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Cold stress regulation of gene expression in plants

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Cold stress adversely affects plant growth and development. Most temperate plants acquire freezing tolerance by a process called cold acclimation. Here, we focus on recent progress in transcriptional, post-transcriptional and post-translational regulation of gene expression that is critical for cold acclimation. Transcriptional regulation is mediated by the inducer of C-repeat binding factor (CBF) expression 1 (ICE1), the CBF transcriptional cascade and CBF-independent regulons during cold acclimation. ICE1 is negatively regulated by ubiquitination-mediated proteolysis and positively regulated by SUMO (small ubiquitin-related modifier) E3 ligase-catalyzed sumoylation. Post-transcriptional regulatory mechanisms, such as pre-mRNA splicing, mRNA export and small RNA-directed mRNA degradation, also play important roles in cold stress responses.

The effect of cold stress

Cold stress, which includes chilling (<20 °C) and/or freezing (<0 °C) temperatures, adversely affects the growth and development of plants and significantly constraints the spatial distribution of plants and agricultural productivity. Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses. Cold acclimation is a process by which plants acquire freezing tolerance upon prior exposure to low non-freezing temperatures. Most temperate plants can cold-acclimate and acquire tolerance to extracellular ice formation in their vegetative tissues. Winter-habit plants (winter wheat, barley, oat, rye, oilseed rape, etc) have a vernalization requirement, which prevents premature transition to the reproductive phase before the threat of freezing stress during winter has passed. Thus, vernalization requirement allows plants to over-winter as seedlings. However, after vernalization and at the end of the vegetative phase, the cold acclimation ability of winter cereals gradually decreases [1]. Many important crops, such as rice, maize, soybean, cotton and tomato, are chilling sensitive and incapable of cold acclimation; moreover, they cannot tolerate ice formation in their tissues. Nevertheless, the temperature threshold for chilling damage is lowered even in chilling-sensitive crops by prior exposures to suboptimal low temperatures [2]. The molecular basis of this acquired chilling tolerance or chilling acclimation is poorly understood, but might be related in part to the cold acclimation process.

Studies on acquired freezing tolerance in *Arabidopsis* have contributed substantially towards the understanding of cold acclimation mechanisms. Cold acclimation involves the remodeling of cell and tissue structures and the reprogramming of metabolism and gene expression [3,4]. This review summarizes the latest work that addresses the following questions: How are cold temperatures sensed and the signal transduced to the nucleus to regulate gene expression? What are the mechanisms of post-translational regulation of transcription factors under cold stress? To what extent are post-transcriptional processes involved in cold acclimation?

Cold stress signaling

Cellular membranes are fluid structures, and cold temperatures can reduce their fluidity, causing increased rigidity. Plant cells can sense cold stress through low temperature-induced changes in membrane fluidity, protein and nucleic acid conformation and/or metabolite concentration (a specific metabolite or redox status). Using a pharmacological approach, plasma membrane rigidification has been shown previously to induce *COR* (*COLD RESPONSIVE*) genes and result in cold acclimation in alfalfa and *Brassica napus* [5,6]. The *Arabidopsis fad2* mutant defective in oleate desaturase exhibits membrane rigidification and activation of diacylglycerol kinase at higher temperatures (18 °C) as compared with the wild type (14 °C) and transgenic *Arabidopsis* overexpressing linoleate desaturase (12 °C) [7]. These results add support to the notion that plant cells can sense cold stress through its membrane rigidification effect. Cold-induced Ca^{2+} increase in the cytosol can also be mediated through membrane rigidification-activated mechano-sensitive or ligand-activated Ca^{2+} channels. Subsequently, calcium signal amplification and phospholipid signaling might be involved in cold-stress signaling [8–11].

Secondary signals, such as abscisic acid (ABA) and reactive oxygen species (ROS), can also induce Ca^{2+} signatures that impact cold signaling. *Arabidopsis* mutants defective in the activation of the molybdenum cofactor of abscisic aldehyde oxidase, namely *aba3/freezing sensitive 1* (*frs1*) [12], also known as *los5* (*low expression of osmotically responsive genes 5*) [13], exhibit hypersensitivity to freezing stress. *los5* mutant plants show a significant reduction in

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the expression of cold and osmotic stress induction of genes [13]. ROS accumulate in cells challenged with various abiotic stresses, and they appear to have a strong influence on cold regulation of gene expression. The *Arabidopsis fro1* (*frostbite1*) mutant, which constitutively accumulates high levels of ROS, exhibits impaired expression of *COR* genes and hypersensitivity to chilling and freezing. *FRO1* encodes the Fe–S subunit of complex I (NADH dehydrogenase) of the respiratory electron transfer chain in mitochondria, and its disruption leads to high levels of ROS generation [14]. Besides their effect on calcium signatures, ROS signals can also exert their effects directly through the activation of redox-responsive proteins, such as transcription factors and protein kinases.

