1 **REVIEW: Post-translational regulation of cold acclimation response**

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Abstract

Cold acclimation is an adaptive response whereby plants from temperate regions increase their capacity to tolerate freezing in response to low-nonfreezing temperatures. Numerous studies have unveiled the large transcriptome re-programming that takes place during cold acclimation in diverse species, and a number of proteins have been identified as important regulators of this adaptive response. Post-translational mechanisms regulating the function of proteins involved in cold acclimation have been, however, much less studied. Several components of the signal transduction pathways mediating cold response have been described to be post-translationally modified. These post-translational modifications, including protein phosphorylation and dephosphorylation, ubiquitination, SUMOylation, N-glycosylation and lipid modification, determine key aspects of protein function such as sub-cellular localization, stability, activity or ability to interact with other proteins. Integrating these post-translational mechanisms within the appropriate spatio-temporal context of cold acclimation is essential to develop new crops with improved cold tolerance. Here, we review available evidence regarding the post-translational regulation of cold acclimation, discuss its relevance for the accurate development of this response, and highlight significant missing data.

Keywords: Post-translational modification, Post-translational regulation, Cold acclimation, Abiotic stress, Freezing tolerance, Signal transduction

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1. Introduction

Post-translational modifications are a common strategy to regulate protein function. More than 200 types of modifications determine critical facets of protein function, including turnover, subcellular localization, activity, or interaction with other proteins [1,2]. The importance of post-translational regulation in plants is suggested by the observation that 10% of the Arabidopsis genome is dedicated to protein phosphorylation and ubiquitination, two of the most frequent post-translational modifications [3,4]. Furthermore, the protein kinase superfamily has been demonstrated to be larger in plants than in other eukaryotes [3], indicating the relevance of protein post-translational modifications in plants. The higher number of protein kinases in plants may be related to the need of plants to adapt to ever-changing environmental conditions [5].

Low temperature is one of the most important environmental challenges that plants face during their life cycle. Plants from temperate climates increase their freezing tolerance after a brief exposure to low non-freezing temperatures, an adaptive response termed cold acclimation [6,7]. During the last 20 years, significant progress has been made in understanding the molecular mechanisms leading to cold acclimation [8,9]. Low temperature is signaled by a swift rise in cytosolic free Ca^{2+} that is subsequently transduced by phosphorylation and transcriptional cascades resulting in a large re-programming of the plant transcriptome. As a consequence, an array of cellular adjustments takes place protecting the photosynthetic machinery, cellular membranes and other cellular structures from cold-induced reactive oxygen species and dehydration [10,11]. A number of proteins have been established as important regulators or effectors of cold acclimation [8]. Thus, an interesting picture is emerging in which cold acclimation is a very complex response that is tightly regulated at all levels, i.e. transcriptionally, post-transcriptionally, translationally and post-translationally [12]. Our current knowledge of these regulatory levels, however, is not uniform. Many papers have addressed the

regulation of cold acclimation at transcriptional and post-transcriptional levels but data concerning the translational and post-translational regulation of this response are quite scarce. Here, we review and discuss results that have been reported on the post-translational mechanisms implicated in regulating freezing tolerance and cold acclimation, and highlight the relevance of post-translational modifications in plant response to low temperature. Most data discussed have been obtained from the model plant *Arabidopsis thaliana* and, in some cases, are difficult to extrapolate to other species such as cereals. We will not consider the post-translational regulation of histones, since this subject has been recently reviewed in the context of the epigenetic control of the cold acclimation response [13,14].

2. Post-translational modifications regulating freezing tolerance and cold acclimation

2.1 Phosphorylation

Phosphorylation mediated by kinases is one of the most ordinary mechanisms by which environmental cues are transduced and protein function is regulated in eukaryotic cells. Approximately, 1-2% of eukaryotic genes encode protein kinases [3], which indicates the significance of phosphorylation when considering the different types of post-translational modifications. Phosphorylation is largely a reversible protein modification, with the level of phosphorylation of a particular substrate depending on the balance of specific kinase and phosphatase activities. Protein phosphorylation may affect several aspects of protein function such as catalytic activity, subcellular localization, stability and/or interaction with other proteins [15].

