freezing injury (Artus et al., 1996; Steponkus et al., 1998). There is evidence that the mature COR15a polypeptide, COR15am, acts directly as a cryoprotective protein that decreases the propensity of lipid bilayers to form deleterious hexagonal II phase lipids (Steponkus et al., 1998), a major type of freeze-induced membrane lesion that occurs in nonacclimated plants (Steponkus et al., 1993).

Whereas overexpression of *COR15a* has a detectable effect on freezing tolerance of chloroplasts and protoplasts, the effect is small (1°C–2°C; Artus et al., 1996; Steponkus et al., 1998). Moreover, it has little if any effect on whole-plant freezing survival (Jaglo-Ottosen et al., 1998). Expression of the entire battery of *COR* genes, which includes the *COR6.6, COR47,* and *COR78* gene pairs (in addition to the *COR15* gene pair), however, does (Jaglo-Ottosen et al., 1998). The ability to express all of the *COR* genes in concert at warm temperature was made possible by the discovery of the CBF family of transcriptional activators (Stockinger et al., 1997; Gilmour et al., 1998), also known as DREB1 proteins (Liu et al., 1998; Shinwari et al., 1998). *COR6.6, COR15a, COR47,* and *COR78,* and presumably other yet to be discovered CRT (C-repeat)/DRE (dehydration responsive element)-regulated *COR* genes, contain in their promoters a cold- and dehydration-responsive DNA regulatory element known as the CRT/DRE (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The first CRT/DRE binding factor to be dis-

covered, CBF1 (CRT/DRE binding factor 1), was initially shown to activate expression of reporter genes in yeast that carried the CRT/DRE element (Stockinger et al., 1997). This indicated that the protein, which has an AP2/EREBP DNA binding motif (Riechmann and Meyerowitz, 1998), was a transcriptional activator. Overexpression of *CBF1* in Arabidopsis was subsequently shown to activate expression of the entire battery of known CRT/DRE-regulated *COR* genes and to enhance whole plant freezing survival without a low temperature stimulus (Jaglo-Ottosen et al., 1998).

Additional studies have shown that *CBF1* is a member of a small gene family encoding nearly identical proteins (Gilmour et al., 1998; Shinwari et al., 1998). The genes, *CBF1*, *CBF2*, and *CBF3* (also known as *DREB1b*, *DREB1c*, and *DREB1a*, respectively), are located in tandem on chromosome 4 (Gilmour et al., 1998; Shinwari et al., 1998). Overexpression of *CBF3* in Arabidopsis, like overexpression of *CBF1*, activates *COR* gene expression and enhances freezing tolerance at warm nonacclimating temperatures (Liu et al., 1998; Kasuga et al., 1999). All three *CBF* genes are cold-induced; *CBF* transcript levels increase within 15 min of transferring plants to low temperature followed at approximately 2 h by accumulation of transcripts for the target CRT/DRE-regulated *COR* genes (Gilmour et al., 1998; Shinwari et al., 1998). The mechanism whereby the *CBF* genes are activated by low temperature is not known but does not appear to involve autoregulation (Gilmour et al., 1998).

Many biochemical changes occur in plants during cold acclimation in addition to *COR* gene expression and are likely to have roles in freezing tolerance. It is well documented that lipid composition changes during cold acclimation in a wide range of plants and there are compelling data to indicate that this contributes to greater freezing tolerance (Steponkus et al., 1993). In a similar manner, the levels of Pro and Suc increase in Arabidopsis (McKown et al., 1996; Wanner and Junttila, 1999) and other plants (Guy et al., 1992; Koster and Lynch, 1992) during cold acclimation and are likely to have roles in freezing tolerance. There is evidence that Pro can protect both membranes and proteins against freeze-induced damage in vitro (Rudolph and Crowe, 1985; Carpenter and Crowe, 1988) and direct evidence that increased levels of Pro enhances whole plant freezing tolerance (Nanjo et al., 1999). Suc and other simple sugars have also been shown to be effective cryoprotectants in vitro (Strauss and Hauser, 1986; Carpenter and Crowe, 1988) and there is correlative evidence indicating a role in freezing tolerance in cold-acclimated plants (Guy et al., 1992; Koster and Lynch, 1992; Wanner and Junttila, 1999). A question thus raised is whether the CBF transcription factors are limited to activating the expression of *COR* genes encoding cryoprotective polypeptides, or alternatively, have a role in activating multiple components

of the cold acclimation response. The results presented here support the latter model.

RESULTS

Identification of Transgenic Arabidopsis Plants That Overexpress *CBF3*

Transgenic Arabidopsis plants that overexpress *CBF3* at normal growth temperature were created by placing the *CBF3* coding sequence under control of the cauliflower mosaic virus (CaMV) 35S promoter and transforming the construct into Arabidopsis plants using the floral dip transformation procedure. Twenty-two independent lines were identified in which the T2 plants segregated 3:1 for kanamycin resistance (the selectable marker carried on the transformation vector). These lines presumably carried a single active T-DNA locus. The kanamycin resistant T2 plants were then screened by western analysis for constitutive expression of *COR15a*, a target gene of the CBF transcriptional activators. Eight lines were identified that constitutively produced the COR15am protein at levels that were equal to or greater than that which occurred in cold-acclimated wild-type plants. However, further analysis indicated that in five of these lines, *COR15a* expression was not uniform among the plants; i.e. although all of the plants were kanamycin resistant, not all produced the COR15am polypeptide. This variation may have resulted from gene silencing events. Regardless, these lines were not used further for this study.

Three independent transgenic lines (A28, A30, and A40) were identified that produced the COR15am polypeptide at high levels uniformly among plants grown at normal temperatures. Northern analysis (Fig. 1) indicated that the transcript levels for *CBF3* were about equal in nonacclimated and cold-treated A28, A30, and A40 plants and were much greater than those observed in either nonacclimated or cold-treated control plants (i.e. non-transformed plants or transgenic plants carrying the transformation vector without an insert). The transcript levels for two target *COR* genes, *COR15a* and *COR6.6*, were also nearly equal in nonacclimated and cold-treated A28, A30, and A40 plants and approximated the levels observed in cold-acclimated control plants (Fig. 1). Western analysis (Fig. 2) indicated that the proteins encoded by *COR15a* and *COR6.6* were present in both nonacclimated and cold-acclimated A28, A30, and A40 plants at 3- to 5-fold higher levels than those found in cold-acclimated control plants.

