

Freezing tolerance revisited – effects of variable temperatures on gene regulation in temperate grasses and legumes

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Climate change creates new patterns of seasonal climate variation with higher temperatures, longer growth seasons and more variable winter climates. This is challenging the winter survival of perennial herbaceous plants. In this review, we focus on the effects of variable temperatures during autumn/winter/spring, and its interactions with light, on the development and maintenance of freezing tolerance. Cold temperatures induce changes at several organizational levels in the plant (cold acclimation), leading to the development of freezing tolerance, which can be reduced/lost during warm spells (deacclimation) in winters, and attained again during cold spells (reacclimation). We summarize how temperature interacts with components of the light regime (photoperiod, PSII excitation pressure, irradiance, and light quality) in determining changes in the transcriptome, proteome and metabolome.

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Introduction

The level of freezing tolerance in temperate perennial plants follows an annual cycle, determined largely by cold acclimation in autumn and deacclimation in spring. Cold acclimation is the exposure to low, nonfreezing temperatures, usually in autumn, which induces transcriptional, morphological and physiological changes resulting in the acquisition of freezing tolerance [1,2]. Freezing tolerance is generally at its peak in mid-winter and drops gradually towards spring. Upon exposure to warmer temperatures in spring, plants deacclimate and lose freezing tolerance. However, this can occur too early in spring or in response to warm spells in mid-winter, when there is still a risk of freezing temperatures [3,4]. Under some conditions,

plants have the ability to reacclimate if temperatures drop again. Cold acclimation, deacclimation and reacclimation are highly complex processes, and although temperature is the main driving force in many species, such as *Arabidopsis thaliana* and temperate grasses, other environmental and physiological factors like light, carbohydrate reserves and metabolism, drought, flooding, nutrient status and atmospheric CO₂ concentration, are interacting with temperature and are influencing these processes [4]. In particular, light conditions have important functions in coordinating freezing tolerance with growth and the seasonal variation in temperature.

The climate change projections predict that the growing seasons will be warmer and longer, especially at higher latitudes. This will affect cold acclimation, which will take place later in the autumn under shorter photoperiods and at lower light intensities [4]. The temperature is also predicted to become more variable. This will increase the frequency of warm spells during winter leading to more frequent acclimation and deacclimation cycles [3,4]. As an example, it has been demonstrated that deacclimation, measured as loss of freezing tolerance (LT₅₀), was less in timothy (*Phleum pratense* L.) than in perennial ryegrass (*Lolium perenne* L.), and that the loss of freezing tolerance increased with increasing deacclimation temperatures (3, 9 or 12 °C during 9 days) [5]. Deacclimation was most rapid in the most winter-hardy cultivar, which obtained the highest initial freezing tolerance.

In this review, we mainly focus on recent studies of low temperature responses in temperate grass species, with additional examples from dicots (perennial legumes and *A. thaliana*) when relevant. We will discuss: (1) interactions between temperature and light on the development of freezing tolerance; (2) interactions between vernalization and photoperiod on deacclimation and the ability to reacclimate; and (3) summarize studies of transcriptomic, proteomic and metabolomic responses to low temperature and release of low temperature. For the gene nomenclature of the main genes discussed in this review, see [Table 1](#).

Effects of interactions between temperature and light on freezing tolerance

Although temperature is a main driver of cold acclimation and deacclimation processes in perennial grasses, light also plays a key role in determining freezing tolerance. Light is the energy source for the accumulation of a range

Table 1**Gene nomenclature of the main genes discussed in this study**

Gene	Description
<i>VRN1</i>	Vernalization 1
<i>CBF</i>	C-repeat binding factor
<i>FT</i>	Flowering locus T
<i>FT1/VRN3</i>	Flowering locus T1/vernalization 3
<i>COR</i>	Cold regulated
<i>CR7</i>	Cold regulated 7
<i>1-SST</i>	Sucrose:sucrose 1-fructosyltransferase
<i>6-SFT</i>	Sucrose:fructan 6-fructosyltransferase
<i>GalS3</i>	Galactinol synthase 3

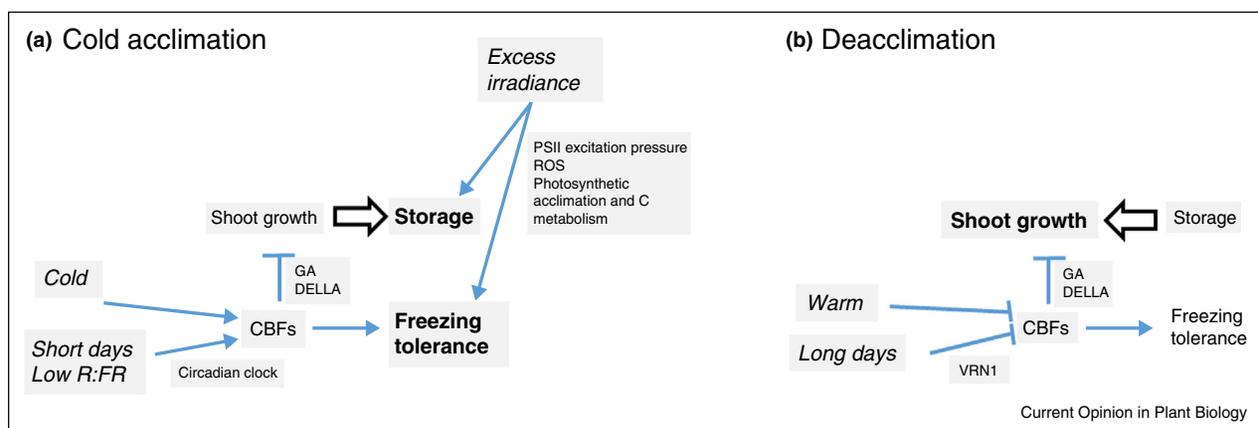
of carbohydrates and other substances that directly affect freezing tolerance as osmolytes and cryoprotectants [1]. In addition, irradiance, photoperiod and light quality elicit signalling processes, sometimes through interactions with temperature on photosynthesis and growth, leading to changes in gene expression and freezing tolerance.