The disruption of phosphorylation has a significant effect on the ability of plants to acclimate to low temperature [16]. Several protein kinases belonging to diverse families, including mitogen activated protein kinases (MAPKs), Ca²⁺-dependent protein kinases (CDPKs), calcineurin-B-Like interacting protein kinases (CIPKs), and receptor-like kinases (RLKs) are involved in regulating cold acclimation. Signaling

pathways mediated by MAPKs have long been recognized as key molecular switches for transduction of environmental and developmental signals. The mechanistic paradigm of a typical MAPK pathway is that plasma-membrane receptors activate MAP kinase kinase kinases (MAP3Ks) that, in turn, elicit a phosphorylation cascade involving other downstream MAPKs culminating in the phosphorylation of effector proteins, which usually are either enzymes or transcription factors [17]. MAPKs have been implicated in plant responses to abiotic stresses including low temperature. For instance, the over-expression of a constitutively active form of OsMPKK6 in rice increases chilling tolerance [18]. MAPKs also have an important role in cold acclimation in freezing tolerant plants such as Arabidopsis. In this way, different lines of evidence link the Arabidopsis MAPK pathway mediated by MEKK1-MKK2-MPK4/6 to cold acclimation (Fig. 1). First, *MEKK1* expression is induced by cold [19] and a N-terminal truncated MEKK1 can interact *in vitro* with MKK2 increasing its activity [20]. Second, *mkk2* mutants have enhanced sensitivity to freezing temperature in both non-acclimated and cold-acclimated plants whilst over-expression of constitutively active *MKK2* increases freezing tolerance even without previous exposure to cold [20]. Finally, MKK2 activity is enhanced by low temperature upregulating MPK4 and MPK6 activity as well as the expression of several cold-regulated genes, which links MAPK signaling to the large transcriptome re-programming that leads to cold acclimation [20]. Nevertheless, the specific downstream proteins targeted by MPK4 and MPK6 in response to low temperature remain to be elucidated. High-throughput phosphoproteomic analysis [21] and yeast-two-hybrid screens [22] have allowed the identification of a collection of putative MPK4 and MPK6 targets that include key transcription factors in cold acclimation in Arabidopsis like ICE1, CBF1, CBF3 and MYB15 (Fig. 1). These proteins represent good candidate targets of MPK4 or MPK6 activity during cold acclimation development. Furthermore, a 1-aminocyclopropane 1-carboxylate synthase isoform that regulates the rate-limiting step of ethylene biosynthesis in Arabidopsis [23], the ACS6, is phosphorylated by MPK6 which results in increased protein stability and higher ACC synthase activity [24] (Fig. 1). Multiple

studies have implicated ethylene in cold signaling, however its precise role in cold acclimation still constitutes a matter of debate [25,26]. It is possible therefore, that one of the outputs of MEKK1-mediated cold signaling is the regulation of ethylene biosynthesis via ACS6 phosphorylation. The Arabidopsis transcription factor ZAT10 is also a substrate for MPK6-mediated phosphorylation; both *in vitro* and *in vivo* [22]. *ZAT10* expression is strongly induced by low temperature [27,28], suggesting that it may have a role in cold response. Transactivation assays suggest that ZAT10 may function as a transcriptional repressor [27] and overexpression of *ZAT10* induces the expression of several oxidative-stress responsive genes [28]. Hence, it is tempting to speculate that ZAT10 could function in response to cold by repressing the transcription of a negative regulator of oxidative-stress responsive genes. Further experiments are needed to determine whether ZAT10 is involved in cold acclimation and, if so, to establish whether the phosphorylation of ZAT10 mediated by MPK6 takes place in response to low temperature and the significance of this post-translational modification in the development of cold acclimation response in Arabidopsis.

