So What's New in the Field of Plant Cold Acclimation? Lots!

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Due to its intrinsically interesting nature and importance to agriculture, considerable effort has been directed at understanding the phenomenon of "cold acclimation," the process whereby certain plants increase in freezing tolerance upon exposure to low temperature. By 1980, thousands of research articles had been published on the topic and significant insights had been gained, including the following: Freezing tolerance is a multigenic trait, freezing injury in most plants and tissues results largely from the severe cellular dehydration that occurs upon ice formation, and the cellular membrane systems are a primary site of freeze-induced injury. What was lacking, however, was a consensus regarding the mechanistic basis of freezing tolerance. Moreover, specific genes with functional roles in freezing tolerance had not been identified and their modes of action had not been determined, critical deficiencies in regard to both a basic understanding of cold acclimation and practical efforts to improve the stress tolerance of agronomic plants.

Now, 20 years later, the cold acclimation research landscape has dramatically changed. Largely through bringing molecular genetic and mutational approaches to bear on the topic, along with the development of Arabidopsis as a model to study the phenomenon, genes with roles in cold acclimation have begun to be identified and their modes of action determined. In addition, low-temperature signaling and regulatory pathways involved in activating the cold acclimation response have begun to be described and the insights gained are beginning to suggest novel approaches to improve the environmental stress tolerance of plants. My objective here is to highlight some of these exciting advances.

THE CBF/DREB1 TRANSCRIPTIONAL ACTIVATORS: REGULATORS OF COLD ACCLIMATION

In 1985, Guy et al. (4) established that changes in gene expression occur with cold acclimation and so opened a floodgate of effort by investigators to identify and characterize cold-responsive genes (22). The underlying hypothesis was that some of these genes were likely to be involved in freezing tolerance and that studies of their regulation and function would provide new insights into the cold acclimation process. Studies on cold-regulated gene expression in Arabidopsis have resulted in the discovery of a family of transcriptional activators, the CBF/DREB1 proteins, that have a key role in cold acclimation.

The CBF/DREB1 Proteins Regulate Expression of Freezing Tolerance Genes

Initial studies on cold-regulated gene expression established that the promoters of certain cold-responsive genes are activated in response to low temperature and dehydration stress (22). Further analysis in Arabidopsis led to the identification of a DNA regulatory element, the C-repeat (CRT) dehydration responsive element (DRE), which has a conserved core sequence of CCGAC, that imparts responsiveness to low temperature and dehydration (24). Transcriptional activators that bind to the CRT/DRE, designated either CBF1, CBF2, and CBF3 (3, 20) or DREB1b, DREB1c, and DREB1a (14), respectively, were subsequently identified. The proteins, which contain the AP2/EREBP-DNA-binding domain, have nearly identical amino acid sequences and are encoded by genes located on chromosome 4 in tandem array (3, 14, 18, 20). Constitutive overexpression of the *CBF1/DREB1b* (6) or *CBF3/DREB1a* (9, 14) genes in transgenic Arabidopsis plants induces the expression of multiple cold-responsive CRT/DRE-containing genes without a low-temperature stimulus (Fig. 1). Moreover, nonacclimated transgenic plants overexpressing either *CBF1/DREB1b* (6) or *CBF3/DREB1a* (9, 14) are more freezing tolerant than nonacclimated control plants. Thus, it has been concluded that the "CBF regulon" (CRT/DRE-containing genes that are induced by the CBF/DREB1 transcription factors) includes genes with roles in cold acclimation.

Given that the CBF/DREB1 proteins can induce transcription of cold-regulated CRT/DRE-containing target genes without a low-temperature stimulus, why aren't the CRT/DRE-regulated genes normally expressed at warm temperatures? The simple answer is that the *CBF/DREB1* genes themselves are cold regulated (3, 14). Within 15 min of transfer to low temperature, CBF/DREB1 transcripts begin to accumulate, followed at 1 to 2 h by accumulation of