Low temperature affects water and nutrient uptake, membrane fluidity and protein and nucleic acid conformation, and it drastically influences cellular metabolism either directly by reducing the rates of biochemical reactions or indirectly through gene expression reprogramming. Metabolic profiling revealed that cold acclimation increases ~75% of the 434 metabolites detected in *Arabidopsis* plants [15,16], although metabolite profiles do not appear to correlate with cold acclimation capacity of *Arabidopsis* [17]. In addition to their role as osmoprotectants and osmolytes, certain metabolites (individual metabolites or redox state) induced during cold acclimation might act as signals for reconfiguring gene expression. For example, cold stress induces the accumulation of proline, a well-known osmoprotectant. Microarray and RNA gel blot analyses have shown that proline can induce the expression of many genes, which have the proline-responsive element (PRE, ACTCAT) in their promoters [18,19].

Transcriptional regulation

Cold acclimation temperatures induce profound changes in the plant transcriptome. In *Arabidopsis*, cold-regulated genes have been estimated to constitute ~4% [20] to 20% of the genome [21]. Significant progress has been made in the past decade in elucidating the transcriptional networks regulating cold acclimation.

ICE1–CBF transcriptional cascade

Cold stress induces the expression of APETALA2/ETHYLENE RESPONSE FACTOR family transcription factors, that is, CBFs (C-repeat binding factors, also known as dehydration-responsive element-binding protein 1s or DREB1s), which can bind to *cis*-elements in the promoters of *COR* genes and activate their expression. Analyses in transgenic plants have shown that ectopic expression of *CBFs* is sufficient to activate the expression of *COR* genes and induce cold acclimation even at warm temperatures [22,23]. CBFs regulate the expression of genes involved in phosphoinositide metabolism, transcription, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone metabolism and signaling and many others with known or presumed cellular protective functions [20,24,25]. *CBF* homologs have been cloned from both cold-tolerant (wheat, barley and *Brassica napus*) and cold-sensitive (rice, maize, tomato and cherry) crops. Transgenic expression of *Arabidopsis CBFs* in different plant species was able to enhance chilling/freezing tolerance, and, conversely, the ectopic expression of *CBFs* from other plant species could enhance the freezing tolerance of transgenic *Arabidopsis* [10,26]. Microarray analysis of transgenic *Arabidopsis* plants ectopically expressing *CBFs* revealed a constitutive expression of downstream cold-responsive transcription factor genes *RAP2.1* and *RAP2.7*, which might control subregulons of the CBF regulon [24]. Thus, CBFs play a pivotal role in gene regulation during cold acclimation in evolutionarily diverse plant species. However, CBF regulons from freezing-tolerant and -sensitive plant species can differ, as evident from microarray analysis of transgenic tomato and *Arabidopsis* plants overexpressing *LeCBF1* and *AtCBF3*, respectively [27]. Winter plants exhibit significant genotypic differences in constitutive freezing tolerance and acquired freezing tolerance; the two traits appear to have independent genetic controls [28]. However, the molecular basis of constitutive freezing tolerance is poorly understood. Transcriptome and metabolome analyses in *Arabidopsis* accessions differing in constitutive freezing tolerance suggest that the CBF pathway might also have a crucial role in constitutive freezing tolerance [17].

In *Arabidopsis*, ICE1 (INDUCER OF CBF EXPRESSION1), a MYC-type basic helix–loop–helix transcription factor, can bind to MYC recognition elements in the *CBF3* promoter and is important for the expression of *CBF3* during cold acclimation. The *ice1* mutant is defective in the cold induction of *CBF3* and is hypersensitive to chilling stress and incapable of cold acclimation. Constitutive overexpression of *ICE1* enhanced the expression of *CBF3*, *CBF2* and *COR* genes during cold acclimation, and increased freezing tolerance of the transgenic *Arabidopsis*. *ICE1* is constitutively expressed and localized in the nucleus, but it induces expression of *CBFs* only under cold stress. This suggests that cold stress-induced post-translational modification is necessary for ICE1 to activate downstream genes in plants [29] (Figure 1). Indeed, cold stress induces phosphorylation of ICE1 (H. Fujii and J-K. Zhu, unpublished). Transcriptome analysis revealed that expression of ~40% of cold-regulated genes, and in particular 46% of cold-regulated transcription factor genes are impaired in the dominant *ice1* mutant. The cold induction of genes involved in calcium signaling, lipid signaling or encoding receptor-like protein kinases are also affected by the *ice1* mutation [20]. Bioinformatics analysis of microarray data on the cold-responsive transcriptome of wild type and mutants or of transgenic *Arabidopsis* plants overexpressing specific transcription factors led to the prediction of a cold-acclimation transcriptional network. In this network, ICE1 is predicted to be a transcriptional inducer of *CBFs* (CBF1–CBF3), *ZAT12*, *NAC072* and the constitutively expressed transcription factor *HOS9* in *Arabidopsis* [30].