Another MAPK component, the MAP3K VH1-interacting kinase (VIK), has been related to cold acclimation in Arabidopsis [29]. Cold acclimation requires metabolic adjustments, including sugar allocation changes between vacuole and cytoplasm [30,31]. These changes might rely upon the VIK-dependent increase of tonoplast transporter TMT1 activity that occurs under low temperature conditions [29]. Several observations support this idea: i) TMT1 is a major contributor to vacuole import of glucose and fructose [30]; ii) VIK interacts with and phosphorylates TMT1 *in vitro* [29]; iii) vacuolar glucose import is activated in the presence of VIK [29]; iv) phosphorylation of TMT1 is enhanced by low temperature [31]. Nonetheless, there is no experimental evidence yet that TMT1 is phosphorylated by VIK *in planta* in response to low temperature and that this phosphorylation increases TMT1 activity and the capacity of Arabidopsis to cold acclimate.

Many developmental and environmental cues are transduced through changes in the levels of cytosolic free Ca^{2+} . Low temperature is not an exception, and specific Ca^{2+} signatures (i.e. specific spatiotemporal oscillations in Ca^{2+} levels) have been described upon cold exposure [32]. Among the proteins that sense Ca^{2+} levels and play a major role in Ca^{2+} -mediated signaling are the CDPKs. These proteins transduce the signal by eliciting a phosphorylation cascade after activation by Ca^{2+} binding [33]. Members of this subfamily of protein kinases have been implicated in plant responses to abiotic stress, including low temperature [34]. Expression analysis revealed that several genes encoding rice CDPKs are induced by low temperature [35]. Moreover, the over-expression of some of them, i.e. OsCDPK7/CPK13, in transgenic rice appears to enhance chilling tolerance [36], indicating that CDPKs have a function in cold response. CDPKs might also have an important role in cold acclimation. Thus, RARE COLD INDUCIBLE 1A (RCI1A) and ALCOHOL DEHYDROGENASE 1 (ADH1), two Arabidopsis proteins encoded by cold-inducible genes [37,38], have been identified as potential targets of CPK3 [39], an Arabidopsis CDPK whose activity increases under low temperature conditions [39]. The study of the precise role of CPK3 in cold acclimation, however, is yet to be addressed. On the other hand, abscisic acid (ABA) has long been recognized as an important component of cold acclimation signaling [40]. ABA mediates the expression of several cold-regulated genes [41,42] and its exogenous application at warm temperatures increases plant freezing tolerance [43]. Furthermore, ABA deficient mutants have decreased capacity to tolerate freezing before and after cold acclimation [44]. Interestingly, the expression of Arabidopsis *CPK4* is induced by cold [45] and CPK4 phosphorylates ABF1 [46], a b-ZIP transcription factor encoded by an ABA- and cold-inducible gene [47]. It is attractive to propose that ABF1 might participate in ABA-mediated cold acclimation, its function being regulated by CPK4. CIPKs are another family of Ca^{2+} -signaling transducers through their interaction with calcineurin-B-like (CBL) proteins. CBLs are Ca^{2+} -binding proteins that can associate with a CIPK partner in response to a rise in cytosolic free Ca^{2+} levels,

enhancing CIPK kinase activity and triggering a phosphorylation cascade [48]. Although the role of CIPKs in freezing tolerance has not been experimentally demonstrated yet, it may be inferred by the fact that CBL1 has been described to act as a negative regulator of freezing tolerance in Arabidopsis [49]. The specific CIPK partner that participates in this CBL1-mediated cold response, however, awaits to be uncovered.

RLKs also mediate cold acclimation. A null mutant for a gene encoding a plasma-membrane RLK, namely the Ca^{2+} /CaM-regulated receptor like kinase 1 (CRLK1), shows reduced cold-induced gene expression and reduced capacity to cold acclimate [50]. Interestingly, CRLK1 and MEKK1 interact *in planta* (Fig. 1) and the increase in MAPK activity that is produced in response to low temperature is dependent on CRLK1 [51].