In *A. thaliana*, cold-induced freezing tolerance is enhanced by short photoperiods and low red to far-red ratios (R:FR), effects that are mediated by the circadian clock through its control of expression of *CBF* transcription factors [6]. Cold temperatures can also act directly on the circadian clock by cold-induced alternative splicing of clock components [7]. Circadian clock genes are largely conserved across dicots and monocots, but some evolution of paralogous genes within monocots has occurred, and some differences in circadian control of physiological and developmental processes have been found [8]. Interestingly, the circadian network was found to be perturbed during cold acclimation in a southern-adapted genotype of perennial ryegrass, but not in a more freezing tolerant northern-adapted genotype [9].

Low temperature limits the rates of enzymatic reactions of photosynthesis more than the rates of electron transfer

reactions. As a result, a certain level of irradiance combined with low temperature creates an energy imbalance in the photosystems, leading to a change in the redox state of photosynthetic electron-transport components [10]. The altered photosystem redox state is thought to act as a cold sensor. As a result of the photosystem II (PSII) over-excitation, reactive oxygen species (ROS) are generated, and act in signalling pathways leading to expression of genes involved in freezing tolerance in winter cereals [11].

Different plant species have different strategies to counteract the energy imbalance in PSII arising under low temperatures with light. Contrary to many woody species, *A. thaliana*, *Brassica napus* and winter cereals have been shown to upregulate their photosynthetic carbon metabolism and maintain quantum yield of CO₂ assimilation [12]. This appears also to be a strategy employed by perennial grasses [13,14], which, combined with cessation of leaf elongation in favour of accumulation of photosynthate storages near meristematic tissues in or close to the ground, ensures energy for stress responses and early spring growth. In fact, in overwintering plants, leaf growth inhibition in response to low temperature is not simply a result of lower metabolic rates, but an actively regulated process, which is coordinated with changes in carbon metabolism [14]. In *A. thaliana*, CBFs can down-regulate growth by down-regulating the content of gibberellic acid, thereby allowing accumulation of DELLA proteins which inhibits growth, and in addition increases freezing tolerance by an unknown mechanism [15]. CBFs can also enhance photosynthetic capacity in *A. thaliana* and *B. napus*, and have been proposed as master-regulators of leaf growth, photosynthetic capacity and freezing tolerance in response to temperature conditions [16]. PSII excitation pressure and circadian control of CBFs appears to account for several layers of interactions between light and temperature affecting these physiological and developmental processes (Figure 1a).

Figure 1

Model for regulation of freezing tolerance and allocation of organic reserves between shoot growth and storage during (a) cold acclimation and (b) deacclimation in *Arabidopsis thaliana*, cereals and perennial grasses. The open arrows indicate allocation of photosynthates.

As mentioned earlier, the expected longer growth seasons with increasing autumn temperatures following climate change, may delay growth cessation, affect acclimation of the photosynthetic apparatus and reduce freezing tolerance of perennial herbaceous plants. This has been investigated in populations of timothy, perennial ryegrass and red clover with different degrees of 'northern' adaptations [17]. The results showed that a rise in autumn temperature decreased both cold acclimation capacity and photoacclimation in these species. As a result, the freezing tolerance was reduced, and reduced significantly more in northern-adapted than in southern-adapted populations of the grass species. Interestingly, the red clover (*Trifolium pratensis*) populations were less affected by temperature changes in the autumn than the grasses.

Effects of interactions between vernalization and photoperiod on deacclimation and the ability to reacclimate

Interactions between temperature and light are likely to be as important during deacclimation as they are during cold acclimation. Deacclimation has been much less studied than cold acclimation, but the relationship between vernalization (the process of becoming competent to flower after a prolonged period of cold) and freezing tolerance in cereals has received some attention. In these species, it has been shown that freezing tolerance and expression of genes involved in freezing tolerance are down-regulated in leaf and stem base tissue when the vernalization requirement is saturated, but before any development of the apex is visible in the microscope [18,19]. There is an interaction between vernalization and photoperiod on this deacclimation and on the ability to reacclimate. In cultivars with a long day requirement for flowering, the negative effect of vernalization on freezing tolerance is stronger when plants are vernalized under long days than under short days, whereas vernalization-insensitive and photoperiod-insensitive cultivars are not able to develop much freezing tolerance at all [20,21]. Also, plants vernalized and deacclimated under long days are less able to reacclimate [22]. The model developed for the interactions between the circadian clock and CBF activity on freezing tolerance and growth described above presents possible explanations for the role of photoperiod in deacclimation and reacclimation in winter cereals (Figure 1b).