It is likely that ICE1 and related proteins also play a critical role in the regulation of the expression of genes important in the chilling tolerance of *Arabidopsis*. The *ice1* mutation renders *Arabidopsis* plants chilling sensitive [29], and it affects the basal transcript levels of 204 of the 939 cold-regulated genes under non-stress conditions [20]. Basal expression of these genes could be important for chilling tolerance of *Arabidopsis*, as

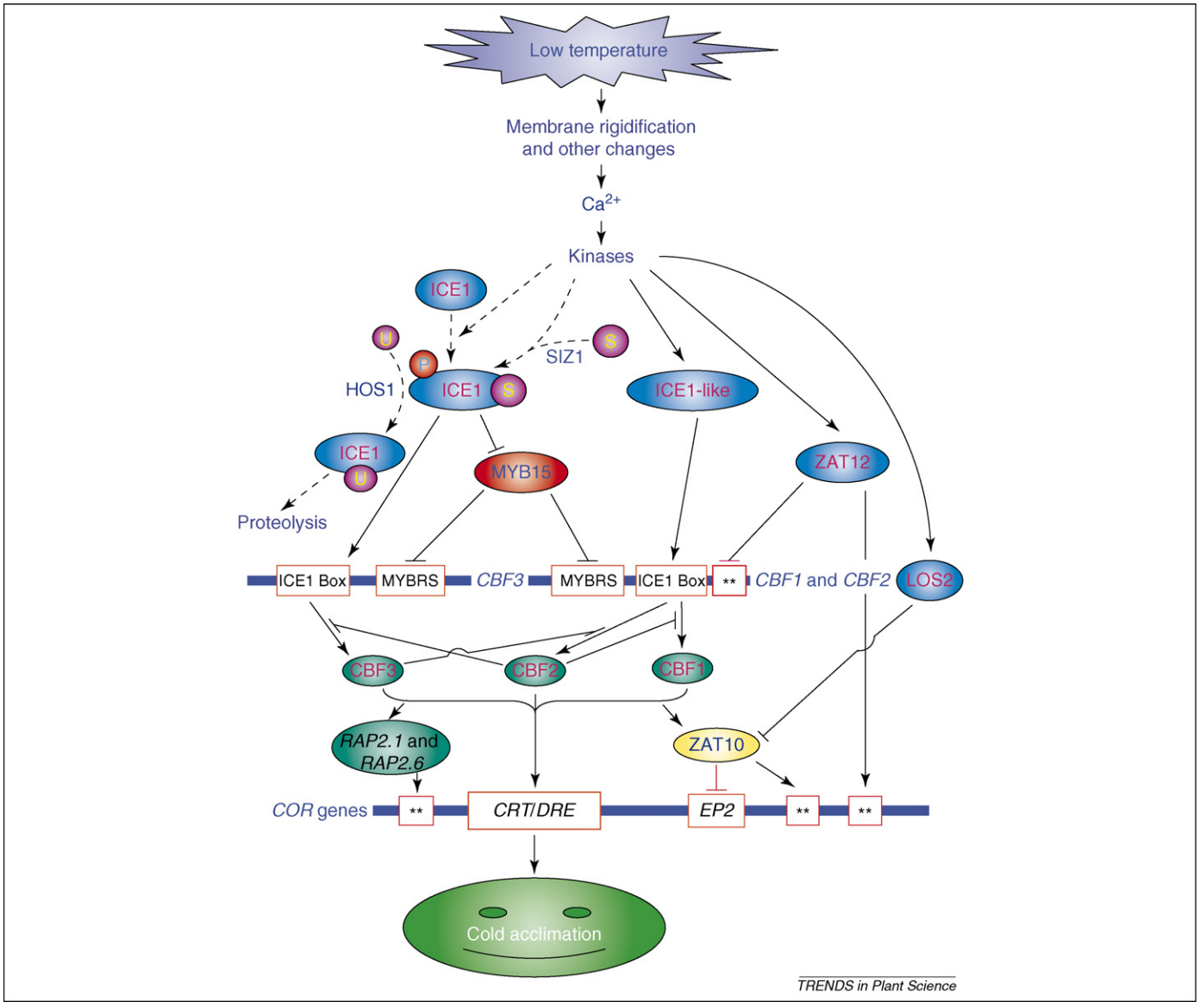

Figure 1. Diagram of cold-responsive transcriptional network in *Arabidopsis*. Plants probably sense low temperatures through membrane rigidification and/or other cellular changes, which might induce a calcium signature and activate protein kinases necessary for cold acclimation. Constitutively expressed ICE1 is activated by cold stress through sumoylation and phosphorylation. Cold stress induces sumoylation of ICE1 at K393, which is critical for ICE1-activation of transcription of *CBFs* and repression of *MYB15*. CBFs regulate the expression of *COR* genes that confer freezing tolerance. The expression of *CBFs* is negatively regulated by MYB15 and ZAT12. HOS1 mediates the ubiquitination and proteosomal degradation of ICE1 and, thus, negatively regulates CBF regulons. CBFs might cross-regulate the each other's transcription. CBFs induce the expression of *ZAT10* (=*STZ*), which might downregulate the expression of *COR* genes. Cold-upregulated LOS2 represses the transcription of *ZAT10*. ZAT10 and ZAT12 are two C2H2 zinc finger transcription factors. Broken arrows indicate post-translational regulation; solid arrows indicate activation, whereas lines ending with a bar show negative regulation; the two stars (**) indicate unknown *cis*-elements. Abbreviations: CBF, C-repeat binding factor (an AP2-type transcription factor); CRT, C-repeat elements; DRE, dehydration-responsive elements; HOS1, high expression of osmotically responsive genes1 (a RING finger ubiquitin E3 ligase); ICE1, inducer of CBF expression 1 (a MYC-type bHLH transcription factor); LOS2, low expression of osmotically responsive genes 2 (a bifunctional enolase with transcriptional repression activity); MYB, myeloblastosis; MYBRS, MYB transcription factor recognition sequence; SIZ1, SAP and MiZ1 (a SUMO E3 ligase); P, phosphorylation; S, SUMO (small ubiquitin-related modifier); U, ubiquitin.

altered expression of these genes in *ice1* is correlated with chilling sensitivity.

Negative regulators of the CBF regulon

Feedback repression of transcription factors that regulate cold-responsive gene expression appears to be a key to maintaining an optimal cold-induced transcriptome. Molecular analysis of a *cbf2* null mutant of *Arabidopsis* suggested that CBF2 is a negative regulator of *CBF1* and *CBF3* expression during cold acclimation [31]. Conversely, *CBF3* might negatively regulate *CBF2* expression, because reduced expression of *CBF3* in the *ice1* mutant is accompanied by an enhanced expression of *CBF2* [29]. These results suggest that cross-regulation and, perhaps, also self-regulation