In addition to the proteins targeted by the kinases discussed above (i.e., ACS6, ZAT10, and TMT1), other proteins having a well established function in cold acclimation are also post-translationally modified by phosphorylation. Unfortunately, however, the protein kinases involved in these modifications and the function of the phosphorylated proteins in cold acclimation has not yet been elucidated. One of these proteins is the photomorphogenic regulator HY5. This transcription factor accumulates upon exposure to low temperature and regulates the expression of approximately 10% of cold-regulated genes [52]. Consistent with these data, *hy5-1* mutant plants have a limited capacity to cold acclimate [52]. Thus, it was concluded that HY5 is a positive regulator of cold acclimation integrating low temperature and light signaling [52]. During photomorphogenesis, HY5 stability and binding affinity for its target promoters is regulated by phosphorylation [53]. Whether HY5 is also phosphorylated in response to low temperature and, if so, which aspects of its function in acclimation

are regulated by this post-translational modification are important questions that need to be answered.

Dehydrins are also proteins involved in cold acclimation that are post-translationally modified by phosphorylation. They accumulate in response to diverse abiotic stresses, including low temperature [54], and act as molecular chaperones, protecting nucleic acids, enzymes, cytoskeleton and membranes from stress-induced damage [54]. In addition, dehydrins can also chelate metal ions and, perhaps, buffer Ca^{2+} levels [54]. Dehydrins are phosphorylated *in vitro* [55] and this phosphorylation is enhanced by cold exposure [56]. Phosphorylation may regulate diverse aspects of dehydrin function, such as their ion binding properties [55] or membrane association capacities [57]. Thus, it is possible that phosphorylation regulates dehydrin function in cold acclimation.

As already mentioned, the phosphorylation level of a particular target protein depends on the balance of kinase and phosphatase activities acting on it. Therefore, dephosphorylation by phosphatases constitutes another important post-translational modification with regulatory implications on protein function. Phosphatases are classified by their substrate preferences as Ser/Thr, Tyr or dual specificity phosphatases. The Ser/Thr subclass has been associated with low temperature signaling and the regulation of cold stress response. The inhibition of Ser/Thr phosphatase activity is necessary for the induction of several cold-responsive genes in different species, including Arabidopsis, alfalfa, potato and tomato [16,58,59]. PP2A, one of the most prevalent Ser/Thr phosphatases, is the primary target for this cold-mediated inhibition [16,58,59]. In Arabidopsis, PP2A activity modulates the phosphorylation levels of ACS6 [60] and, therefore, ethylene biosynthesis [24,60]. Thus, available evidence suggests that PP2A may be a negative regulator of low temperature response, likely by regulating ethylene biosynthesis. The involvement of PP2A function in freezing tolerance and cold acclimation, however, is

yet to be analyzed. Other Ser/Thr phosphatases such as PP2CA, which functions as a negative regulator of ABA signaling, also have a relevant task in cold acclimation. Hence, silencing *PP2CA* expression in *Arabidopsis* reduces the duration of low-temperature exposure required for full development of cold acclimation [61]. This phenotype correlates with an earlier and higher induction of those cold-responsive genes whose upregulation by low temperature is mediated by ABA [61]. Since *PP2CA* expression is increased in response to low temperature, it was proposed that PP2CA acts as a negative feedback regulator during the development of ABA-mediated cold acclimation. The absence of *PP2CA* expression would suppress this negative feedback accelerating the development of cold acclimation [61]. Unfortunately, the proteins targeted by PP2CA during the development of cold acclimation have not yet been determined.

2.2 Ubiquitination

Ubiquitin is a small polypeptide often attached to a lysine residue in a number of target proteins. The ubiquitin polypeptide also contains lysine residues that can also act as substrates of further ubiquitin conjugation. The number of ubiquitin molecules attached to a target protein determines its fate. Polyubiquitinated proteins are usually targeted to degradation by the 26S proteasome [62]. Conversely, monoubiquitination affects the subcellular localization or activity of the target proteins [63]. Ubiquitination involves the concerted action of three enzymes. First, an E1 enzyme activates the ubiquitin molecule in an ATP-dependent fashion. Then, ubiquitin conjugates with an E2 enzyme that interacts with an E3-ubiquitin ligase enzyme, which provides a scaffold for the ubiquitination reaction and specificity to the whole process by interacting with the ubiquitination target. There are more than 1400 genes in the *Arabidopsis* genome encoding E3 ubiquitin ligases, illustrating the relevance of ubiquitination in regulating protein function [4]. E3 ubiquitin ligases can be classified in two groups defined by the presence of either Homology to E6-Associated Carboxyl Terminus (HECT) or Really

