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Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation

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Temperate plants are capable of developing freezing tolerance when they are exposed to low nonfreezing temperatures. Acquired freezing tolerance involves extensive reprogramming of gene expression and metabolism. Recent full-genome transcript profiling studies, in combination with mutational and transgenic plant analyses, have provided a snapshot of the complex transcriptional network that operates under cold stress. Ubiquitination-mediated proteosomal protein degradation has a crucial role in regulating one of the upstream transcription factors, INDUCER OF CBF EXPRESSION 1 (ICE1), and thus in controlling the cold-responsive transcriptome. The changes in expression of hundreds of genes in response to cold temperatures are followed by increases in the levels of hundreds of metabolites, some of which are known to have protective effects against the damaging effects of cold stress. Genetic analysis has revealed important roles for cellular metabolic signals, and for RNA splicing, export and secondary structure unwinding, in regulating cold-responsive gene expression and chilling and freezing tolerance.

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Introduction

Cold stress is a major environmental factor that limits the agricultural productivity of plants. Low temperature has a huge impact on the survival and geographical distribution of plants. Plants differ in their tolerance to chilling (0–15 °C) and freezing (<0 °C) temperatures. Plants from temperate regions are chilling tolerant, although most are not very tolerant to freezing but can increase their freezing tolerance by being exposed to chilling temperatures, a process known as cold acclimation [1]. By contrast, plants of tropical and subtropical origins, including many crops such as rice, maize and tomato, are sensitive to chilling stress and largely lack the capacity for cold acclimation.

Most molecular studies on plant responses to cold stress are focused on the mechanism of cold acclimation rather than on chilling tolerance. Nevertheless, recent evidence indicates that some of the molecular changes that occur during cold acclimation are also important for chilling tolerance [2,3•]. In other words, it appears that chilling tolerance that is exhibited by temperate plants is not entirely constitutive, and that at least part of it is developed during exposure to chilling temperatures.

Numerous physiological and molecular changes occur during cold acclimation [4]. Among them, the transcriptional activation and repression of genes by low temperature are of central importance [4]. The reprogramming of gene expression results in the accumulation not only of protective proteins but also of hundreds or more of metabolites, some of which are known to have protective effects. This review summarizes recent work on cold-responsive gene expression and metabolite accumulation in *Arabidopsis*. In addition, we discuss some intriguing findings on the role of metabolic status and posttranscriptional RNA processing in controlling cold-responsive gene expression and chilling and freezing tolerance.