Effects of *CBF3* Overexpression on Vegetative Growth, Time to Flowering, and Freezing Tolerance
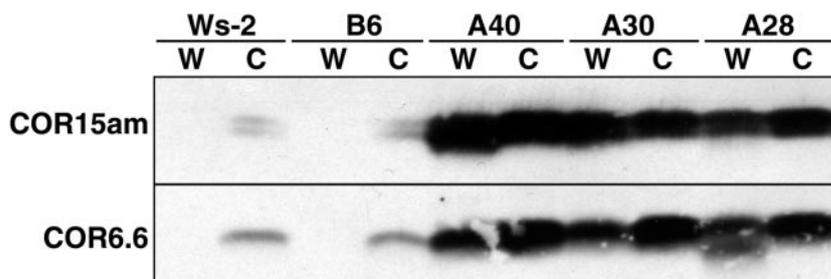
Liu et al. (1998) have reported that transgenic Arabidopsis plants overexpressing *CBF3* (*DREB1a*) have a "dwarf" phenotype. This was also true of the A28, A30, and A40 transgenic plants. After the same num-
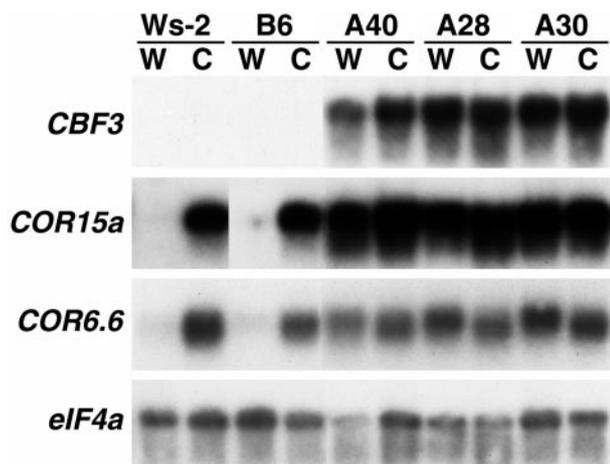
Figure 1. Transcript levels of *CBF3* and target *COR* genes in transgenic plants overexpressing *CBF3*. Northern analysis of total RNA (20 μg for *CBF3*; 5 μg for the other genes) prepared from control Arabidopsis Ws-2 and B6 plants and from *CBF3*-expressing A40, A28, and A30 plants. Plants were either grown at 20°C (W) or at 20°C and then cold treated at 5°C for 7 d (C). *eIF4a* (eukaryotic initiation factor 4a) is a constitutively expressed gene used as a loading control (Metz et al., 1992). Upon long exposure, *CBF3* transcripts can be detected in the total RNA samples prepared from cold-treated Ws-2 and B6 plants (not shown).
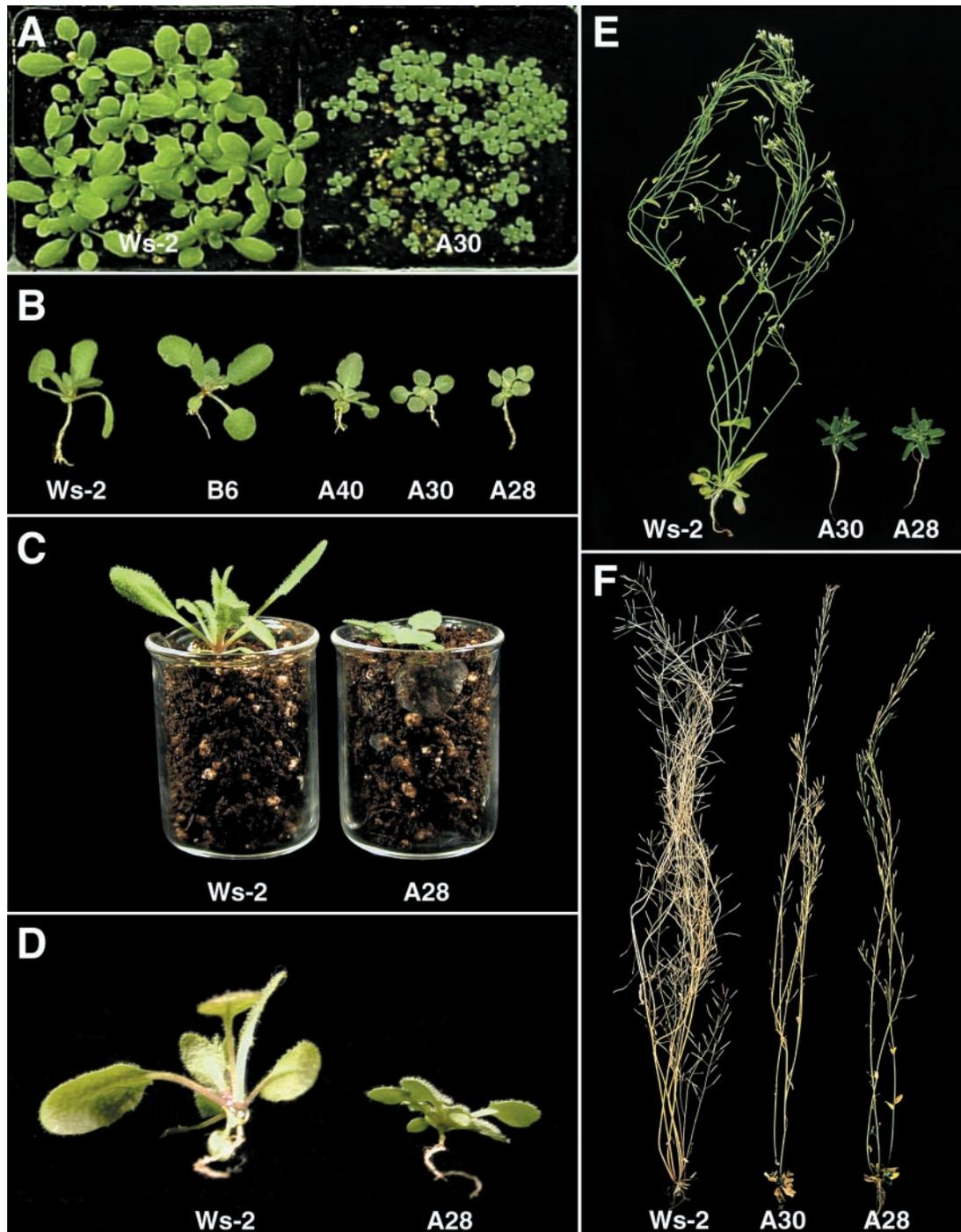
ber of days of vegetative growth at normal temperature, the size of the leaves and overall dimensions of the *CBF3*-expressing plants were considerably less than those of the control plants (Fig. 3, A and B). Additional effects on growth that were not previously noted were also evident. One was that *CBF3*-expressing plants had a pronounced prostrate growth habit; whereas the leaves of the control plants generally had an upright stature, those of the transgenic plants laid flat to the soil (Fig. 3C). The *CBF3*-expressing plants also had much shorter petioles when compared with those of the control plants (Fig. 3D) and the leaves had a slight bluish-green tint (Fig. 3, A and B). Also, there was a substantial difference in time to flowering between the control and *CBF3*-expressing plants; i.e. control plants bolted and formed flowers well before the *CBF3*-expressing plants did (Fig. 3E). In one experiment, for instance, the control plants began to bolt at 17 d, whereas the A40, A30, and A28 plants took 21, 26, and 28 d, respectively, to initiate bolting

