Temperature sensing and cold acclimation
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The fundamental question in cold acclimation is how do plants perceive the low but nonfreezing temperatures that activate cold acclimation responses. New findings in the past year suggest that changes in membrane fluidity, cytoskeleton rearrangement, and calcium influxes are among the earliest events taking place in plants upon exposure to low nonfreezing temperatures. In the cyanobacterium Synechocystis PCC 6803, temperature change is detected by at least two separate sensors. One of these measures membrane fluidity using a classical two-component system involving histidine kinases and a response regulator in a His-to-Asp phosphorelay. Although these Synechocystis results may not be directly relevant to cold acclimation, they can guide our thinking as we search for biological thermometers in higher plants.

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Abbreviations
ABA abscisic acid
CAS cold-acclimation-specific gene
CBF CRT-binding factor
COR cold regulated gene
crn cold-inducible RNA helicase
CRT C-repeat
des fatty-acid-desaturase gene
DRE dehydration-responsive element
DREB DRE-binding protein
esk1 eskimo1
Hik histidine kinase
RD responsive-to-desication gene
Rer1 Response regulator1
35S cauliflower mosaic virus 35S promoter
sfr sensitive-to-freezing

Introduction
Temperature is a major environmental factor that changes from season to season and undergoes daily fluctuations and short, erratic lows and highs. As sessile organisms, plants must be able to sense transient fluctuations as well as seasonal changes in temperature and to respond to these changes by actively adjusting their biology to fit the subsequent temperature regime. One of the best-characterized plant temperature responses is the ability of plants to increase their freezing tolerance in response to a period of low nonfreezing temperatures prior to the onset of winter. This process is known as cold acclimation. In nature, low nonfreezing temperatures in late fall or early winter are the main triggers of cold acclimation [1], although light quality and photoperiod may also be involved in this response. In the laboratory, cold acclimation is induced by exposing plants to low nonfreezing temperatures (2–6 °C) under moderate light. Biologically, cold acclimation is complex, involving numerous changes in gene expression, metabolism, and morphology [2]. These changes include the increased expression of many genes [1], the reduction or cessation of growth, transient increases in abscisic acid (ABA) concentrations, changes in membrane lipid composition, the accumulation of compatible osmolytes (such as proline, betaine, polyols and soluble sugars), and increased levels of antioxidants [3]. In short, almost every cellular process is altered during cold acclimation. It has, however, been a great challenge to separate the processes that are critical for enhanced freezing tolerance from those that merely respond to low temperature.

During freezing of plant tissues, ice forms first in the extracellular compartment, reducing its water potential and leading to loss of water from the cells by osmosis. Thus, dehydration is a major component of freezing stress. This explains the overlapping relationship between cold acclimation and dehydration responses both in terms of the biochemical changes noted above and in the transcriptional regulation of the responses (see below). This relationship was explored in last year’s review by Shinozaki and Yamaguchi-Shinozaki [4] and has also been highlighted by mutational analyses in Arabidopsis [5,6,7,8].

How do plants perceive the low temperatures that induce cold acclimation? How do they transduce these signals to activate the complex set of changes needed to bring about increased freezing tolerance? Is there one signal pathway that activates a master switch that controls cold acclimation or are there multiple pathways that interact and modulate the acclimation response? These are the key questions that must be answered to improve our understanding of cold acclimation. In this short review, we interpret recent discoveries as suggesting that multiple signaling pathways and probably multiple temperature sensors are used in higher plants to initiate and control cold acclimation. The search for temperature sensors will be the primary focus of cold acclimation research in the next few years. Other reviews [1,3,4,9] serve as excellent resources for those who want to learn more about cold acclimation.

Gene expression regulated by low nonfreezing temperatures
As we have said, many genes are transcriptionally upregulated during cold acclimation [1]. Some of these genes encode proteins with known enzymatic functions. Nevertheless, the importance of many of these enzymes and pathway products to cold acclimation remains questionable and the possibility of redundant processes exists. For example, mutants that are deficient in a single cold-induced enzyme often show no change in their ability to develop freezing tolerance.
Most cold-upregulated enzyme-encoding genes are expressed at 24°C but their transcript levels are 2–5-fold greater at 4°C. One class of cold-regulated genes is extremely strongly induced by cold acclimation (typically 50- to 100-fold). The proteins encoded by this class are not enzymes and their precise physiological functions remain a matter of debate, although at least some probably act as cryoprotective proteins or dehydrins [1]. Because genes of this class are easily identified by differential screening, they have been characterized by several groups and have been given different names such as COR for cold regulated, LTI for low-temperature-induced, CAS for cold-acclimation-specific and RD for responsive-to-desiccation genes. We refer to these genes as COR in the following discussion. The COR proteins have no known enzymatic activity, but many of them are hydrophilic and remain stable upon boiling in dilute aqueous solution [1]. These unusual characteristics suggest that they may function as cryoprotectants.