have an important role in the expression levels of *CBFs* during cold acclimation (Figure 1). Furthermore, *CBFs* are negatively regulated by an upstream transcription factor, MYB15 (an R2R3-MYB family protein) in *Arabidopsis*. *MYB15* is expressed even in the absence of cold stress, and MYB15 can bind to MYB recognition elements in the promoters of *CBFs*. *myb15* T-DNA knockout mutant plants show enhanced expression of *CBFs* during cold acclimation and enhanced freezing tolerance, whereas transgenic *Arabidopsis* overexpressing

MYB15 show a decreased expression of *CBFs* and a reduction in freezing tolerance. Thus, MYB15 is an upstream transcription factor that negatively regulates the expression of *CBFs* [32] (Figure 1). Interestingly, ICE1 can negatively regulate *MYB15* as indicated from the increased *MYB15* transcript level in *ice1* mutant compared with wild-type plants under cold stress [29,32]. Yeast two-hybrid and *in vitro* pull-down assays showed that MYB15 can interact with ICE1, but the functional significance of ICE1–MYB15 interaction in cold acclimation is unknown [32].

In *Arabidopsis*, a cold-induced C2H2 zinc finger transcription factor gene, *ZAT12*, also appears to function as a negative regulator of *CBFs* (Figure 1). The circadian clock-regulated, rhythmic expression pattern of *ZAT12* is ~180° out of phase with the rhythms of *CBF2* and *RAV1* [33]. Transgenic overexpression of *ZAT12* decreases the expression of *CBFs* under cold stress. Transcriptome analysis of *ZAT12*-overexpressing *Arabidopsis* revealed that the ZAT12 regulon consists of at least 24 COS (COLD STANDARD SET) genes, of which nine are cold-induced and 15 are cold-repressed genes [34]. Molecular analysis of the *los2* mutant of *Arabidopsis* revealed that another C2H2 zinc finger protein, *ZAT10*/*STZ*, might act as a negative regulator of CBF-target genes. LOS2, a bifunctional enolase, binds to the MYC recognition elements in the *ZAT10* promoter *in vitro* and *los2* mutant plants showed an enhanced and more sustained induction of *ZAT10* during cold stress [35]. Thus LOS2 appears to be a negative regulator of *ZAT10* expression during cold acclimation. Transient expression assays showed that ZAT10 could repress the expression of *RD29A*, a target gene of CBFs [35]. CBFs might have a role in mediating or modulating cold-stress induction of *ZAT10* because transgenic plants overexpressing *CBF3* showed an enhanced expression of *ZAT10* [25]. Furthermore, impairment of *CBF3* expression caused by the *ice1* mutation also led to a significant decrease in the cold induction of *ZAT10*, as is evident from microarray data [10,29]. Thus, ZAT10 could be a subregulon of CBFs and might regulate a subset of genes involved in cold acclimation (Figure 1). ZAT10 and ZAT12 might serve as converging nodes in abiotic stress-regulated transcriptional networks, because these transcription factors are induced by cold and other abiotic stresses, and transgenic plants overexpressing these genes exhibit enhanced osmotic and oxidative stress tolerance [36,37].