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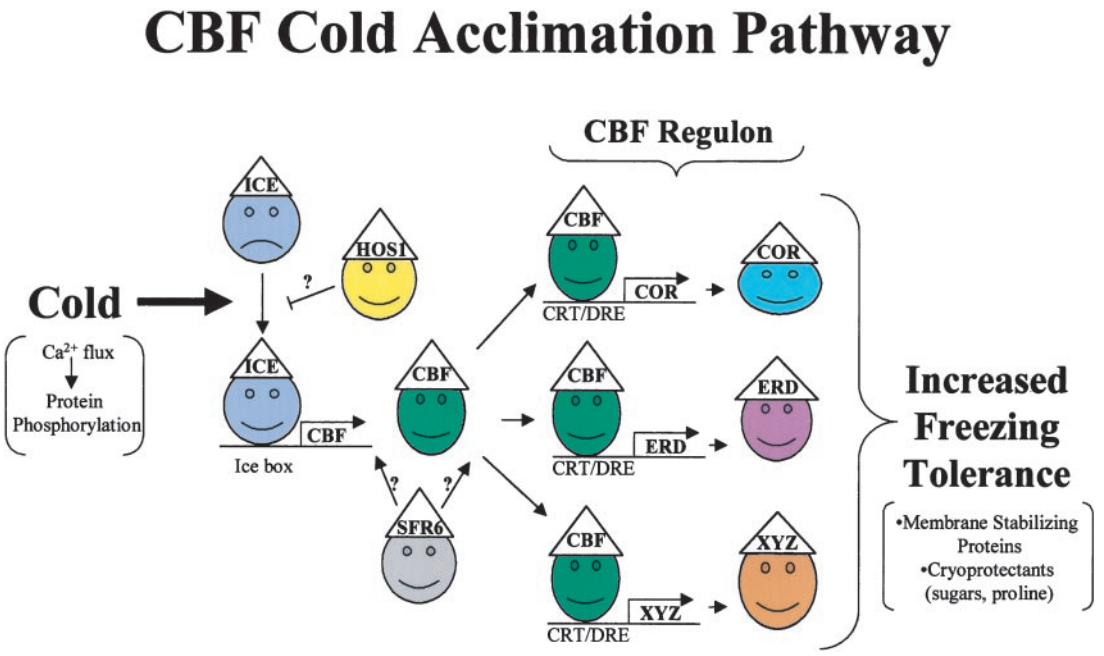

Figure 1. Model of the Arabidopsis CBF cold acclimation pathway. Low temperature leads to rapid induction of the *CBF/DREB1* genes that in turn results in expression of the CBF regulon of CRT/DRE-regulated genes. Action of the CBF regulon, which includes *COR*, *ERD*, and presumably yet to be discovered ("*XYZ*") cold-regulated genes, results in an increase in plant freezing tolerance. Cold-induced expression of the *CBF/DREB1* genes has been proposed (3) to involve the action of a regulatory protein present at warm temperature designated ICE (inducer of CBF expression). Low temperature is envisioned to either activate the ICE protein or other protein(s) with which it interacts (3). Such activation may involve alterations in protein phosphorylation caused by a cold-induced influx of calcium (see text). The SFR6 protein appears to act between *CBF/DREB1* transcription and induction of the CRT/DRE-regulated genes (11) whereas HOS1 appears to act upstream of CBF transcription (13).

transcripts for the target CRT/DRE-regulated genes. The mechanism whereby the *CBF/DREB1* genes are activated by low temperature is not yet known, but appears to involve the action of cold-responsive promoters (18) that are not subject to autoregulation (3). Thus, Gilmour et al. (3) have hypothesized the existence of a transcription factor, designated ICE, that acts at the CBF/DREB1 promoters (Fig. 1). As envisioned, the protein would be present at normal growth temperature, but be in an inactive state. Upon exposing plants to low temperature, ICE (or a protein that it interacts with) is proposed to become activated and stimulate transcription of the *CBF/DREB1* genes followed by induction of the CBF regulon (Fig. 1).