(Table I). The *CBF3*-expressing plants went on to form flowers and set seed, although as noted by Liu et al. (1998), the final plant mass and seed yield were considerably less than that obtained with control plants (Fig. 3F). The lower yield of seed was due at least in part to the *CBF3*-expressing plants producing fewer axillary shoots. The delay in flowering observed in the *CBF3*-expressing plants, significantly, did not "simply" involve a slower overall growth rate, but appeared to involve a developmental delay in flowering. In one experiment, for instance, the control plants produced an average of 4.5 and 4.6 leaves per rosette, whereas the A40, A30, and A28 transgenic plants produced 6.0, 9.7, and 12.5 leaves per rosette, respectively (Table I).

Overexpression of *CBF3* (*DREB1a*) (Liu et al., 1998; Kasuga et al., 1999), like overexpression of *CBF1* (Jaglo-Ottosen et al., 1998), has been reported to increase the freezing tolerance of nonacclimated plants. Similarly, we found that nonacclimated control plants were killed by freezing at −6°C for 24 h whereas nonacclimated *CBF3*-expressing plants were not; results for Arabidopsis (L.) Heynh. ecotype Wassilewskija (Ws)-2 and A30 plants are shown in Figure 4A. To quantify the increase in freezing tolerance we carried out electrolyte leakage tests. The results indicated that the freezing tolerance of nonacclimated *CBF3*-expressing plants was greater than that of the nonacclimated control plants; nonacclimated control plants had an EL_{50} (temperature that caused a 50% leakage of electrolytes) of approximately −4.5°C, whereas the three *CBF3*-expressing lines had EL_{50} values of approximately −8°C (Fig. 4B). The freezing tolerance of cold-acclimated *CBF3*-expressing plants was significantly greater than that of both nonacclimated *CBF3*-expressing plants and cold-acclimated control plants. *CBF3*-expressing plants that had been cold acclimated for 7 d had EL_{50} values of −11°C and lower (Fig. 4, C and D). In these particular experiments, the cold-acclimated control plants had EL_{50} values of approximately −6°C (Fig. 4, C and D). In other experiments, we have obtained EL_{50} values as low as −8°C, but have never observed cold-acclimated control Ws-2 plants with EL_{50} values as low as those obtained with the cold-acclimated *CBF3*-expressing plants.

Figure 2. Protein levels of COR15am and COR6.6 in transgenic plants overexpressing *CBF3*. Western analysis of total soluble protein (50 μg) prepared from control Arabidopsis Ws-2 and B6 plants and from *CBF3*-expressing A40, A30, and A28 plants. Plants were either grown at 20°C (W), or grown at 20°C and then cold treated at 5°C for 7 d (C). Protein transfers were treated with antiserum made to recombinant COR15am and COR6.6 polypeptides (Gilmour et al., 1996).

Figure 3. Growth characteristics of *CBF3*-expressing transgenic plants. A, Non-transformed Ws-2 plants and transgenic *CBF3*-expressing A30 plants after 2 weeks growth at 20°C. B, Non-transformed Ws-2 and transgenic B6 "control" plants and transgenic *CBF3*-expressing A40, A30, and A28 plants after 15 d growth at 20°C. C and D, Non-transformed Ws-2 plants and transgenic *CBF3*-expressing A28 plants after 16 d growth at 20°C. E, Non-transformed Ws-2 plants and *CBF3*-expressing A30 and A28 plants after 5 weeks growth at 20°C. F, As in E except after 9 weeks of growth at 20°C.

Overexpression of *CBF3* Affects Pro Metabolism

Pro levels increase during cold acclimation in Arabidopsis and other plants (Koster and Lynch, 1992; Alberdi et al., 1993; McKown et al., 1996; Wanner and Junttila, 1999). We reasoned that if the increase in Pro that occurs in Arabidopsis with cold acclimation was brought about by genes that were regulated by the CBF activators, then overexpression of *CBF3* might result in elevated levels of Pro in nonacclimated plants. This was the case. Under nonacclimating

Table I. *Effects of CBF3 expression on time to flowering and rosette leaf number*

| Plants	Time to Flowering (d)[a]	Rosette Leaves Per Plant (n)
Ws-2	17	4.5 (8)
B6	17	4.6 (8)
A40	21	6.0 (7)
A30	26	9.7 (15)
A28	28	12.5 (8)

[a] Period of time between planting seeds in soil and appearance of first flower buds in a population of "*n*" plants.

growth conditions, the free Pro levels in the *CBF3*-expressing plants were approximately 5-fold higher than they were in the control plants, levels which were about the same as those found in cold-acclimated control plants (Fig. 5). The Pro levels in the *CBF3*-expressing plants increased further (approximately 2-fold) upon cold acclimation and were 2- to 3-fold higher than those found in the cold-acclimated control plants (Fig. 5).