CBF-independent regulons

Microarray analysis has shown that CBFs regulate only ~12% of the cold-responsive transcriptome [24]. Hence, non-CBF transcription factors might regulate the remaining large portion of cold-responsive genes. In soybean, the cold-stress inducible C2H2-type zinc finger protein SCOF1 appears to induce the expression of *COR* genes, probably by enhancing the DNA binding activity of the cold-inducible basic leucine zipper transcription factor, G-Box-binding factor 1 [38]. The *eskimo1* (*esk1*) mutant of *Arabidopsis* accumulates constitutively high levels of proline and is constitutively freezing tolerant [39]. *ESK1* encodes a DUF231 (domain of unknown function231) protein. *ESK1* expression is not altered by cold stress. Transcriptome analysis identified 312 genes with altered expression in *esk1* mutant, of which only 12 genes show increased expression in both the *esk1* mutant and *CBF2*-overexpressing transgenic *Arabidopsis* plants. Thus, the freezing tolerance imparted by the recessive *esk1* mutation might have a distinct molecular basis from that of CBFs-conferred cold acclimation [40]. The mechanism by which ESK1 regulates freezing tolerance is yet to be understood.

By employing a genetic screen for deregulated expression of the P_{RD29A}*::LUC* reporter gene, two constitutively expressed transcription factors, HOS9 (a homeodomain protein) and HOS10 (an R2R3-type MYB), were identified. *hos9* mutant plants are less tolerant to freezing both before and after cold acclimation, although the cold induction of *CBFs* is similar to that of wild-type *Arabidopsis*. Microarray analysis revealed that the HOS9 regulon is distinct from that of the CBFs [41]. Moreover, the *hos10-1* mutant has much less freezing tolerance despite an enhanced expression of some *COR* genes under stress. Furthermore, HOS10 appears to regulate positively expression of *NCED3* (9-*cis*-epoxycarotenoid dioxygenase). Thus, HOS10 might regulate ABA-mediated cold acclimation [42]. Overexpression of a cold-stress-inducible rice transcription factor, *MYB4* (an R2R3-type MYB), in transgenic *Arabidopsis* enhances the expression of *COR* genes, proline levels and freezing tolerance [43]. Additionally, the cold-, drought- and salt- upregulated *OsMYB3R-2* (an R1R2R3 MYB) appears to regulate positively cold and other abiotic stress tolerance by a CBF-independent pathway in rice [44].

Microarray analysis led to the identification of the cold-stress-inducible AP2 family transcription factor gene *RAV1* (*RELATED TO ABI3*/*VP1*) [24,34] that might regulate plant growth under cold stress. *RAV1* is downregulated by epibrassinolide, and transgenic *Arabidopsis* overexpressing *RAV1* exhibits a retardation of lateral root and rosette-leaf development, whereas antisense *RAV1* plants show an early-flowering phenotype [45].

Post-transcriptional regulation

In addition to transcriptional regulation, gene expression is regulated post-transcriptionally at pre-mRNA processing, mRNA stability, export from nucleus and translation steps. Recent studies revealed that post-transcriptional regulation plays critical roles during cold acclimation.

RNA processing and export from the nucleus

Pre-mRNA splicing is a crucial nuclear process for the synthesis of functional mRNAs of intron-containing genes, and this process is coupled with nuclear export of mRNAs. Furthermore, alternative splicing in response to developmental and environmental cues enables cells to synthesize different proteins from a single gene. In wheat, two early *COR* genes (a ribokinase and a C3H2C3 RING-finger protein) were shown to be regulated by intron retention in their mature mRNAs under cold stress [46]. By using the P_{RD29A}*::LUC* genetic screen, Lee *et al.* [47] identified *STABILIZED1* (*STA1*), a nuclear pre-mRNA splicing factor, as a regulator of pre-mRNA splicing that is of particular importance to cold tolerance in *Arabidopsis*. Cold stress upregulates the expression of *STA1*. The *sta1* mutant is defective in the splicing of the cold-induced *COR15A*

pre-mRNA and is hypersensitive to chilling, ABA and salt stresses. These results showed that a fully functional STA1 is required for splicing and turnover of specific transcripts, and, under cold stress, there is an increased demand for this factor [47]. The serine/arginine-rich (SR) proteins are part of the spliceosome and act as splicing regulators in eukaryotes. In *Arabidopsis*, cold and heat stresses regulate the alternative splicing of pre-mRNAs of many SR genes, which might produce different isoforms of SR proteins with altered splicing functions under stress conditions [48].