Cold-responsive transcriptional cascades

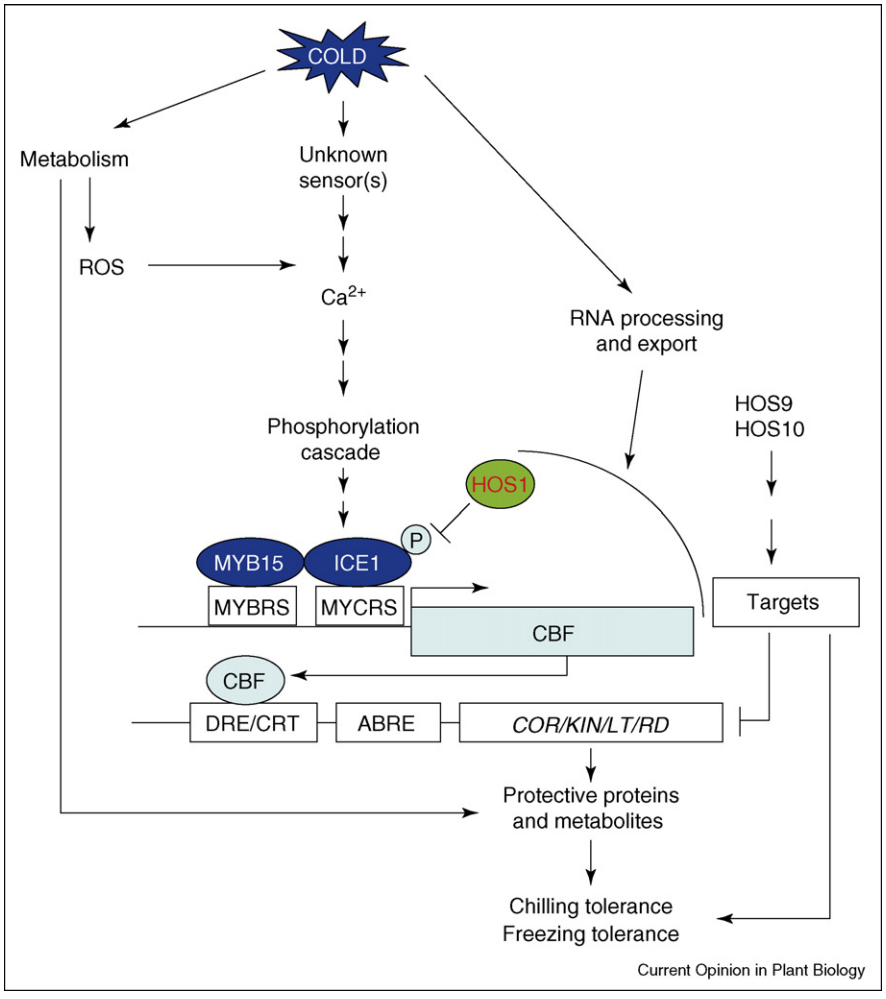
Various differential screening and cloning studies over the years have led to the identification of a core set of robustly cold-regulated plant genes, which are known as *COR* (cold-regulated), *KIN* (cold-induced), *LTI* (low-temperature-induced) or *RD* (responsive to dehydration) genes (reviewed in [4]). C-repeat (CRT)-binding factors (CBFs), also known as dehydration-responsive-element-binding proteins (DREBs), are upstream transcription factors in the APETALA2 (AP2)/ETHYLENE RESPONSE FACTOR (ERF) family that bind to the promoter *cis*-element and activate the expression of these cold-responsive genes [4]. The *CBF* genes are induced early and transiently by cold. Ectopic expression of the CBFs in *Arabidopsis* results in constitutive expression of downstream cold-inducible genes, even at warm temperatures, and in increased freezing tolerance. Several *cis*-elements in the CBF2 promoter have been found to be involved in the cold induction of CBF2, although the transcription factors that bind to these elements have yet to be identified [5]. Inducer of CBF Expression1 (ICE1), a bHLH (basic helix–loop–helix) protein, is an upstream transcription factor that binds to the CBF3 promoter and is required to activate CBF3 expression upon cold stress [6]. Recently,

an R2R3-type MYB transcription factor, AtMYB15, was found to interact with ICE1 and to play a negative role in regulating the expression of *CBF* genes under cold stress [7•]. It appears that cold induction of the three *CBF* genes is controlled by a set of redundant and interacting bHLHs (ICE1 and other related bHLHs) and MYB transcription factors. Some of these transcription factors cross-regulate each other [7•]. Similarly, CBF2 was known to regulate CBF1 and CBF3 negatively [8], and *ZAT12* negatively regulates the expression of the *CBF* genes [9••].

The signal transduction pathway that is responsible for cold stress activation of the ICE1–CBF–COR transcriptional cascade remains to be defined. It involves as-yet-unknown cold sensor(s), calcium and inositol 1,4, 5-triphosphate, phosphatidyl inositol 4,5-phosphate and other phospholipid second messengers, calcium-sensor(s) and calcium-dependent protein kinases, and a mitogen-activated protein (MAP) kinase (MAPK) cascade (reviewed in [10]). The cold-activated *Arabidopsis* MAP kinase kinase 2, and probably its downstream MAPK4 and MAPK6, are important for the expression of the CBF regulon and for freezing tolerance [11]. In *Arabidopsis* plants, ICE1 is phosphorylated in response to cold treatment (H Fujii, J-K Zhu, unpublished). Therefore, transcription cascades that are directed by ICE1 and ICE1-like bHLH proteins are probably activated by the cold sensing and signaling pathway via protein phosphorylation (Figure 1). The cold-induction of CBFs is gated by the circadian clock [12•], but the step at which the input from circadian rhythm is integrated into the cold-response pathway is unknown.

The availability of DNA microarrays has provided opportunities to examine genome-wide cold-regulated gene transcripts [9••,13••,14]. Using Affymetrix *Arabidopsis*

Figure 1



Schematic illustration of the cold response network in *Arabidopsis*. Cold sensing and signaling leads to the activation of multiple transcriptional cascades, one of which involves ICE1 and CBFs. The ubiquitin E3 ligase HOS1 negatively regulates ICE1. Metabolism, and RNA processing and export, affect cold tolerance via cold signaling and/or cold-responsive gene expression. The constitutive HOS9 and HOS10 regulons have a role in the negative regulation of CBF-target genes. MYBRS, MYB recognition sequence; MYCRS, MYC recognition sequence.