The Pro biosynthetic enzyme Δ^1-pyrroline-5-carboxylate synthase (P5CS) has a key role in determining Pro levels in plants (Yoshiba et al., 1997). Because of this, and that *P5CS* transcript levels have been shown to increase in Arabidopsis in response to low temperature (Xin and Browse, 1998), it was of interest to determine whether *P5CS* transcript levels were elevated in the *CBF3*-expressing plants. Northern analysis indicated that they were; *P5CS* transcript levels were approximately 4-fold higher in nonacclimated *CBF3*-expressing plants than they were in nonacclimated control plants and were about equal to those found in the control plants that had been cold-treated for 1d (Fig. 6). The *P5CS* transcript levels in 7-d cold-acclimated *CBF3*-expressing plants were 2- to 3-fold higher than in cold-acclimated control plants (Fig. 6), a finding that was consistent with the 2- to 3-fold higher levels of Pro found in the cold-acclimated *CBF3*-expressing plants (Fig. 5).

Overexpression of *CBF3* Affects Sugar Metabolism

The accumulation of simple sugars is another commonly observed biochemical change that occurs with cold acclimation in Arabidopsis and other plants (Guy et al., 1992; Koster and Lynch, 1992; McKown et al., 1996; Wanner and Junttila, 1999). Thus, it was of interest to determine whether *CBF3* expression affected the sugar levels in plants. This was tested by determining the amount of total soluble sugars in control and *CBF3*-expressing plants at both nonacclimating and cold-acclimating temperatures. The results indicated (Fig. 7) that the levels of total sugars in nonacclimated *CBF3*-expressing plants were approximately 3-fold higher than those in nonacclimated control plants. Upon cold acclimation, sugar levels went up in both the control and *CBF3*-expressing plants approximately 2-fold; thus, sugar levels in the *CBF3*-expressing plants remained ap-
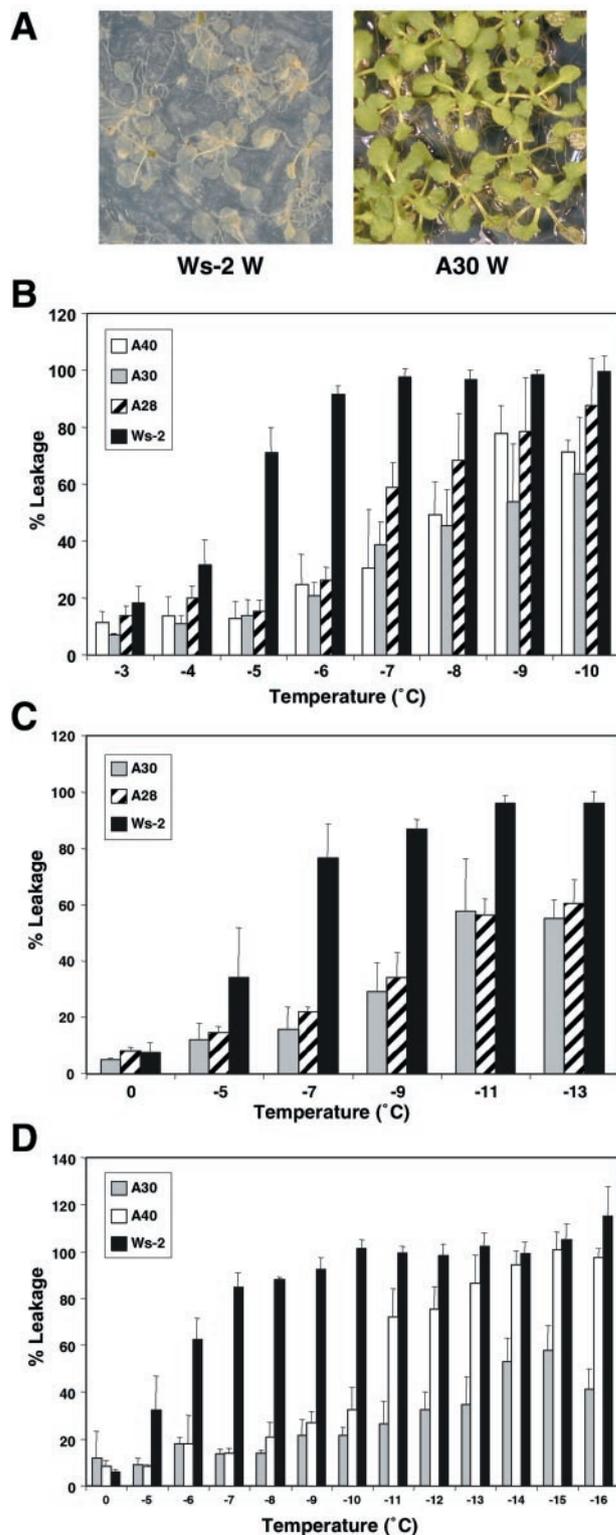
Figure 4. Effect of *CBF3* overexpression on plant freezing tolerance. A, Seedlings of control Arabidopsis Ws-2 plants and *CBF3*-expressing A30 plants were grown at 20°C on solid medium and then frozen at −2°C for 24 h followed by 24 h at −6°C. B, Control Arabidopsis Ws-2 plants and *CBF3*-expressing A40, A30, and A28 plants were grown at 20°C, and the freezing tolerance of leaves was measured using the electrolyte leakage test. C and D, Same as B except that plants were grown at 20°C followed by 7-d-cold acclimation at 5°C.
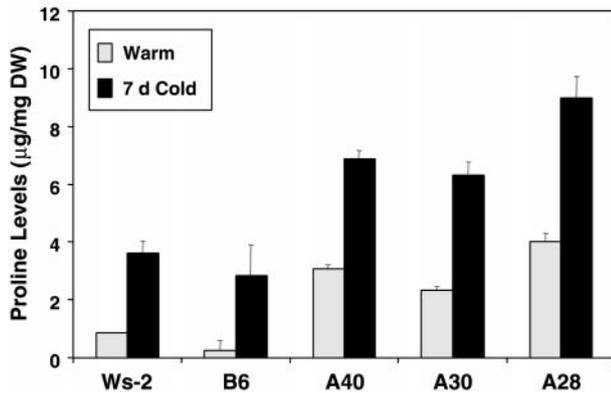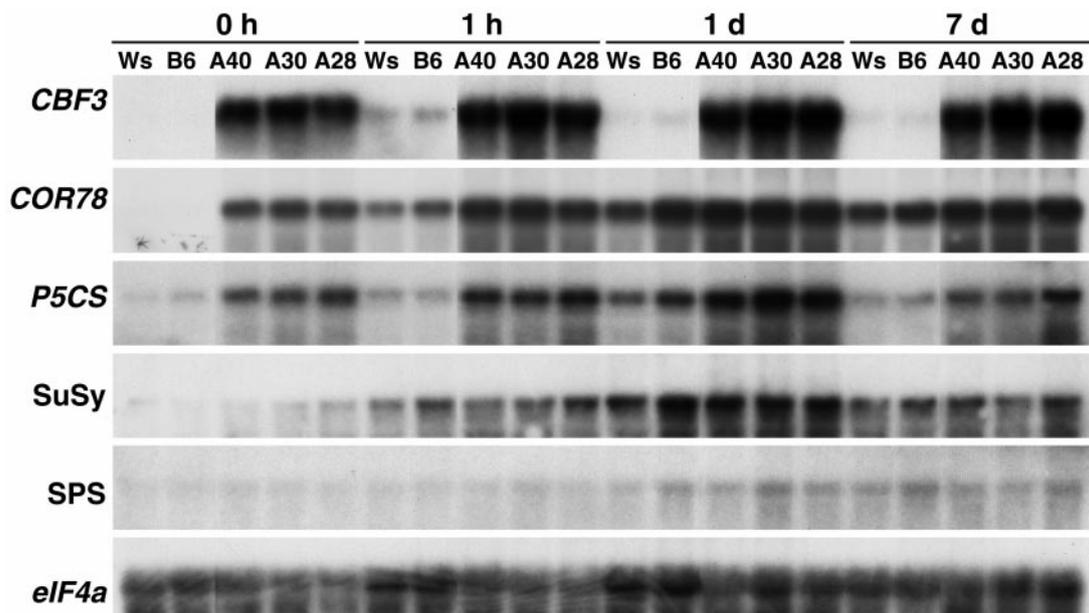
Figure 5. Effect of *CBF3* expression on Pro levels. Free Pro levels were determined in leaf tissue from control Arabidopsis Ws-2 and B6 plants and *CBF3*-expressing A40, A30, and A28 plants grown at 20°C (warm), or plants grown at 20°C and cold-treated at 5°C for 7 d (7-d cold).