246 Interesting New Gene (RING) domains that are essential for the ubiquitination reaction [64]. Abiotic
247 stresses alter the expression of more than 500 E3-ubiquitin ligase genes [4], suggesting that
248 ubiquitination is a very important regulatory mechanism that facilitates adaptation to adverse
249 environmental conditions, including low temperature. Currently, only RING-finger ubiquitin ligases
250 have been implicated in cold acclimation, although it cannot be ruled out that HECT ubiquitin ligases
251 may also play an important role in the development of this adaptive response.

252
253 HOS1, a RING-finger E3 ubiquitin ligase from Arabidopsis, interacts with and ubiquitinates ICE1 [65]. It
254 was the first E3 ubiquitin ligase to be related to cold acclimation (Fig. 1). Overexpression of *HOS1*
255 decreases the capacity of Arabidopsis to cold acclimate [65]. The B-helix-loop-helix (bHLH) ICE1
256 transcription factor is a key regulator of the cold acclimation response. It activates the expression of
257 *CBF3*, another important transcription factor involved in cold acclimation, and controls the expression
258 of approximately 40% of cold-responsive genes [66]. Interestingly, ICE1 stability *in planta* decreases in
259 response to low temperature in a HOS1-dependent manner [65]. Hence, it was concluded that HOS1
260 participates in a negative feedback regulation of the cold acclimation process in Arabidopsis, mediating
261 ICE1 degradation after the onset of low temperature response [65]. COP1 is another Arabidopsis RING-
262 finger E3 ubiquitin ligase that participates in cold acclimation. It interacts with and ubiquitinates HY5,
263 which, as mentioned earlier, is also required for the complete development of cold acclimation [52],
264 regulating its degradation [67]. Under cold conditions, COP1 is depleted from the nucleus leading to
265 increased HY5 stability [52]. COP1, therefore, would act as a negative regulator of Arabidopsis cold
266 acclimation although the molecular mechanism by which low temperature controls COP1 activity has
267 not yet been identified.

CHIP is an Arabidopsis U-box (closely related to RING finger) E3 ubiquitin ligase with a function in cold signaling. *CHIP* gene expression is induced by low temperature, and *CHIP* overexpression results in increased sensitivity of Arabidopsis to chilling stress, as evidenced by a reduced growth and a high membrane damage in cold-exposed *CHIP*-overexpressing lines [68]. A PP2A A subunit was identified as a putative CHIP-interacting protein using a yeast-two-hybrid screen. *In vitro* experiments showed that CHIP ubiquitinates phosphatase PP2A A subunits [58]. Transgenic lines overexpressing *CHIP* showed no change in PP2A protein levels but a higher PP2A activity under cold conditions than wild-type plants [58], indicating that PP2A ubiquitination by CHIP increases PP2A activity rather than tagging this enzyme to degradation. As mentioned above, PP2A may be a negative regulator of cold response by controlling ethylene biosynthesis. Thus, it is possible that CHIP negatively modulates Arabidopsis response to low temperature through its role in ethylene biosynthesis via regulation of PP2A activity. Nevertheless, further research is needed to explore the impact that this regulation may have in cold acclimation.