Overexpression of a single COR gene, COR15a, has been demonstrated to offer modest protection against freezing to chloroplasts and protoplasts that are derived from nonacclimated transgenic plants [10]. Because it is localized in the chloroplast stroma, the mature COR15am protein is proposed to function by deferring the formation of the freezing-induced hexagonal II lipid phase to lower temperature [11]. This might be achieved through the alteration of the intrinsic curvature of the inner membrane of the chloroplast envelope [11]. No measurable improvement in freezing tolerance has been observed, however, in COR15a transgenic lines at the whole-plant level [10,12]. Overexpression of other cold-induced genes in transgenic plants has also resulted in little or no enhancement of freezing tolerance [13,14]. In all likelihood therefore, individual components of freezing tolerance can only work within the context of a broader cold-acclimation response.

The COR genes have been particularly useful in investigating the signal pathways that are associated with cold acclimation using molecular-genetic approaches in Arabidopsis. Gene-fusion studies have uncovered cis-acting elements in the promoter region of COR genes [1,4]. These elements are named either dehydration-responsive elements (DREs) or low-temperature-responsive elements (LTERs). Both DREs and LTERs contain a core amino-acid sequence of CCCGAC, which has been named the C-repeat (CRT). The DRE/CRT elements have been used as bait to isolate two groups of DRE/CRT-binding proteins using the yeast one-hybrid system [15,16]. These proteins are called DREBs (DRE-binding proteins) or CBFs (CRT-binding factors). Five DRE/CRT-binding proteins have been isolated [16]. They are classified as two groups, DREB1 and DREB2. Three DREB1 proteins are encoded by genes tandemly repeated on Arabidopsis chromosome 4 in the order DREB1B (CBF1), DREB1A (CBF3), and DREB1C (CBF2). There are two DREB2 proteins, DREB2A and DREB2B. All five DREB proteins bind specifically to DRE/CRT and function as transcription factors to activate the expression of COR genes.

Overexpression of DREB1B/CBF1 in transgenic Arabidopsis induced the expression at warm temperatures of the entire battery of COR genes that have the common DNA-elements in their promoter region [12,16]. When freezing tolerance in the absence of cold acclimation was assayed by an ion-leakage test, transgenic tissues were able to tolerate temperatures 3.3°C below those tolerated by nontransgenic control plants. It is now clear that much better freezing tolerance is achieved by overexpression of DREB1A (CBF3) [16,17**]. Transcripts of DREB1A/CBF3 are present at very low levels in wild-type Arabidopsis plants even after 7 d of cold acclimation at 5°C. Transgenic plants containing a cauliflower mosaic virus 35S promoter (35S):CBF3 construct, however, displayed both very high levels of CBF3 transcript and an over-induction of COR polypeptides to levels 3–5-fold higher than those found in fully acclimated wild-type plants [18**]. In that study, nonacclimated wild-type plants exhibited an EL50 (i.e. the temperature that causes a 50% leakage of electrolytes) of approximately –4.5°C, which was improved to –6°C after a 7-d acclimation at 5°C. The three independent CBF3-overexpressing lines had EL50 values of approximately –8°C without any cold treatment. After 7 d at 5°C, however, the EL50 values of the CBF3-overexpressing plants increased dramatically to temperatures of –11°C or lower. One of the cold-acclimated CBF-overexpressing lines was so cold tolerant that its tissues did not suffer 50% leakage of electrolytes even at the lowest temperature (~16°C) used in these experiments. Thus, by overexpressing a single regulator it is possible to improve freezing tolerance to well beyond that which can be achieved naturally by the normal mechanisms of cold acclimation.

This raises the question of why cold acclimation at 5°C does not lead directly to the maximum frost tolerance that is physiologically possible? The answer may be that cold acclimation signaling has evolved to modulate the level of freezing tolerance so that it is sufficient for the conditions found within the geographic range of a given ecotype but does not unnecessarily limit the plant’s competitiveness [2]. Consistent with this hypothesis, the transgenic plants overexpressing DREB1A/CBF3 have severely compromised growth and development even under the benign growth conditions of controlled environments [16,18**]. A first step toward overcoming (or at least ameliorating) such deleterious effects was taken in Shinozaki’s group by placing the DREB1A/CBF3 coding sequence under control of a COR gene promoter (i.e. rd29A). In comparison with 35S::DREB1A transgenics, rd29A::DREB1A plants had greatly improved appearance although they retained a slight growth retardation relative to wild-type controls — again under controlled-environment conditions [17**].