Role of the CBF Regulon in Dehydration Stress Tolerance

As temperatures drop below freezing, ice formation is generally initiated in the extracellular spaces of plants and, because the chemical potential of ice is less than that of liquid water, there is movement of unfrozen water from inside the cell to the extracellular spaces where it freezes. This process continues until an equilibrium in chemical potential is achieved. If the freezing temperature is −10°C, the unfrozen liquid will have an osmolarity of about 5 and typically greater than 90% of the osmotically active water will have moved out of the cell. It is clear, then, that freezing tolerance must include tolerance to severe dehydration stress. Given this, it is not unreasonable to think that the mechanisms of freezing and drought tolerance might include the action of common genes. Shinozaki and Yamaguchi-Shinozaki and coworkers (9, 14) have shown that Arabidopsis plants overexpressing *CBF3/DREB1a*, and consequently the CBF regulon, are not only more freezing tolerant than control plants, but are also more tolerant of dehydration stress caused by either drought or high salinity. Thus, the "biological rationale" for why the CRT/DRE imparts responsiveness to both low temperature and dehydration stress is obvious. However, the genes encoding the CBF/DREB1 activators are not responsive to dehydration stress (14). So what accounts for the dehydration responsiveness of CRT/DRE regulatory element? The Shinozaki labs have provided an explanation for this apparent "paradox" (14). They have identified a gene, *DREB2a*, that encodes an AP2/EREBP domain protein that binds to the CRT/DRE and is induced in response to drought and high salinity.

Functions of the CBF Regulon

The results summarized above indicate that a fundamental function of the CBF regulon is to protect

cells against freezing and other stresses involving dehydration. How does the CBF regulon accomplish this? At present, our knowledge in this area is scant as only six CRT/DRE-controlled genes have been identified (*KIN1*, *COR6.6/KIN2*, *COR15a*, *COR47/RD17*, *COR78/RD29a*, and *ERD10*; 6, 9) and direct evidence for the mode of action of only one, *COR15a*, is available (1, 19). Overexpression of *COR15a*, which encodes a polypeptide that is targeted to the stromal compartment of the chloroplasts, increases the freezing tolerance of chloroplasts in nonacclimated Arabidopsis plants by 1°C to 2°C (1). This effect appears to result from the mature *COR15a*-encoded polypeptide, COR15am, decreasing the propensity of membranes to form deleterious hexagonal II phase lipids upon freeze-induced dehydration (19). Whether other CRT/DRE-controlled genes encode proteins that participate in stabilizing membranes remains to be determined. The function of the CBF regulon, however, does not appear to be "limited" to the action of membrane-stabilizing proteins. Arabidopsis plants overexpressing *CBF3* not only have elevated levels of COR proteins, but also have elevated levels of Pro and total sugars (2). Increased levels of Pro and sugars occur with cold acclimation in a wide variety of plants and are thought to contribute to the enhancement of freezing tolerance, in part, through stabilizing membranes. Thus, the CBF/DREB1 regulatory proteins appear to be "master switches" that integrate activation of multiple components of the cold acclimation response.

Use of CBF/DREB1 Genes to Improve Environmental Stress Tolerance

A major challenge in coming years will be keeping food production in pace with increasing world population. Developing crops with increased environmental stress tolerance will greatly help in this regard as abiotic stresses limit the geographical locations where crops can be grown and cause significant losses in plant productivity on an annual basis. The results described above suggest the possibility of using the Arabidopsis *CBF/DREB1* genes, or homologs from other plants, to optimize expression of CBF regulons in agronomic crops and thereby enhance freezing, drought, and salt tolerance. There is preliminary evidence that overexpression of the Arabidopsis *CBF* genes in canola (*Brassica napus*) results in expression of target CRT/DRE-regulated genes and increases freezing tolerance in both nonacclimated and cold-acclimated plants (7). Whether the "CBF cold acclimation pathway" is operative in more distantly related plants remains to be determined. In addition, the best strategy to optimize *CBF/DREB1* expression is not yet certain. Placing the *CBF/DREB1* genes under control of a strong constitutive promoter may not be the best approach as overexpression of *CBF3/DREB1a* in Arabidopsis using the cauliflower mosaic virus 35*S* promoter can cause a severely stunted phenotype (2, 14). Using stress-inducible (9) or other conditional promoters may be a better approach to improve stress tolerance without causing negative agronomic effects.