proximately 3-fold higher in the control plants. Total soluble sugars notably accounted for as much as 20% of the total dry weight of plant material in the cold-acclimated *CBF3*-expressing plants.

Analysis of the total soluble sugars by gas chromatography was performed on samples prepared from Ws-2 and B6 control plants and A28, A30, and A40 *CBF3*-expressing plants. The results indicated that *CBF3* expression brought about increased levels of several sugars, namely Glc, Fru, Suc, and raffinose. A representative experiment comparing nonacclimated

and cold-acclimated Ws-2 and A28 plants is presented in Figure 8. The results indicate that the levels of Glc, Fru, Suc, and raffinose increased in the Ws-2 control plants upon cold acclimation by approximately 2- to 5-fold depending on the particular sugar. In nonacclimated A28 plants, the levels of the four sugars were equal to or higher than they were in the cold-acclimated Ws-2 plants and increased further upon cold acclimation. In the cold-acclimated A28 plants, Suc plus raffinose accounted for approximately 12% of the total dry weight and Glc plus Fru accounted for approximately 8%.

Two enzymes that have key roles in determining the levels of Suc in plant cells are Suc-phosphate-synthase (SPS) and Suc synthase (SuSy; Winter and Huber, 2000). Transcript levels for genes encoding both SPS (Strand et al., 1997) and SuSy (Déjardin et al., 1999) have been shown to increase in Arabidopsis in response to low temperature. Thus, it was of interest to determine whether CBF3 had an effect on the expression of these genes. As previously reported, we found that the transcript levels for both the SPS and SuSy genes increased in response to low temperature (Fig. 6). However, these increases did not appear to involve the CBF transcriptional activators as there was little if any difference in the transcript levels for the SPS and SuSy encoding genes in the *CBF3*-expressing and control plants (Fig. 6). Thus, the effects of CBF3 on sugar levels do not appear to be mediated by altering transcription of the SPS or SuSy encoding genes.

Figure 6. Effect of *CBF3* expression on transcript levels of genes involved in Pro and sugar metabolism. Northern analysis of total RNA (20 μg for *CBF3*; 5 μg for other genes) isolated from control Arabidopsis Ws-2 and B6 plants and from *CBF3*-expressing A40, A30, and A28 plants. Plants were grown at 20°C then cold-treated at 5°C for the times indicated. The blots were hybridized with probes for *CBF3*, *COR78*, *P5CS2*, Suc synthase (SuSy), Suc-phosphate synthase (SPS), and *eIF4a*, a constitutively expressed gene used as a loading control (Metz et al., 1992).
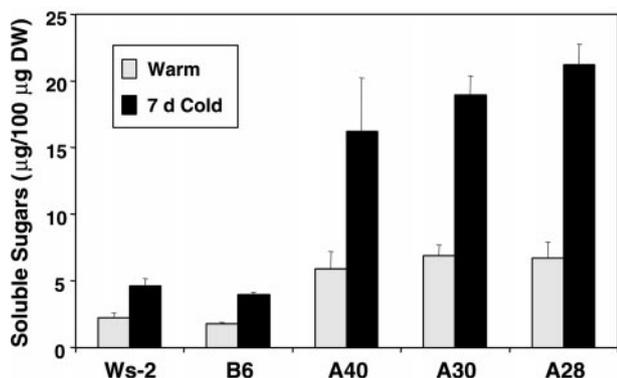
Figure 7. Effect of *CBF3* expression on levels of total soluble sugars. Total soluble sugars were determined for leaf tissue from control Ws-2 and B6 plants and *CBF3*-expressing A40, A30, and A28 plants grown at 20°C (warm) or plants grown at 20°C and cold treated at 5°C for 7 d (7-d cold).