Whether or not the DREB1 transcription factors turn out to regulate all or only a subset of the cold acclimation responses, it is clear that their identification and characterization are revolutionary advances. Cold-induced genes appear not to confer useful cold tolerance when expressed
individually. However, now it is possible to contemplate a time when we will be able to induce strong and controllable frost tolerance by expressing suites of genes via the transgenic expression of regulatory proteins.

**Multiple signaling pathways mediate cold acclimation**

We will not fully understand cold acclimation and freezing tolerance until we have a complete picture of the signaling processes that are involved in cold acclimation. Many lines of evidence indicate that parallel or branched signaling pathways activate distinct suites of cold-acclimation responses. For example, studies of COE gene expression indicate that both ABA-dependent and ABA-independent pathways are involved in cold acclimation [19–21], whereas analysis of the eski1 (esk1) mutant of Arabidopsis revealed that considerable freezing tolerance can be achieved in the absence of COE gene expression [2]. Support for a cold-acclimation model involving multiple pathways also comes from the analysis of sensitive-to-freezing (sfr) mutants that are not able to fully acclimate [22,23]. Most of the sfr mutants retain over 50% of the wild-type capacity to cold acclimate. The simplest explanation for this is that each sfr mutation blocks one signaling pathway. Therefore, each mutant is still able to partially cold acclimate through signaling pathways that are not disrupted. Map-based cloning of the sfr loci and of genes corresponding to constitutively freezing-tolerant mutants, such as eski [2], will undoubtedly contribute to our understanding of the complexities of cold-acclimation signaling.

Of course, the number of pathways an investigator sees depends on what level in the signaling cascade he or she is looking at. The eski1 mutant contains elevated levels of proline and soluble sugars that are believed to contribute to the constitutive freezing tolerance of this mutant [2]. Plants overexpressing CBF3 (DREB1A) contain increased proline and soluble sugars as well as exhibiting constitutive expression of the CBF genes [18••]. Hence, the expectation is that DREB1A/CBF3 acts in a pathway that leads into both the COE pathway and the ESK1 pathway. Does this mean that there is a single temperature sensor and a trunk signal pathway on which DREB1A/CBF3 acts as a master switch? Not nearly enough data is available to provide a definitive answer this question, but we suggest that the weight of evidence argues against a single master switch.

Overexpression of DREB1A/CBF3 results in considerable freezing tolerance in vivo as well as that demonstrated in ion-leakage assays performed on excised leaves [16,17••,18••]. However, the experiments of Gilmour et al. [18••] clearly demonstrate that transgenic plants that overexpress DREB1A/CBF3 can develop much greater levels of freezing tolerance following cold acclimation at 5°C for 7 d. A straightforward explanation for these observations is that DREB1A/CBF3 activates a subset of the total cold acclimation responses and that a high degree of freezing tolerance is achieved because the DREB1A/CBF3-responsive components are hyperactivated in the transgenic plants. (An alternative interpretation of the results is presented by Gilmour et al. [18••] and in [9].)

**Hunting for the temperature sensors**

A central issue in considering whether a single pathway or multiple pathways control cold acclimation is the question of whether there is one temperature sensor in plant cells or several sensors that may respond to the same or different physical manifestations of a drop in temperature? It is now clear that, in some cases at least, a change in membrane fluidity is the physical alteration that is used as the basis of a biological thermometer. In higher plants, the concept of the thermometer as a sensor that detects the reduction in membrane fluidity occurring at low temperature is developed only at a crude physiological level. Thus, Örvar et al. [24*] used measurements of cell viability and cas30 gene expression as reporters of cold signaling in alfalfa cell cultures. When dimethyl sulfoxide (DMSO) was used to reduce the membrane fluidity of cells at 25°C, there was a modest increase in cas30 transcript levels and in the freezing tolerance of cells. Conversely, pretreatment of cells with benzyl alcohol (a reagent that increases membrane fluidity) before cold acclimation at 4°C reduced the expression of cas30 and the ability of cells to fully cold acclimate. In this model, membrane fluidity leads to cold acclimation through changes in cytoskeleton organization and the induction of Ca2+ influx into the cells. Other evidence also indicates that Ca2+ fluxes are an important early step in low temperature signaling [25•,26]. These experiments are noteworthy because they provide the first evidence in higher plants to show that a temperature sensor may be responding to changes in the fluidity of the plasma membrane. Nevertheless, we are a long way from being able to extend these observations to a mechanistic explanation at the biochemical and genetic levels.