The transduction of environmental signals into the nucleus to alter transcription and the export of mRNAs and small RNAs to the cytoplasm through the nuclear pore complex (NPC) of the nuclear envelop are crucial to gene regulation in eukaryotes. NPCs are composed of several proteins collectively called nucleoporins (NUPs). Export-competent messenger ribonucleoproteins consist of mRNA cargo and nucleocytoplasmic shuttling nuclear proteins, such as the RNA export factors, DEAD-box protein 5 (Dbp5) and NUPs [49]. The NUP107–160 complex involved in mRNA export in animals and yeast has identifiable homologs in the *Arabidopsis* proteome [50]. Recently, AtNUP160 was found to have a critical role in cold acclimation. Expression of *AtNUP160* is ubiquitous in all tissues and is not regulated by cold stress. *Arabidopsis atnup160-1* mutant plants are impaired in the cold induction of *CBFs* and several other genes involved in cold acclimation. The mutant plants are hypersensitive to chilling and freezing stresses. The AtNUP160–GFP fusion protein is localized at the nuclear rim. Although the *atnup160-1* mutant is impaired in poly(A) mRNA export at both warm and cold temperatures, poly(A) mRNA accumulation in the nucleus in the *atnup160-1* mutant is higher under cold stress. Thus, these phenotypes of the *atnup160-1* mutant suggest a connection between the function of the NPC and chilling and freezing tolerance [51].

The DEAD-box family of RNA helicases is involved in RNA metabolism, such as transcription, RNA processing, RNA decay and nucleocytoplasmic transport [49]. A role for such a helicase in mRNA export and plant abiotic stress responses was revealed from the analysis of the *los4* (*l*ow *expression of osmotically responsive genes 4*) mutant of *Arabidopsis*. The chilling stress sensitive *los4-1* mutants showed a reduced expression of P_{RD29A}*::LUC* and *CBF3* and a delayed expression of *CBF1* and *CBF2* during cold acclimation. *LOS4* encodes a DEAD-box RNA helicase [52]. Later, the *cryophyte*/*los4-2* mutant (allelic to *los4-1*) was isolated, which showed a superinduction of *CBF2* under cold stress and an enhanced cold tolerance. The LOS4–GFP fusion protein was found to be enriched in the nuclear rim. Consistent with the cold-sensitive phenotype of *los4-1* mutant, mRNA export is significantly lower in *los4-1* under both normal and cold-stress conditions. By contrast, the cold-tolerant but heat-sensitive *cryophyte*/*los4-2* mutant shows normal mRNA export under cold stress but is defective in mRNA export from the nucleus at warm temperatures [53]. In pea, DNA helicase 45 (*PDH45*) and *PDH47* are upregulated by various abiotic stresses including cold stress and ABA [54]. Transgenic tobacco plants overexpressing *PDH45* show an enhanced tolerance to salt stress [55]. These observations suggest that DEAD-box RNA helicases are critical for mRNA export, and this and/or other functions of the helicases are important for plant tolerance to cold and other abiotic stresses.

Nuclear trafficking involves the nucleocytoplasmic shuttling of NUPs, transport receptors (karyopherins) and nuclear export factors. Karyopherins have a critical role in Ran GTPase-dependent nuclear export and import [56]. By employing the P_{RD29A}*::LUC* genetic screen, we have also identified an importin β-domain/karyopherin protein, SAD2, involved in nucleocytoplasmic trafficking under cold, osmotic stress and ABA treatments. *sad2-1* null mutant plants show an enhanced expression of P_{RD29A}*::LUC* and several endogenous ABA- and stress-responsive genes under various stresses including cold stress. It appears that the nuclear import of ABA-signaling factor(s) has a specific requirement for SAD2, because a T-DNA knockout of *At3g59020*, a *SAD2* paralog, does not mimic the ABA-hypersensitive phenotype of SAD2 [57]. SAD2 might be involved in the nucleocytoplasmic trafficking of NUPs/nuclear export factors/small RNAs, which in turn are critical for cold- and abiotic stress responses.

Transcription by RNA polymerase II (Pol II) is coordinated with pre-mRNA processing, which is necessary for the formation of functional mRNAs and for mRNA export. The C-terminal domain (CTD) of Pol II plays a vital function in these processes in a manner dependent on its phosphorylation state [58]. Pol II CTD phosphatases control the transcription and pre-mRNA processing by dephosphorylation of Pol II. In *Arabidopsis*, one of the CTD phosphatases, FIERY2 (FRY2)/CPL1, affects the expression of *COR* genes [59,60]; indeed, *fry2* mutants show an enhanced expression of *CBFs* and *COR* genes under cold stress and ABA treatment. Because *fry2* mutants are hypersensitive to freezing stress, even though the expression of *CBFs* is not reduced, FRY2 might function in cold acclimation primarily through non-CBF regulons [60].