DISCUSSION

The increase in freezing tolerance that occurs with cold acclimation is thought to involve the activation of multiple freezing tolerance mechanisms. Here we show that overexpression of the CBF3 transcriptional activator results in multiple biochemical changes that are commonly observed to occur in plants during cold acclimation. We show specifically that CBF3 overexpression in Arabidopsis results in the accumulation of COR polypeptides, the accumulation of Pro, and the accumulation of soluble sugars including Suc, raffinose, Glc, and Fru. There is evidence to indicate that each of these "classes" of biochemical alterations—COR polypeptides (Thomashow, 1998), Pro (Rudolph and Crowe, 1985; Carpenter and Crowe, 1988; Nanjo et al., 1999), and sugars (Strauss and Hauser, 1986; Carpenter and Crowe, 1988; Koster and Lynch, 1992; Wanner and Junttila, 1999)—contribute to an enhancement of freezing tolerance. It is interesting that we also found that Arabidopsis plants overexpressing *CBF3* displayed a prostrate growth habit, a phenotype that has been associated with cold acclimation, and increased freezing tolerance in other plants (Omran et al., 1968; Roberts, 1990). Taken together, these findings lead us to propose that Arabidopsis *CBF3* is a key regulatory gene that acts to integrate the activation of multiple mechanisms that work in concert to enhance freezing tolerance. Presumably *CBF1* and *CBF2* have overlapping, if not identical, roles to those of *CBF3*.

How does CBF3 bring about the biochemical changes that we observed? In the case of *COR* gene induction, the answer is known; CBF3 binds to CRT/DRE regulatory elements located in the promoters of these genes and activates their expression (Gilmour et al., 1998). Whether CBF3 also binds to the promoters of genes directly involved in sugar and Pro synthesis remains to be determined. However, our results indicate that *CBF3* overexpression causes an increase in the transcript levels for at least one of the two known *P5CS* Pro biosynthetic genes (Strizhov et

al., 1997). A CBF3-induced increase in *P5CS* gene expression thus seems likely to contribute to the increase in Pro levels observed in the *CBF3*-expressing plants. An examination of the promoter region of the *P5CS2* gene (Strizhov et al., 1997; GenBank accession no. X86788) reveals that the core conserved sequence of the CRT/DRE regulatory element, CCGAC (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994), is present twice within 350 nucleotides upstream of the ATG start codon. Whether CBF3 binds to these sequences and activates expression of the *P5CS2* gene, however, remains to be determined. It should be noted that Kasuga et al. (1999) did not observe any effect of CBF3 (DREB1A) expression on the levels of *P5CS* transcripts, nor did they observe an increase in *P5CS* transcript levels in response to low temperature, as we observed here (Fig. 6) and as Xin and Browse (1998) previously reported. The reason for this difference is not immediately obvious, but may be due, in part, to the *P5CS* probes used; we used a cDNA for the *P5CS2* gene whereas Kasuga et al. (1999) used a probe for *P5CS1*.

Xin and Browse (1998) have identified an Arabidopsis gene, *ESKIMO1* (*ESK1*), that affects the levels of Pro and sugars and has a major effect on freezing tolerance. Whereas wild-type plants grown at normal temperatures had a median lethal temperature $(LT)_{50}$ of $-2.8°C$ in a whole plant freeze test, plants carrying an *esk1* mutation had a median lethal temperature of $-10.6°C$. The concentrations of free Pro and total sugars were elevated in the *esk1* mutant plants about 30-fold and 2-fold, respectively. In addition, *P5CS* transcript levels in nonacclimated *esk1* plants were
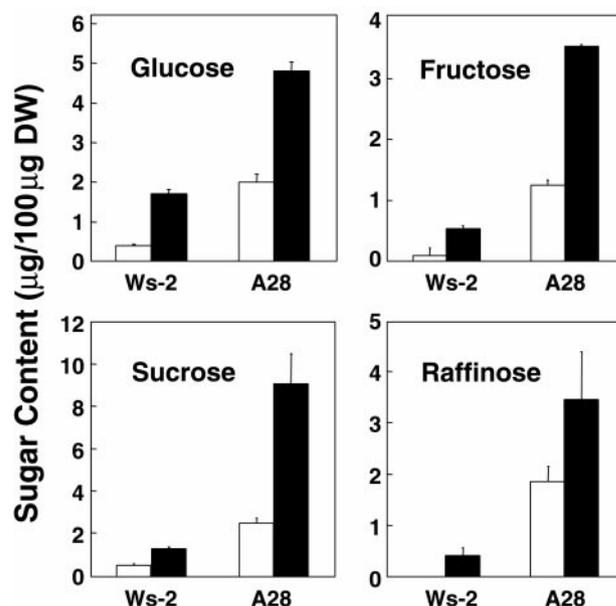
Figure 8. Effect of *CBF3* expression on the levels of specific soluble sugars. Total sugars were prepared from leaf tissue of control Ws-2 plants and CBF3-expressing A28 plants grown at 20°C (white bars) or at 20°C followed by 7 d at 5°C (black bars) and the levels of individual sugars determined by gas chromatography.

approximately 8-fold higher than in nonacclimated wild-type plants. The *esk1* mutation, significantly, did not affect expression of the *COR* genes; the transcript levels for *COR6.6*, *COR15a*, *COR47*, and *COR78* remained low under normal growth conditions in the *esk1* plants and were highly induced in response to low temperature. Therefore, Xin and Browse (1998) reasonably argued that it is not appropriate to consider cold acclimation as a simple, linear signaling pathway activating the full set of processes responsible for increasing freezing tolerance. Instead, they proposed a model in which parallel or branched signaling pathways activate "distinct suites" of cold acclimation responses. As envisioned, activation of one pathway would be able to result in considerable freezing tolerance without support from other components. Because *esk1* plants did not overexpress the *COR* genes, Xin and Browse (1998) proposed that *esk1* defines a signaling pathway of cold acclimation that is distinct from that which mediates expression of the *COR* genes.