FBP7 is an Arabidopsis F-box protein that participates in a multimeric RING-finger E3 ubiquitin ligase complex [69]. The expression of *FBP7* is induced by cold, and FBP7 activity is required for *de novo* cold-induced protein synthesis [69]. Interestingly, the FBP7 yeast homolog co-purifies with the elongation factor EF2 [69]. Therefore, it may be hypothesized that the Arabidopsis FBP7 would interact with the Arabidopsis EF2 homolog, known as LOS1. Although such interaction and its concomitant ubiquitination remain to be tested, it is worth pointing out that cold-induced protein synthesis is repressed in the Arabidopsis *los1-1* mutant in the same way as in the *fbp7* mutant [70]. Considering that LOS1 protein levels are not altered in the *fbp7* mutant [69], FBP7 would regulate other aspects of LOS1 such as its activity or subcellular localization. Since LOS1-mediated cold-induced protein synthesis is required for

proper cold acclimation [70], it can be conjectured that FBP7 activity has a relevant role in cold acclimation through its presumed regulation of LOS1 function.

Ubiquitination is a reversible post-translational modification [4] and, therefore, de-ubiquitination enzymes (ubiquitin proteases) are also involved in modulating protein function [71,72]. The participation of ubiquitin proteases in plant responses to abiotic stress, and more specifically in freezing tolerance and cold acclimation, however, remains to be explored.

2.3 SUMOylation

SUMOylation is another important type of post-translational modification in which SUMO, a small polypeptide very similar to ubiquitin, is also covalently bound to a lysine residue of a target protein. The conjugation of SUMO to a target protein takes place in a series of biochemical steps reminiscent of those of ubiquitination (see section 2.2). Thus, SUMOylation also requires the concerted action of three SUMO ligase enzymes, E1, E2 and E3. However, whereas a wide array of E3 ubiquitin ligases have been identified in plant cells, only a few of the equivalent E3 SUMO ligases seem to be encoded by plant genomes, which suggests that specificity may be determined at the target level [73,74]. As in the case of ubiquitination, SUMOylation provokes a conformational change in the target protein that may affect its interaction with other proteins, its subcellular localization or its stability [73,74]. Regulation of abiotic stress responses by SUMOylation was suggested by the observation that SUMO conjugates increase when plants are exposed to adverse environmental conditions [75], including low temperature [76]. SIZ1 is one of the three E3 SUMO ligases encoded by the Arabidopsis genome and is responsible for the augmentation in SUMO conjugates that takes place in response to low temperature. The loss of function of SIZ1 causes a reduction in both constitutive and cold-induced freezing tolerance, demonstrating that SIZ1 is a positive regulator of freezing tolerance and cold acclimation in Arabidopsis

[76]. SIZ1 SUMOylates ICE1, a key transcription factor in cold acclimation response (see section 2.1), both *in vitro* and *in vivo* [76]. *In vitro* experiments indicated that SUMOylation of ICE1 may prevent ICE1 ubiquitination, suggesting that ICE1 levels may be determined by a balance of SUMOylation and ubiquitination [76]. SUMOylation, as ubiquitination, is a reversible protein modification [75]. Consequently, de-SUMOylation can also have a relevant role in regulating protein function. In this way, Arabidopsis SUMO proteases ULP1c and ULP1d have been implicated in salt stress response [77]. A putative role for SUMO proteases in cold response, however, has not yet been reported.

Besides SUMO, four other ubiquitin-like protein modifiers, i.e. RUB, NEDD8, ATG8 and ATG12, have been identified in plants [73]. The significance of the conjugation of these ubiquitin-like modifiers in the regulation of plant stress responses awaits to be determined.

2.4 Protein lipid modifications

Lipid modifications are important modulators of protein function, often determining subcellular localization, protein-protein interactions and conformational changes [78]. Three types of protein lipid modifications have been observed: prenylation, S-acylation and N-myristoylation [78]. The relevance of plant lipid modifications in plant responses to abiotic stresses is exemplified by the phenotype displayed by the Arabidopsis *era1* mutant. *era1* plants lack farnesyl-transferase activity, have an enhanced response to ABA and are drought-tolerant [79]. Lipid modifications may also be relevant for the normal development of cold acclimation. As already discussed, CBLs mediate Ca²⁺ signaling under abiotic stress conditions and one of them, CBL1, acts as a negative regulator of cold acclimation [49]. CBL1 can be N-myristoylated and S-acylated, both lipid modifications being required for its plasma membrane localization [80]. Mutations blocking any of these modifications disrupt the role of CBL1 in the salt stress response [80]. Whether they also affect the capacity of Arabidopsis to cold acclimate has

not yet been tested. N-myristoylation is also required for tonoplast and plasma membrane localization of CPK3 [39], an Arabidopsis protein kinase that is activated by low temperature and can target proteins involved in cold response such as RCI1A and ADH1 [37,38] (see section 2.1). N-myristoylation, therefore, may be an important modulator of CPK3 function in cold acclimation.