THE *SFR* AND *ESKIMO1* FREEZING TOLERANCE GENES

Warren and colleagues (15) have used chemical mutagenesis to identify seven Arabidopsis genes, designated *SFR* (sensitivity to freezing), that affect cold acclimation. Mutant alleles of five of these genes (*sfr1*, *2*, *4*, *5-1*, *5-2*, and *6*) have no obvious adverse effects on the ability of plants to cope with low nonfreezing temperatures, but decrease the level of freezing tolerance that plants attain with cold acclimation. The identities of the *SFR* genes are not known. However, *SFR6* has a role in regulating expression of the CBF regulon (11). To be specific, transcripts for multiple CRT/DRE-regulated genes do not accumulate to normal levels during cold acclimation in *sfr6* plants. This finding offers a simple explanation for why *sfr6* plants are defective in cold acclimation. However, the reason why accumulation of transcripts for CRT/DRE-regulated genes is impaired in *sfr6* plants is not known. It is significant that it does not result from a defect in cold responsiveness of the *CBF* genes because transcripts for *CBF1*, *2*, and *3* accumulate normally in the *sfr6* mutant in response to low temperature (11). Thus, the SFR6 protein appears to act somewhere between CBF transcription and induction of the CBF regulon (Fig. 1).

Xin and Browse (23) have used chemical mutagenesis to isolate cold acclimation "constitutive" mutants. One mutation, *eskimo1* (*esk1*), results in increased freezing tolerance in both nonacclimated and cold-acclimated plants. Nonacclimated *esk1* mutant plants have a 30-fold higher level of Pro than nonacclimated wild-type plants; a 2-fold higher level of total soluble sugars; and a 3-fold higher level of transcripts for *RAB18*, a cold-responsive gene encoding a dehydrin protein. It is presumed that the increases in Pro and sugars, and potentially the RAB18 protein, contribute to the enhancement of freezing tolerance. It is significant that the *esk1* mutation does not affect expression of four CRT/DRE-regulated cold-responsive genes tested. Thus, Xin and Browse (23) proposed that there are parallel or branched signaling pathways that activate "distinct suites" of cold acclimation responses and that activation of one pathway can bring about considerable freezing tolerance without support from other components. Because *esk1* plants do not overexpress CRT/DRE-regulated cold-responsive genes, they proposed that *esk1* defines a cold acclimation signaling pathway that is distinct from the CBF cold acclimation pathway. The mechanism of *ESK1* action is not known, but the fact that the two available *esk1* alleles are

recessive suggests that *ESK1* may act as a negative regulator of cold acclimation.

COLD ACCLIMATION SIGNAL TRANSDUCTION

There is mounting evidence that calcium is an important second messenger involved in activating the cold acclimation response. In both Arabidopsis (10) and alfalfa (*Medicago sativa*; 16), cytoplasmic calcium levels increase rapidly in response to low temperature due in part to an influx of calcium from extracellular stores. This increase in calcium is required for plants to fully cold acclimate and for maximal cold induction of at least some CRT/DRE-regulated genes (10, 21). Little is known about the steps between calcium influx and the activation of gene expression, but it appears that protein phosphorylation may be involved (17); transcript levels of the alfalfa cold-responsive *cas15* gene increase at normal growth temperatures in plants treated with the protein phosphatase inhibitor okadaic acid and do not accumulate to normal levels upon low-temperature treatment in plants treated with the protein kinase inhibitor staurosporine. Moreover, low temperature causes a rapid and dramatic decrease in protein phosphatase 2A activity that is dependent on calcium influx (17). Thus, low temperature may lead to an influx in calcium that inhibits phosphatase 2A activity that, in turn, leads to the phosphorylation of one or more proteins involved in inducing genes involved in cold acclimation. The protein kinase(s) responsible for inducing cold-regulated genes and activating freezing tolerance mechanisms is not known, but there are interesting candidates (22) including an alfalfa mitogen-activated protein kinase, designated $p44^{mmk4}$, that becomes activated within 10 min of alfalfa plants being exposed to low temperature (8).