Our findings with the *CBF3* overexpressing plants do not contradict the general cold acclimation model of Xin and Browse (1998), but suggest a more integrated circuitry for cold acclimation signaling. In particular, our results provide evidence that the synthesis of COR proteins, Pro and sugars are coordinately regulated and that *CBF3* has a key role in this regulation. The fact that the *esk1* mutation affects the levels of *P5CS* expression, but not *COR* gene expression, can be explained in ways that do not necessitate the existence of distinct signaling pathways for Pro and COR protein synthesis. The two available *esk1* alleles are recessive and, as mentioned above, cause an 8-fold increase in *P5CS* transcript levels. Thus, *ESK1* appears to be a negative regulator of *P5CS* transcription at warm temperatures. One simple possibility is that ESK1 is a transcriptional repressor that binds to the promoter of one or both of the Arabidopsis *P5CS* genes (Strizhov et al., 1997) at warm temperature and keeps transcription at a relatively low level. At low temperature, CBF3 could either directly bind to the *P5CS* promoter(s) and overcome repression by ESK1, or induce the expression of some other protein that inactivates ESK1. In these scenarios, the *esk1* mutation would not affect expression of the *COR* genes because the *COR* genes would not have the ESK1 binding sequence. Of course, more complex models could also explain the current findings. The fundamental point is that the new information provided by this study argues for an integrated control of cold acclimation with CBF3, and presumably the other CBF proteins as well, having a key role in inducing multiple biochemical and physiological changes that act to increase plant freezing tolerance.

The finding that *CBF3* overexpression results in accumulation of COR polypeptides, Pro, and sugars suggests that a considerable portion of the cold acclimation response falls under control of the *CBF*

genes. However, our results also indicate that the freezing tolerance of the *CBF3* overexpressing plants increased further in response to low temperature (Fig. 4). Thus, overexpression of *CBF3* at warm temperature did not result in maximum freezing tolerance even though expression of its target genes, as judged by the levels of *COR* proteins (Fig. 2), was somewhat greater than that which occurred in cold-acclimated control plants. These findings could signify that *CBF3* controls only a subset of low temperature-induced freezing tolerance genes and that additional non-*CBF3*-controlled genes might be induced in response to low temperature and activate additional freezing tolerance mechanisms. This would be akin to the parallel pathway model proposed by Xin and Browse (1998). However, it remains possible that *CBF3* actually regulates all of the genes that are important in cold acclimation, but that the activities of the proteins encoded by these genes might be greater at low temperature; the proteins might be activated in response to phosphorylation catalyzed by a temperature-regulated protein kinase, for instance, or their action affected by changes in metabolite pool levels brought about by slower plant growth at low temperature. There is also the more trivial explanation that expression of *CBF3* under control of the CaMV 35*S* promoter might not completely mimic the activation of *CBF3* by low temperature.

The effect that *CBF3* overexpression has on flowering is curious. Time to flowering is controlled by multiple physiological and environmental factors (Koornneef et al., 1998; Levy and Dean, 1998). In some plants, including Arabidopsis, the transition to flowering is responsive to vernalization, a long period (weeks) of low temperature treatment. In "facultative" plants such as Arabidopsis, the effect of vernalization is to shorten the time to flowering (the magnitude of the effect varies greatly among Arabidopsis ecotypes). If *CBF3* expression at warm temperature was fully mimicking exposure to low temperature, then one might think that if it had any effect on flowering, that it would decrease the transition time and cause a corresponding decrease in the number of rosette leaves per plant. Our results, however, indicate that *CBF3* expression had the opposite effect; it increased the time to flowering and increased the number of rosette leaves per plant. A mechanism to explain this effect is not immediately obvious but would seem to be consistent with the notion that *CBF3* expression does not control all low temperature responses in Arabidopsis.

We previously reported that *CBF1* overexpression induced *COR* gene expression and increased plant freezing tolerance without a low temperature stimulus (Jaglo-Ottosen et al., 1998). A significant difference in that study and the results presented here is that we did not note any obvious effect of *CBF1* overexpression on plant growth. A likely explanation for this difference is suggested by the results of Liu et

al. (1998). These investigators found a positive correlation between the level of *CBF3* (*DREB1a*) expression, the level of expression of the target gene *COR78* (*RD29a*), and the degree to which the plants were stunted in growth. In our previous experiments (Jaglo-Ottosen et al., 1998), the level of COR15am protein in nonacclimated *CBF1*-expressing plants was approximately equal to the levels of COR15am in control plants that had been cold-acclimated for 7 d. Here, the COR15am levels in the nonacclimated *CBF3*-expressing plants was greater than in control plants that had been cold-acclimated for 7 d (Fig. 2). Thus, it seems likely that the lack of effect on growth and development observed with the *CBF1*-expressing plants was due to lower levels of CBF-induced target gene expression. It should be kept in mind, however, that it has not yet been established that all three *CBF* genes control the same sets of genes. A detailed comparison between the levels of *CBF1*, *CBF2*, and *CBF3* expression in transgenic Arabidopsis plants, the levels of target gene expression assessed on a genomic scale through microarray analysis, and a detailed analysis of the growth and development of the plants should help resolve this issue.

MATERIALS AND METHODS

Plant Growth

Arabidopsis (L.) Heynh. ecotype Wassilewskija (Ws)-2 and transgenic plants in the Ws-2 background were grown in controlled environment chambers at 20°C under constant illumination from cool-white fluorescent lights (100–150 μmol m^{-2} s^{-1}) in Baccto planting mix (Michigan Peat, Houston). Pots were subirrigated with deionized water as necessary. All seeds were cold-treated (5°C) for 4 d immediately after planting to ensure uniform germination. Plants were cold acclimated by placing pots at 5°C under continuous light (20–60 μmol m^{-2} s^{-1}) as described (Gilmour et al., 1988).

Constructs and Plant Transformation

A 910-bp *Bam*HI/*Hin*dIII fragment from a cDNA clone containing the whole coding region of *CBF3* (Gilmour et al., 1998) was inserted into the *Bgl*II and *Hin*dIII sites of the binary transformation vector pGA643 (An et al., 1988). The resulting plasmid, pMPS13, which contains the *CBF3* coding sequence under control of the CaMV 35*S* promoter, was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Arabidopsis plants were transformed with plasmid pMPS13 or the transformation vector pGA643 using the floral dip method (Clough and Bent, 1998). Transformed plants were selected on the basis of kanamycin resistance. Homozygous T3 or T4 plants were used in all experiments.