Small RNAs have potentially a large share in plant stress responses

Small non-coding RNAs of ~21 to 24 nucleotides in length, namely microRNAs (miRNAs) and short interfering RNAs (siRNAs), are ubiquitous repressors of gene expression in animals and plants. The function of small RNAs in plant development and genome defense is relatively well established, but their role in abiotic stress responses was discovered only recently [61,62]. miRNAs and siRNAs regulate gene expression by directing the cleavage or translational repression of complementary target mRNAs or by inducing transcriptional silencing of target genes. Abiotic-stress-induced miRNAs and siRNAs would downregulate target genes that probably encode negative regulators or determinants of stress response, whereas the downregulation of those miRNAs or siRNAs might result in the accumulation of their target mRNAs that probably encode positive regulators or determinants of stress tolerance [63]. Microarray analysis indicated that ~17% of cold-upregulated genes encode transcription factors, whereas only 7% of cold-downregulated genes encode transcriptional regulators [20]. Hence, it is conceivable that post-transcriptional regulation might serve as a major mode of downregulation of genes during cold acclimation. Because

miRNAs regulate various developmental processes under non-stress environments [64], it is possible that plants might employ the same miRNAs to regulate growth and development under cold stress as well. Consistent with this notion, microarray data from Genevestigator [65] suggest that many of the miRNA target genes involved in regulation of growth and development of *Arabidopsis* might be responsive to cold stress.

Cold, ABA, dehydration and salt stress upregulate the expression of miR393, miR397b and miR402. Furthermore, miR319c shows possibly a cold-stress-specific upregulation. By contrast, the expression of miR389a.1 is downregulated by cold, ABA, dehydration and salt stresses [61]. In accordance with the upregulation of miR393 under cold stress, its target genes (putative E3 ubiquitin ligase SCF complex F-box proteins) appear to be downregulated by cold stress in *Arabidopsis* (Genevestigator, https://www.genevestigator.ethz.ch). Thus, cold-upregulated miR393 might target the cleavage of E3 ubiquitin ligase mRNAs, leading to less proteolysis of these E3 ubiquitin ligase target proteins (probably positive regulators of cold tolerance) during cold acclimation. One of the targets of cold-upregulated miR393 encodes an F-box protein (At4g03190) [61], which is similar to Glucose repression resistance 1 (GRR1), a yeast protein involved in glucose repression. As sugar metabolism is affected by various abiotic stresses, plants can use sugar status as a signal to modulate growth and development in response to abiotic stresses. Cold-stress-upregulated miR393 might target the *At4g03190* mRNA for degradation as *At4g03190* transcript level decreases under cold stress and, thus, miR393 might integrate sugar signaling with stress responses [63]. Many adverse environmental conditions including cold temperatures can induce oxidative stress in plants. superoxide dismutases (SODs) form the first line of defense against superoxide radicals. In *Arabidopsis*, oxidative stress downregulates the expression of miR398, whereas the transcript levels of its target genes *CSD1* and *CSD2* are enhanced. Transient co-expression assay and transgenic plants overexpressing miR398-resistant *CSD2* showed that miR398 targets the *CSD* mRNAs for degradation *in vivo*. Because miR398 and its target sites on the *CSD* mRNAs are conserved across plant species, miR398 might have a ubiquitous role in oxidative stress management under various abiotic stresses [66].

Many of the ~2000 genes in convergent overlapping gene pairs in *Arabidopsis* are regulated by various abiotic stresses and can potentially generate nat-siRNAs (natural antisense transcripts-generated siRNAs). Some of these siRNAs might regulate gene expression during cold acclimation. Recent findings from our laboratory have demonstrated that nat-siRNAs, which are derived from a *cis* natural antisense transcript pair of *SRO5* and *P5CDH*, regulate osmolyte catabolism and oxidative stress management under salt stress in *Arabidopsis* [62]. Transcriptome analysis revealed that the *NRPD1A* (*At1g63020*) required for nat-siRNA and heterochromatic siRNA biosynthesis is upregulated by cold stress [20]. The upregulation of NRPD1A might impact the cold-responsive transcriptome through increased generation of some siRNAs.