Fortunately, a far better-developed model for temperature signaling has emerged from the work of Murata’s group on the cyanobacterium *Synechocystis* PCC6803, and this model provides a useful basis for discussing concepts for temperature sensors in higher plants. When *Synechocystis* is shifted from a growth temperature of 34°C to 22°C, transcripts of three of the four fatty-acid-desaturase genes (des), desA (Δ12), desB (ω3), and desD (Δ6), are induced approximately ten-fold, whereas the transcript level of the desC (Δ9) gene remains constant [27]. The increased expression of fatty acid desaturases is believed to be an adaptive response of the cyanobacterium to low temperature that modulates the degree of lipid desaturation and, thus, membrane fluidity. Several years ago, Vigh et al. [28] showed that transcription of desA can also be induced at 34°C by reducing membrane fluidity through the Pd-catalyzed hydrogenation of fatty acids in the plasma membrane of *Synechocystis* PCC6803.

In hunting for temperature sensors, Murata’s group focused on the possibility that the sensors could be two-component regulators and took advantage of the convenience of the *Synechocystis* system for targeted gene replacement [29••].
The genome of *Synechocystis* has been completely sequenced and shown to contain 43 putative histidine kinase (Hik) genes [30]. To identify temperature sensors, the histidine kinases were systematically inactivated by targeted insertional mutagenesis [29**]. To monitor the changes in the induction of the *desB* gene in response to low temperature in the disruption mutants, a reporter construct containing the bacterial luciferase gene (*luxAB*) fused to the *desB* promoter was used [29**]. In these experiments, two histidine kinases, Hik33 and Hik19, were identified as necessary for the low-temperature induction of *desB*. *Synechocystis* contains more than 10 copies of the chromosome per cell and hence the isolation of disruption mutants requires extended selection for the antibiotic marker of the insertion cassette. After six months culture on 20µg/ml spectinomycin, polymerase-chain-reaction (PCR) analysis revealed that wild-type alleles of Hik33 and Hik19 were still present at low levels in the mutants. Levels of the corresponding proteins were, however, apparently reduced substantially so that low-temperature sensing was blocked. To identify other components of the temperature-sensing pathway, Suzuki *et al.* [29**] performed random insertional mutagenesis using a spectinomycin-resistance cassette. From approximately 20,000 spectinomycin-resistant lines, 18 mutants were isolated that exhibited reduced luciferase activity in response to a down-shift in temperature. One of the genes identified in this way, *Rer1* (*Response regulator1*), encodes a putative protein with domains that are typical of a response element in a two-component regulator. The acceptable working-model presented by Suzuki *et al.* [29**] is that Hik33 is activated by reduced membrane fluidity, allowing the autophosphorylation of Hik33 and the subsequent transfer of a phosphate group to Hik19 and finally to Rer1. Traditional approaches [31] and, recently, microarray analysis using the Hik-mutants [32**] have demonstrated that many genes besides the desaturases are regulated by this thermosensor system. Many of these genes encode proteins that are involved in RNA stability and translation in *Synechocystis*.

Further experiments with the *Synechocystis* mutants provide evidence for at least one additional temperature sensor in this cyanobacterium. Mutations in Hik33 and Hik19 genes greatly reduced (but did not eliminate) the cold induction of *desB* and *desD* genes and reduced the cold induction of *crh* (*cold-inducible RNA helicase*), but had no effect on the cold induction of the *desA* gene. Thus, a separate sensor apparently operates to regulate the cold induction of the *desA* gene and to partially control *desB*, *desD* and *crh* expression (Figure 1). It is also worth noting that the response regulator, Rer1, which functions downstream of Hik33 and Hik19, only regulates the cold induction of *desB* and not of *desD* or *crh*, which presumably are regulated by additional response elements [29**]. A genome-wide microarray analysis [32**] identified 70 genes in *Synechocystis* that are induced (24 genes) or repressed (46 genes) by a temperature shift from 34°C to 22°C. In the ∆Hik33 mutant, cold-induced transcriptional changes for 14 of these genes were eliminated, changes for 35 genes were reduced, whereas 21 genes showed the same level of induction or repression in the ∆Hik33 mutant as in wild-type cells. These results further support the two-temperature-sensors model (Figure 1). Apparently, each sensor regulates a unique set of genes and the two sensors share the regulation of a third set of genes.

Of course, the temperature-sensing process described for *Synechocystis* involves a temperature shift from 34°C→22°C whereas plants undergoing cold acclimation are responding to temperatures below 10°C. Nevertheless, the evidence for (at least) two independent temperature sensors in this prokaryote establishes a *prima facie* case for considering the possibility of multiple sensors in higher plants, which have more complex cellular organization and differentiated tissues and organs.