24K GeneChips representing about 24 000 *Arabidopsis* genes, 655 genes were identified statistically as cold upregulated and 284 as cold downregulated [13••]. This study suggests that cold stress triggers a multitude of transcriptional cascades because many of the early cold-responsive genes encode transcription factors that probably activate the genes that are induced after longer periods of exposure to the cold. Most of these transcription factors are induced in response to cold, and only one transcription factor was downregulated during early cold stress, suggesting that cold responses in plants are initiated mainly by transcriptional activation rather than by repression of genes. In addition to genes that are involved directly in stress protection or in metabolism, growth and development, several genes that are involved in RNA metabolism and genes encoding chromatin remodeling proteins were found to be cold regulated [13••]. A comparison of cold-responsive transcript profiles between wildtype and *ice1* mutant plants strengthens the notion that ICE1 plays an important role in cold-responsive gene regulation and cold tolerance in plants [13••]. The dominant negative *ice1* mutation affects the cold induction of a large number of genes, including many transcription factors. In addition, the *ice1* mutation alters the basal transcript levels of many cold-responsive genes [13••].

In a separate study using Affymetrix 24 K GeneChips, 514 genes were found to be cold-responsive, including 302 upregulated and 212 downregulated genes [9••]. A comparison with the gene expression profiles of plants that ectopically express CBF2 revealed that 85 of the cold-upregulated genes and eight of the cold-downregulated genes belong to the CBF2 regulon. Therefore, in addition to the CBF regulon, many other regulons exist in the cold responsive transcriptome. Nevertheless, of the 25 most highly upregulated genes, 21 are regulated by ectopic expression of CBF2. Furthermore, the ZAT12 regulon includes substantially fewer cold-responsive genes than the CBF regulon. These results support a major role for CBFs in configuring the low temperature transcriptome in *Arabidopsis*. It is interesting to note that the ectopic expression of *CBF* genes activates the expression of several other cold-responsive transcription factors, such as the RELATED TO AP2 proteins RAP2.1 and RAP2.6, which presumably control subregulons of the CBF regulon [15].

Gene expression changes that are associated with cold deacclimation, i.e. recovery from cold stress, have also been examined recently [14]. Using an *Arabidopsis* 7000 cDNA microarray and the Agilent 22 K oligonucleotide array, 292 genes were identified as being upregulated and 320 as downregulated during deacclimation. Consistent with the notion that cold-stress-induced genes are needed for cold tolerance whereas cold-repressed genes are important for active growth, many of the genes that are downregulated during deacclimation are cold-inducible genes, and conversely many of the genes that are upregulated during deacclimation are cold repressed.

The importance of regulons that are not cold responsive

Recently, forward genetic analysis in *Arabidopsis* identified two transcription factors, HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENEs 9 (HOS9) and HOS10, that are required for basal freezing tolerance [16,17•]. The *HOS9* and *HOS10* genes encode homeodomain and MYB (AtMYB8) transcription factors, respectively, and their transcript levels are not cold responsive. Loss-of-function mutations in these genes cause significant decreases in basal and acquired freezing tolerance. Interestingly, the mutants show stronger or earlier cold-induction of several CBF-target genes, such as *RD29A* and *COR15A*, but no effects on the expression of CBFs. These results suggest a crucial role in freezing tolerance for regulons that are not cold responsive, and these presumably constitutive regulons have a negative effect on the cold-responsive CBF regulon.

The importance of CBF-independent pathways is also supported by analysis of mutants that have increased freezing tolerance. Mutations in ESKIMO1 (ESK1), a protein of unknown function, result in constitutive freezing tolerance, but the genes that are affected by the *esk1* mutation are distinct from those of the CBF regulon [18•]. Similarly, mutations in the transcriptional adaptor protein ADA2 also cause constitutive freezing tolerance but not constitutive expression of *COR* genes [19].