Post-translational regulation

Controlled proteolysis of transcriptional regulators has an important role in shaping the cold-responsive transcriptome in plants, as evidenced from studies on *HOS1* (*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1*). The *Arabidopsis hos1* mutation causes superinduction of *CBFs* and its downstream target genes under cold stress [67]. *HOS1* encodes a RING finger ubiquitin E3 ligase, the nuclear localization of which is also enhanced by cold stress [67,68]. Ubiquitin E3 ligases are known to confer substrate specificity for regulated proteolysis by the ubiquitination/26S proteasome pathway. Hence, it was proposed that HOS1 might target upstream signaling components or transcriptional regulators of *CBFs* for proteolysis to regulate negatively the expression *CBFs* [67]. Recently, ICE1 was identified as a target of HOS1 [68]. HOS1 physically interacts with ICE1 and mediates the ubiquitination of ICE1 both *in vitro* and *in vivo*. Polyubiquitination and proteolysis of ICE1 after 12 h of cold stress was found only in wild type but not in *hos1* mutants. Furthermore, transgenic *Arabidopsis* plants overexpressing *HOS1* show a substantial reduction in GFP–ICE1 protein levels, and a reduction in transcript levels of *CBFs* and CBF regulon genes, and hypersensitivity to freezing stress. These results demonstrate that HOS1 ubiquitinates ICE1 to regulate negatively the expression of ICE1 target genes (Figure 1), and is thus critical for the de-sensitization of plant cells to cold stress [68].

Sumoylation is a post-translational protein modification where SUMO (small ubiquitin-related modifier) proteins are conjugated to protein substrates in a process dependent on SUMO E3 ligases, whereas desumoylation is the removal of SUMO proteins from their target proteins by SUMO proteases. Sumoylation might protect target proteins from proteasomal degradation because sumoylation prevents ubiquitination [69]. Sumoylation/desumoylation of proteins has been shown to have a pivotal role in plant responses to abiotic and biotic stress responses and in ABA and salicylic acid signaling [70]. Recently, a role for sumoylation in cold acclimation was found through studies on the *Arabidopsis* SUMO E3 ligase, SIZ1 (SAP and Miz1) [70]. SIZ1 is required for the accumulation of SUMO conjugates during cold stress, and the *siz1* null mutant is hypersensitive to chilling and freezing stresses. The *siz1* mutation significantly reduces cold-induction of *CBFs* and its target *COR* genes (*COR15A, COR47* and *KIN1*), but it enhances the cold induction of *AtMYB15*, a negative regulator of *CBFs*. In contrast to HOS1, which promotes the proteolysis of ICE1, SIZ1 mediates SUMO conjugation to K393 of ICE1 during cold acclimation, and this reduces polyubiquitination of ICE1. SIZ1-mediated sumoylation is blocked by a K393R substitution in ICE1 [ICE1(K393R)]. Transgenic *Arabidopsis* plants overexpressing *ICE1* but not *ICE1(K393R)* exhibit an enhanced cold induction of *CBFs* and increased freezing tolerance. Further, similar to *ice1* mutant plants, *ICE1(K393R)* overexpressing transgenic plants exhibit a moderate increase in *MYB15* expression under cold stress, and display a hypersensitivity to freezing stress. These results suggest that SIZ1-mediated sumoylation might facilitate ICE1 stability and activity, which is necessary for *CBF* expression and

MYB15 repression to fine-tune the transcription of *COR* genes during cold acclimation (Figure 1) [70].

Conclusions and perspectives

Cold stress affects virtually all aspects of cellular function and it is therefore perhaps not surprising that plant cold acclimation responses are highly integrated into cellular function at all levels. Despite their complexity, recent technical advances in genetic analysis tools, gene expression and small RNA profiling, proteomics and metabolomics have made it possible to dissect the complex processes involved in cold acclimation. Cold stress regulates the plant transcriptome through transcriptional, post-transcriptional and post-translational mechanisms. The ICE1–CBF transcriptional cascade has an important role in cold acclimation in diverse plant species. In addition to the direct induction of *CBF* expression, ICE1 also appears to regulate negatively *MYB15*, an upstream negative regulator of *CBFs*. ICE1 protein level and activity are regulated post-translationally by HOS1-mediated ubiquitination and proteolysis and by SIZ1-mediated sumoylation. Several CBF-independent regulons that are critical for cold acclimation have also been identified. Recently, post-transcriptional regulatory mechanisms, such as pre-mRNA splicing, mRNA export and mRNA degradation directed by small RNAs, have been found to be important for cold responses. These mechanisms have been discovered mainly from studies on cold acclimation of the constitutively chilling-tolerant reference plant *Arabidopsis* at vegetative stages. The reproductive stage, more specifically the maturation of pollen, is most sensitive to cold stress. Expression of the majority of pollen-specific genes is not changed substantially in response to cold stress and many of the *COR* genes implicated in cold tolerance of leaf tissues are not induced significantly in the pollen [71]. Roots and leaves also exhibit different gene expression changes during cold acclimation: 86% of the cold-induced genes are not shared between roots and leaves [72]. These results suggest that the cold-regulated transcriptional networks might also differ in different tissues. Transgenic analyses have shown that overexpression of *Arabidopsis CBFs* in chilling-sensitive plants such as tomato and rice enhances the chilling tolerance of these plants [73,74]. Although these plants have homologs of *CBFs*, they are still sensitive to chilling stress. Molecular genetic analysis of chilling tolerance in model crop plants, such as rice, will be important to identify key regulators of chilling tolerance in vegetative and reproductive stages of plants.

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