Western Analysis

Total soluble protein was obtained essentially as described (Gilmour et al., 1996) by grinding leaf material (approximately 100 mg) in 0.4 mL of extraction buffer (50 mM PIPES (1,4-piperazinediethanesulfonic acid), pH 7.0, 25 mM EDTA) containing 2.5% (w/v) polyvinyl-polypyrrolidone and removing insoluble material by centrifugation (16,000g × 20 min). Protein concentration in the supernatant was measured using the dye-binding method of Bradford (1976) with bovine serum albumin as the standard. Protein samples (50 μg of total protein) were fractionated by Tricine (*N*-[tris(hydroxymethyl)methyl]glycine) SDS/PAGE (Schägger and von Jagow, 1987) and transferred to 0.2 micron nitrocellulose membranes by electroblotting (Towbin et al., 1979). COR15am and COR6.6 were detected using the enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK) with antiserum raised to recombinant COR15am and COR6.6 (Gilmour et al., 1996).

RNA Hybridization and cDNA Probes

Total RNA was extracted from Arabidopsis plants as described previously (Gilmour et al., 1988). Northern transfers were prepared and hybridized as described (Hajela et al., 1990) using high stringency wash conditions (Stockinger et al., 1997). ^{32}P-labeled probes were prepared by random priming (Feinberg and Vogelstein, 1983). A gene-specific probe to *CBF3* was made to the 3' end of the cDNA clone by PCR as described previously (Gilmour et al., 1998). Arabidopsis cDNA clones encoding Arabidopsis Suc synthase (182C20T7), corresponding to the *SUS1* gene (Martin et al., 1993), and Δ^1-pyrroline-5-carboxylate synthase (125M17T7), corresponding to the *P5CS2* gene (Strizhov et al., 1997), were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus). Probes made against the *P5CS2* coding sequence would be expected to cross-hybridize with transcripts from the highly similar *P5CS1* gene (Strizhov et al., 1997) and thus serve as a measure of total *P5CS* transcripts. A cDNA encoding a putative Suc-phosphate synthase gene from Arabidopsis was obtained from James Zhang (Mendel Biotechnology, Hayward, CA).

Pro Analysis

Lyophilized leaf material (30 mg) was extracted with 3 mL of deionized water at 80°C for 15 min. The samples were shaken for approximately 1 h at room temperature and then allowed to stand overnight at 4°C. The extracts were filtered through glass wool and analyzed for Pro content using the acid ninhydrin method (Troll and Lindsley, 1955). Briefly, 500 μL of the aqueous extract was mixed with 500 μL of glacial acetic acid and 500 μL of acid ninhydrin reagent (12.5 mg of ninhydrin, 0.3 mL of glacial acetic acid, 0.2 mL of 18 N orthophosphoric acid) and heated at 100°C for 45 min. After cooling, 500 μL of the reaction mix was partitioned against toluene (2 mL) and the absorbance of the organic phase was determined at 515 nm. The resulting values were compared with a standard curve constructed using known amounts of Pro (Sigma, St.

Louis). Pro levels in certain samples were confirmed by amino acid analysis using an amino acid analyzer at the Macromolecular Structure Facility in the Biochemistry Department at Michigan State University.

Sugar Analysis

Total soluble sugars were extracted from lyophilized leaf material (20 mg) in 80% (v/v) ethanol (2 mL) at 80°C for 15 min. The samples were shaken for approximately 1 h at room temperature and allowed to stand overnight at 4°C. Extracts were filtered through glass wool and chlorophyll removed by shaking samples (0.4 mL) with water (0.4 mL) and chloroform (0.4 mL). The aqueous extract was assayed for sugar content using the phenol-sulfuric acid assay (Dubois et al., 1956). Certain samples were analyzed using a miniaturized version of the gas chromatography method described in Everard et al. (1994) with the following modifications. Vacuum dried carbohydrates were solubilized overnight in 0.5 mL of dry pyridine containing 30 mg mL^{-1} hydroxylamine-HCl and 0.1 mg mL^{-1} phenyl β-D-glucoside (derivatization efficiency internal standard). After heating at 75°C for 1 h, samples were cooled to room temperature and derivatized by the addition of 0.5 mL of hexamethyldisilazane and 0.05 mL of trifluoroacetic acid. Precipitates were allowed to settle for 1 h at room temperature prior to transferring the liquid phase to automatic sampler vials (0.3 mL). Samples were analyzed on a Hewlett-Packard 6890 gas chromatograph fitted with a 0.25-μm film, 30 m × 320 μm DB17 capillary column (J&W Scientific, Folsom, CA), and a flame ionization detector. The chromatography details were as follows: 1-μL injections with a 1:50 split ratio; injector and detector temperatures 305°C; initial column temperature 150°C with a ramp of 3°C min^{-1} to 200°C, followed immediately by a 20°C min^{-1} ramp to 290°C with a 10-min hold at this final temperature. The carrier gas was hydrogen at a linear velocity of 46 cm s^{-1}.

Whole Plant Freeze Test

Ws-2 and A30 seedlings were grown (13 and 20 d, respectively) on Gamborg's B-5 medium containing 0.2% (w/v) Suc under sterile conditions in Petri dishes. The plants were tested for freezing tolerance by first placing the plates at −2°C in the dark for 3 h followed by ice nucleation with sterile ice chips. The plates were incubated an additional 21 h at −2°C, then the temperature of the freezer was turned down to −6°C, and the plates were left at this temperature for an additional 24 h. The plates were taken from the freezer and placed at 4°C in the dark for 18 h, followed by 2 d at 22°C under cool-white fluorescent lights (40–50 μmol m^{-2} s^{-1}) with an 18-h photoperiod. The plates were then scored for freezing damage.

Electrolyte Leakage Freeze Test

Electrolyte leakage freeze tests were performed essentially as described (Uemura et al., 1995) with minor mod-

ifications. Tubes (16 × 12 mm) containing 3 to 4 leaves were placed in a low temperature bath set at −2°C in a randomized design. The randomization was performed with the aid of the SAS system (SAS Institute, Cary, NC). Ice chips were added to each tube after 1 h of incubation. Each tube was capped with foam plugs and incubated a further 1 h at −2°C. The bath temperature was then lowered 1°C every 20 min. Tubes were removed at each temperature and incubated an additional hour at that same temperature in a separate bath. Tubes were placed on ice after removal from the bath until all tubes had been removed. The samples were then thawed overnight at 2.5°C, and electrolyte leakage was measured as described (Gilmour et al., 1988).

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