Regulatory roles of protein ubiquitination in cold stress responses

HOS1, a negative regulator of the *CBF* regulon, was identified from a genetic screen for mutants with deregulated expression of CBF target genes [20,21]. The cold induction of *CBF* genes and their downstream *COR* genes is enhanced in loss-of-function *hos1* mutant plants [20]. *HOS1* encodes a 915-amino acid protein that contains a short motif near the amino terminus that is similar to the Really Interesting New Gene (RING)-finger domain found in the Inhibitor of Apoptosis (IAP) group of animal proteins [21]. *In vitro* ubiquitination assays demonstrated that *Arabidopsis* HOS1 is a functional RING-finger protein that has ubiquitin E3 ligase activity. HOS1 physically interacts with ICE1, suggesting that HOS1 might ubiquitinate ICE1 and target it for proteosomal degradation. Indeed, both *in vitro* and *in vivo* ubiquitination assays showed that HOS1 mediates the polyubiquitination of ICE1 [22••]. Cold-induced degradation of the ICE1 protein was observed in *Arabidopsis* plants and this degradation is blocked by the *hos1* mutation, indicating that HOS1 is required for the degradation of ICE1, which functions to attenuate cold responses in *Arabidopsis* [22••]. ICE1 and perhaps related transcription factors that control the expression of *CBF*

genes are present in the absence of cold stress, but probably undergo certain posttranslational modification(s) (e.g. phosphorylation) in response to cold stress, thereby becoming active in switching on the expression of *CBF* genes [6]. The active, modified form of ICE1 might be more efficiently recognized by HOS1 and then degraded through the ubiquitination/proteasome pathway.

Protein synthesis under cold stress might also be regulated by ubiquitination. A cold- and heat-upregulated *Arabidopsis* F-box protein, AtFBP7 (***At1g21760***), is required for protein synthesis under temperature stress [23]. The mechanism of AtFBP7 function is not known.

RNA processing and nucleocytoplasmic transport play crucial roles in plant responses to cold stress

Both cold-stress-induced transcripts and constitutively expressed transcripts need to be processed, exported to the cytoplasm and kept in conformations that are competent for translation. RNA can fold into extensive secondary structures that could interfere with its function, and this interference is exacerbated by cold temperatures. In bacteria, nucleic-acid-binding cold shock proteins (CSPs) accumulate at cold temperatures and function as transcription antiterminators or translational enhancers by destabilizing RNA secondary structure [24]. Some CSP-domain-containing proteins in plants are upregulated by cold stress, and might function as RNA chaperones in the regulation of translation [25•,26]. A different cold-responsive nucleic-acid-binding protein, a zinc-finger-containing glycine-rich RNA-binding protein from *Arabidopsis* designated atRZ-1a, is also upregulated by cold stress, and genetic analysis supports its function in freezing tolerance [27].

Another group of RNA chaperones, RNA helicases, are involved in every step of RNA metabolism. In cyanobacteria, a cold-induced DEAD-box RNA helicase was suggested to unwind cold-stabilized secondary structure in the 5′-untranslated region of RNA during cold stress [28]. Compared to other organisms, plants have the largest number of DEAD-box RNA helicase genes. One of these helicases, which is encoded by the *Arabidopsis LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES4* (*LOS4*) gene, is essential for plant tolerance of chilling and freezing stress [2]. LOS4 is required for efficient export of RNA from the nucleus to the cytoplasm [29••]. The *Arabidopsis* nucleoporin AtNUP160/SUPPRESSOR OF AUXIN RESISTANCE1 (SAR1) also controls RNA export, and is crucial for chilling and freezing tolerance [3•]. Both LOS4 and AtNUP160 proteins are enriched at the nuclear rim [2,3•]. Defects in the nucleocytoplasmic transport of RNA seem to affect cold tolerance preferentially, because the *los4* and *atnup160* mutant plants do not have severe growth or developmental phenotypes, nor are they strongly altered in tolerance of other abiotic stresses. Interestingly, the *los4* and *atnup160* mutants both have defects in the cold regulation of the CBF regulon. A crucial regulator of the CBF regulon might be more strongly impacted by RNA export than other genes in general. It is also possible that the nuclear pore complex might undergo remodeling under cold temperatures to accommodate altered RNA structures or possibly higher RNA export demand caused by cold stress. LOS4 and AtNUP160, and perhaps other nucleoporins, might be important for this nuclear pore remodeling. Although a higher or modified demand for RNA export is still speculative, there is evidence of an increased demand for RNA splicing under cold stress. The *Arabidopsis* protein STABILIZED1 (STA1) is a pre-mRNA splicing factor similar to the yeast Prp1p and Prp6p [30•]. *STA1* expression is strongly upregulated by cold stress. A hypomorphic *sta1* mutant allele causes mis-splicing of RNAs under cold conditions and the mutant plants are extremely chilling sensitive, suggesting that pre-mRNA splicing is of particular importance for cold tolerance in plants.