2.5 N-Glycosylation

Many proteins localized at the endomembrane system or at the extracellular space are post-translationally modified by the addition of an oligosaccharide at an asparagine residue. This modification, termed N-glycosylation, takes place at the endoplasmatic reticulum and involves the concerted action of multiple enzymes [81]. N-glycosylation may modify protein stability, activity and subcellular localization [81]. The N-glycosylation pattern of 22 rice proteins is modified in response to low temperature [82]. Interestingly, among these proteins there is an enolase whose Arabidopsis homolog, named LOS2, is an important regulator of cold acclimation [83]. The *los2* mutation abolishes the low temperature-induction of an array of important cold-responsive genes and impairs cold acclimation [83]. LOS2 is a bifunctional enzyme with both enolase and transcriptional repressor activities [83]. It binds to the promoter of *ZAT10*, downregulating its expression levels [83]. As previously discussed, *ZAT10* is a cold-inducible gene that encodes a transcription factor implicated in regulating the expression of oxidative-stress responsive genes [28] (see section 2.1). Thus, it is possible that N-glycosylation may regulate the activity of LOS2 as a transcriptional repressor of *ZAT10*. Nevertheless, it remains to be demonstrated whether the N-glycosylation levels of LOS2 changes in response to low temperature.

3. Anticipated post-translational modifications that may regulate cold acclimation

There is experimental evidence suggesting the occurrence of yet uncharacterized post-translational mechanisms that control the function of key players in cold acclimation response. As described above, *ICE1* is an Arabidopsis bHLH transcription factor that binds to the MYC elements present in the *CBF3* promoter inducing its expression and, subsequently, the development of cold acclimation [84]. The *ICE1* gene is constitutively transcribed and translated under control temperature, but the ICE1 protein is not able to induce the expression of *CBF3* in these conditions [84]. Likewise, the overexpression of *ICE1* does not result in high levels of *CBF3* transcripts under control temperatures [84]. These observations suggest that, to be active, ICE1 requires additional regulation by unknown elements that are only present when plants are exposed to cold. ICE1 is both SUMOylated and ubiquitinated, but these post-translational modifications have been proposed to regulate ICE1 levels rather than activity [76] (see sections 2.1 and 2.3). Therefore, there should be another post-translational mechanism that enables ICE1 to activate *CBF3* expression under low temperature conditions. High-throughput proteomic analysis has identified ICE1 as a putative target of MAPK-mediated phosphorylation [21]. Alternatively, ICE1 might be transactivated in response to low temperature through its interaction with other transcription factors. Indeed, *in vitro* experiments showed that ICE1 interacts with the negative cold-response regulator MYB15 [85] (Fig. 1).

The increase of PP2A activity by CHIP ubiquitin ligase (see section 2.2) only takes place when plants are exposed to low temperature since overexpression of *CHIP* does not affect PP2A activity at warm temperatures [58]. This observation indicates that there should be additional yet unidentified post-translational mechanisms regulating CHIP activity that are induced by low temperature.