Two-component regulators are present in plants, and it is likely that such a system forms the basis of an osmotic sensor in *Arabidopsis* [33,34]. The Hik33 and Hik19 genes do not, however, have recognizable homologues in higher plants, although putative homologues of Hik33 have been identified in the chloroplast genomes of *Porphyra purpurea* and *Cyanidium caldarium* [29**]. For this reason, it is not useful to speculate on whether higher plants employ histidine kinases or two-component response regulators as temperature sensors and signal transducers. Equally plausible arguments could be made for fluidity affecting Ca²⁺

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Figure 1

Molecular and mutational analysis of temperature signaling in *Synechocystis* PCC 6803 demonstrates the existence of at least two temperature sensors, one of which involves Hik33 and Hik19, in this prokaryote. Two temperature sensors are depicted as thermometers in the plasma membrane. Downstream components, Hik19 and Rer1, are known for the Hik33 sensor. See text for details. Redrawn from Suzuki *et al.* [29**].
channels in the membrane [25*] and inducing Ca^{2+} fluxes that activate a mitogen-activated-protein (MAP) kinase (or other) signaling cascade(s) [26,35]. It is noteworthy, however, that of the approximately 1000 protein kinases encoded by the Arabidopsis genome, only about 12 are considered to be potential histidine kinases of two-component systems [36**]. Projects to systematically characterize whole families of Arabidopsis genes (e.g., the Arabidopsis 2010 Project) will soon make it possible to test knockout mutants corresponding to candidate genes in cold-acclimation signaling.

Conclusions and perspectives
Research on cold acclimation has reached a most exciting stage. The DREB1/CBF transcription factors have been shown to be major regulators of increased freezing tolerance. The compelling questions now are: how many thermometers are there (and by implication how many signaling pathways)? What are the thermometers and how do they work? In this respect, the molecular and mutational analysis of the two-component temperature sensor in Synchocystis points towards new and exciting prospects for understanding the initiation and execution of cold acclimation in plants.

Update
Seki et al. [37*] recently produced a microarray of 1300 Arabidopsis cDNAs and used it to investigate changes in transcript levels in response to dehydration or cold (i.e. 4°C). In addition, a comparison between wild-type Arabidopsis and plants overexpressing DREB1A (i.e. 35S::DREB1A transgenics) was used to identify DREB1A target genes [16,17**]. Within the microarray dataset, 19 genes were identified as cold-inducible. Of these, 12 were identified as DREB1A target genes. On the basis of these very few genes, we would infer that 50–60% of cold-inducible genes are regulated through DREB1A (CBF3) signaling while one or more additional pathways regulate the remaining genes. We cannot yet determine, however, whether the multiple pathways are branches from a single temperature sensor or operate in parallel from different sensors. The search for the thermometer(s) continues.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest
19. The authors describe the first step toward overcoming the detrimental effects associated with constitutive expression of DREB1A in transgenic plants. Use of the rd29A cold-induced promoter to drive DREB1A expression allowed high levels of freezing, drought and salt tolerance in transgenic plants. These plants grew almost as well as wild-type plants in non-stressful conditions.


The results presented here suggest that a temperature sensor in higher plants may respond to plasma membrane fluidity, as is the case in cyanobacteria. Cytoskeleton changes and Ca$^{2+}$ fluxes are also proposed to be components of the response.


Decrease in temperature leads to a rapid rise in cytosolic free calcium concentration ([Ca$^{2+}$]). The rate of cooling is shown to be the main determinant of the extent of [Ca$^{2+}$] increase.


At last, the molecular mechanisms for a thermometer! Mutational analysis provides evidence of (at least) two distinct temperature sensors in the cyanobacterium, Synechocystis PCC6803. Sequence analysis suggests that one sensor is a two-component regulator.


Use of a genome-wide microarray (involving 97% of all genes for Synechocystis PCC6803) identifies 70 genes that exhibit changes (of 2-fold) in transcript levels following a 34°C→2°C temperature shift. Regulation of these genes is apparently shared between Hik33 and at least one other temperature sensor. More than 70 genes must cold regulated as some genes have been shown by gel-blot analysis to be strongly induced by cold (e.g. 8- to 10-fold increase in transcript levels for desB [29]) are not identified by this microarray assay.


No commentary necessary.


A microarray analysis of cold-induced gene expression in Arabidopsis provides additional evidence for multiple signaling pathways and identifies new cold-induced genes. Many genes are shown to be induced by both cold and dehydration.