The low-temperature metabolome and interplay between metabolic status and cold stress signaling

Cold stress causes dramatic changes to plant metabolism, as a result not only of general reductions in enzyme activities and reaction rates in the cold [1] but also of active reconfiguration of the metabolome [31,32]. The protective functions of metabolites such as sucrose, trehalose, fructan, maltose, galactinol, proline and glycine-betaine are well documented [33–35]. Recently, vitamin E was found to be important for chilling tolerance as vitamin-E-deficient *Arabidopsis* mutants are chilling sensitive because of defective export of photoassimilate [36].

Large-scale profiling of metabolites by gas chromatography-mass spectrometry (GC-MS) has revealed sweeping changes in the plant metabolome in response to cold stress [31,32]. Of more than 400 polar metabolites monitored by Cook *et al.* [32], 75% increased upon cold treatment. Remarkly, cold-induced changes in the metabolome can be largely mimicked by ectopic expression of *CBF* genes at warm temperature, demonstrating a prominent role for the CBF regulon in the reconfiguration of the low-temperature metabolome. Consistent with an important role for the CBF regulon and the metabolites in cold tolerance, an *Arabidopsis* strain that is incapable of cold acclimation (Cape Verde Islands-1 ecotype) has a weak CBF regulon and is deficient in CBF-regulated metabolites [32].

The active reconfiguration of the metabolome is achieved by cold-regulated gene expression changes, which in turn are controlled by low-temperature signaling. Metabolism is not, however, a passive target of cold signaling. It can also regulate cold signaling and cold-responsive gene expression. At least three types of metabolic signals might

be important for cold signaling. First, soluble sugars can serve as important signaling molecules [37]. Second, the tetrapyrrole intermediate Mg-protoporphyrin (Mg-ProtoIX) was recently found to accumulate in plants under cold conditions and binds to the translational elongation factor 2 protein. *Arabidopsis* mutants that are deficient in Mg-ProtoIX are impaired in cold-responsive gene expression and have reduced freezing tolerance (A Strand, pers. comm.). Consistent with a putative chloroplastic signal for cold regulation of gene expression, barley mutants that are affected in chloroplast development are impaired in cold-responsive gene expression [38]. The third type of metabolic signal for cold regulation might be reactive oxygen species (ROS). The *Arabidopsis frostbite1* mutant is impaired in the mitochondrial electron transfer chain and hyperaccumulates superoxide and hydrogen peroxide [39]. The high levels of ROS in this mutant might serve as the metabolic signal that causes reduced cold-induction of *COR* genes and decreased freezing tolerance [39]. Another *Arabidopsis* mutant, *chy1*, which is defective in a peroxisomal β-hydroxyisobutyryl-CoA hydrolase needed for fatty acid β-oxidation and valine catabolism, also accumulates high levels of ROS (C-H Dong, J-K Zhu, unpublished). The *chy1* mutant shows a reduced cold induction of *CBF* genes, and is defective in chilling and freezing tolerance that can be rescued by ectopic expression of *CBF3* under a constitutive promoter. ROS levels and subcellular distribution are very dynamic. ROS is possibly a ubiquitous metabolic signal that modulates many cellular processes, including cold responses.

Conclusions and future directions

Large-scale profiling of gene transcripts and metabolites has permitted a glimpse of the sweeping changes to the plant transcriptome and metabolome that take place under cold stress. Genetic analysis has revealed the unexpected involvement of metabolic signals and of RNA processing and export in cold stress signaling and tolerance. The precise mechanism of these involvements remains to be defined. Information on the low-temperature transcriptome, proteome and metabolome is expected to continue to increase in the near future. This information is necessary for our understanding of the complex network of molecular changes that are important for chilling and freezing tolerance. The power of forward and reverse genetic studies, particularly when combined with biochemical analysis, needs to be exploited further. Additionally, innovative approaches are needed to identify and establish the function of the cold sensors and signaling components that are responsible for the activation of cold-responsive transcriptional cascades.

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