One of the earliest events during the development of cold acclimation is the increase of maltose levels in leaves, which contributes to protect the photosynthetic machinery [86–88]. Maltose may act either

as a compatible solute or as a substrate for carbohydrate accumulation [86,87]. The increase of maltose precedes any change in the transcript levels corresponding to the genes involved in maltose biosynthesis or degradation, suggesting that maltose accumulation could be regulated by post-translational mechanisms [87]. DPE2 is a cytosolic transglucosidase that mediates the conversion of maltose to glucose in Arabidopsis [89]. Recently, it was shown that DPE2 becomes inactivated by low temperature, allowing maltose accumulation, and that *dpe2* mutants display increased capacity to cold acclimate [90], indicating that DPE2 is a negative regulator of cold acclimation in Arabidopsis. Cold inactivation of DPE2 is very fast and correlates with a change in DPE2 subcellular localization [90]. The molecular mechanism governing the subcellular localization of DPE2 is still unknown. It is possible that cold signaling results in DPE2 being post-translationally modified, which could regulate its subcellular localization. The possibility, however, that a DPE2-interacting partner may determine the subcellular localization of DPE2 in response to low temperature cannot be discarded.

4. Conclusions and perspectives

The past decade brought significant progress in the elucidation of how cold acclimation is controlled at both transcriptional and post-transcriptional levels. However, a relative scarcity of data still exists about the post-translational mechanisms governing this adaptive response, hampering the progress of research in this area. For instance, although transcriptional profiling has revealed that cold acclimation involves major changes in the Arabidopsis transcriptome requiring the concerted action of a collection of transcription factors [66,91], the post-translational modifications that determine key functional aspects of these factors, including their promoter binding properties [53], transcriptional activity [92], cellular partitioning [93], turnover [53] and their ability to form dimers with other transcription factors [15], have not been sufficiently studied. ICE1 or HY5 are excellent examples of the complexity with which the function of transcription factors having essential roles in cold acclimation may be post-

translationally regulated. Elucidating the post-translational mechanisms controlling the function of key factors in cold acclimation should be a priority when studying the regulation of this adaptive response. In addition to transcription factors, other signaling components mediating cold acclimation, including Ca^{2+} sensors, kinases and enzymes involved in hormone biosynthesis, are also regulated at the post-translational level. In many cases, however, the significance of these post-translational modifications in cold acclimation awaits further study. Low temperature elicits the changes in the cellular metabolism that ultimately allow cold acclimation. Many of these metabolic changes are also regulated at the post-translational level. Both the cold-induced increase of TMT1 activity and the change in subcellular partitioning of DPE2 are good examples. Finally, many post-translational modifications are reversible, constituting very versatile mechanisms that allow plants to finely adjust their responses to environmental challenges including low temperature.

Despite the relevance of post-translational modifications for protein function, most of the modifications described in this review have been identified in functional studies of individual proteins. It has not been until recently that proteome-wide analyses of post-translational modifications related to low temperature response and cold acclimation have been reported [82,90]. The combination of results generated from large-scale proteomic analysis with detailed genetic studies of particular key effectors of cold acclimation should provide critical data to elucidate the molecular mechanisms that govern this adaptive response. Understanding the post-translational regulation of cold acclimation is essential to address further important tasks like the generation of new crops with improved cold tolerance.

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Figure legends

Figure 1: Multiple post-translational mechanisms overlap with transcriptional regulation of gene expression to fine-tune the development of cold acclimation response. After low temperature perception, cold stress is signaled by kinase-mediated pathways resulting in the phosphorylation of an array of transcription factors that are central to the transcriptome re-programming leading to cold acclimation. Further post-translational modifications, including protein ubiquitination, SUMOylation, N-Glycosylation and lipid modification, converge over these factors creating an intricate network of regulatory mechanisms. The modulation of ICE1 activity is a paradigmatic example of the complexity with which the function of transcription factors with important roles in cold acclimation may be post-translationally regulated. Low temperature elicits a MAPK pathway that culminates with the phosphorylation of ICE1. SUMOylation and ubiquitination also contribute to adjust ICE1 activity during the development of cold acclimation. In addition to transcription factors, other signaling components mediating this adaptive response are also regulated at the post-translational level. Thus, ACS6 is phosphorylated following MAPK activation, which results in ethylene biosynthesis that contributes to the hormonal regulation of cold acclimation. Solid lines represent established pathways while dashed lines represent theoretical pathways. Black arrows indicate transcriptional regulations and blue arrows represent post-translational regulations. Arrowheads denote positive regulation and endlines represent negative regulation.

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Figure 1