Zhu and colleagues (5, 12) have identified two genes, *HOS1* and *HOS2* (high expression of osmotically responsive genes), that appear to encode negative regulators of low-temperature signal transduction. Mutations in these genes result in "superinduction" of *COR78/RD29a* and certain other CRT/DRE-regulated genes in response to low temperature. The *HOS1* gene has recently been identified by positional cloning and shown to encode a protein with a RING-finger motif (13). The precise function of HOS1 is not known, but transcription of the *CBF/DREB1* genes was found to be superinduced in *hos-1* plants. Thus, Zhu and colleagues (13) have proposed that HOS1 is a negative regulator that functions upstream of *CBF/DREB1* transcription (Fig. 1).

CONCLUDING REMARKS

Cold acclimation research, in my view, has entered a "golden age." Tremendous advances have been made in our understanding of cold acclimation in the past two decades and there is every reason to believe that the next 20 years will bring even more spectacular and meaningful insights. Through a blending of classical genetics and biochemistry with the powerful new approaches of proteomics and structural, functional, and comparative genomics, I think it is likely that a core set of "first principles" will soon emerge that will allow for the rational design of plants with increased environmental stress tolerance. Such knowledge will not only be exciting and a profound scientific achievement, but will greatly aid efforts in agriculture to continue providing food to feed the world.

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LITERATURE CITED

1. **Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin CT, Thomashow MF** (1996) Proc Natl Acad Sci USA **93:** 13404–13409
2. **Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF** (2000) Plant Physiol **124:** 1854–1865
3. **Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF** (1998) Plant J **16:** 433–442
4. **Guy CL, Niemi KJ, Brambl R** (1985) Proc Natl Acad Sci USA **82:** 3673–3677
5. **Ishitani M, Xiong L, Lee H, Stevenson B, Zhu J-K** (1998) Plant Cell **10:** 1151–1161
6. **Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF** (1998) Science **280:** 104–106
7. **Jaglo-Ottosen KR, Kleff S, Salazar MP, Thomashow MF** (2000) (Abs # 452; http://ww.rycomusa.com/aspp2000/public/P33/0652.html), ASPP Annual Meeting, Plant Biology 2000, 15–19 July, 2000, San Diego, CA
8. **Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H** (1996) Proc Natl Acad Sci USA **93:** 11274–11279
9. **Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K** (1999) Nat Biotechnol **17:** 287–291
10. **Knight H, Trewavas AJ, Knight MR** (1996) Plant Cell **8:** 489–503
11. **Knight H, Veale EL, Warren GJ, Knight MR** (1999) Plant Cell **11:** 875–886
12. **Lee H, Xiong L, Ishitani M, Stevenson B, Zhu J-K** (1999) Plant J **17:** 301–308
13. **Lee H, Xiong L, Ishitani M, Stevenson B, Zhu J-K** (2000) (abstract no. 107). 11th International Conference on Arabidopsis Research, http://www.wisc.edu/union/info/conf/arabidopsis/Arab_abs.pdf, p. 54

14. **Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K** (1998) Plant Cell **10:** 1391–1406
15. **McKown R, Kuroki G, Warren G** (1996) J Exp Bot **47:** 1919–1925
16. **Monroy AF, Dhindsa RS** (1995) Plant Cell **7:** 321–331
17. **Monroy AF, Sangwan V, Dhindsa RS** (1998) Plant J **13:** 653–660
18. **Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K** (1998) Biochem Biophys Res Commun **250:** 161–170
19. **Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF** (1998) Proc Natl Acad Sci USA **95:** 14570–14575
20. **Stockinger EJ, Gilmour SJ, Thomashow MF** (1997) Proc Natl Acad Sci USA **94:** 1035-1040
21. **Tähtiharju S, Sangwan V, Monroy AF, Dhindsa RS, Borg M** (1997) Planta **203:** 442–447
22. **Thomashow MF** (1999) Annu Rev Plant Physiol Plant Mol Biol **50:** 571–599
23. **Xin Z, Browse J** (1998) Proc Natl Acad Sci USA **95:** 7799–7804
24. **Yamaguchi-Shinozaki K, Shinozaki K** (1994) Plant Cell **6:** 251–264