VRN1, an inducer of the transition to generative development in cereals and other temperate grass species is gradually upregulated during vernalization [23]. In long days, *VRN1* interacts with *FT1/VRN3* in the induction of flowering. Several studies indicate associations between the expression of *VRN1* and *FT1*, and expression of cold-regulated genes and freezing tolerance. Using near-isogenic lines of *Triticum aestivum* and *Hordeum vulgare* and a *T. monococcum* deletion mutant, it has been shown that, under 16 h photoperiod, the *VRN1* (all species) and *FT1* (*H. vulgare*) loci control expression of *VRN1*, *COR14B*

(a target of CBF transcription factors) and other cold regulated-genes [19,21,24]. In these studies high levels of *VRN1* expression, whether vernalization-induced for winter alleles or long day-induced for spring alleles, was associated with down-regulation of the cold-regulated genes and freezing tolerance. Spring alleles of *FT* were also associated with higher expression of *VRN1*, lower expression of *COR14B* and lower freezing tolerance in *H. vulgare* [24]. From these studies it is not entirely clear whether it is *VRN1* and *FT1* themselves, or very closely linked genes, that are responsible. However, using a transgenic approach combined with chromatin immunoprecipitation sequencing and RNA sequencing, Deng *et al.* [25] showed that in barley grown at 16 h photoperiod, *VRN1* binds to the promoter of several *CBF* genes and down-regulates *CBF1* (while *VRN1* was shown to be a direct inducer of *FT*). After short-term cold exposure, when the expression level of *VRN1* is still very low, Oliver *et al.* [26] found similar kinetics in the initial transcription of *VRN1* and *COR14B* upon cold exposure (24 h) in *H. vulgare*. Under short photoperiods, the positive correlation between expression of *VRN1* and *COR14B* remained after long-term cold treatment in *T. monococcum* [21] and *Festuca pratensis* [27^{*}]. Oliver *et al.* [26] suggested that *VRN1* and *COR14B* might be regulated by similar mechanisms in early cold acclimation, possibly through the action of CBF transcription factors. In a study of genetic material of *F. pratensis* [27^{*}] grown at 8 h photoperiod, we found positive correlation between expression of *VRN1*, *CBF6* and *COR14B*, both after 2 weeks of cold acclimation and after 2 weeks of reacclimation of vernalized and deacclimated plants, but not in the vernalized plants before deacclimation and reacclimation. Moreover, several studies show that *CBF6* and *COR14B* are down-regulated by prolonged cold, but only under long photoperiods (see discussion in Ref. [27^{*}]). Taken together, these results suggest that *VRN1* and *CBFs* are co-regulated during early cold acclimation of temperate grasses, but that *VRN1* down-regulates *CBFs* as vernalization proceeds and photoperiods increase. This interaction between vernalization and photoperiod may be mediated by the circadian clock.

Transcriptome responses during cold acclimation, deacclimation and reacclimation

Several studies on transcriptome responses during cold acclimation have been conducted in temperate grasses [28^{**},29–31], while few studies have attempted to understand the molecular changes during deacclimation and reacclimation [27^{*},32^{**},33^{**}].

Induction of genes involved in cell wall and carbohydrate metabolism, redox homeostasis during cold acclimation is important for the acquisition of freezing tolerance in temperate grasses [28^{**},34]. Families of transcription factors that are induced during cold acclimation (MYB, bZIP, AP2/EREBP, WRKY, and NAC) are likely to play

important roles in coordinating genes involved in protective mechanisms [35–37]. Transcriptome analysis of cold acclimated wheat plants revealed 68 genes, including CBF, WRKY and zinc-finger transcription factors, being more than fivefold upregulated by freezing stress [38]. Freezing tolerance is induced by cold acclimation (exposure to low, non-freezing temperatures) and sub-zero acclimation (exposure to temperatures slightly below zero) in most temperate plant species [39]. Sub-zero acclimation is quite distinct from cold acclimation in terms of gene expression. *Le et al.* [39] showed that genes encoding proteins with a putative role in cell wall biosynthesis, for example cellulose-synthase like D2, xyloglucan endotransglucosylase/hydrolase 19, and a glycosyl hydrolase superfamily protein, and genes encoding transcription factors like Myb domain protein 108, Dof-type zinc finger DNA-binding family protein and integrase-type DNA-binding superfamily protein, were upregulated in *A. thaliana* when exposed to sub-zero temperatures. The early responsive to dehydration (ERD) protein-related gene, belonging to the CBF regulon, seems to play a role by increasing freezing tolerance only during cold acclimation [39]. A large number of genes encoding phosphatases and kinases were also differentially expressed during cold acclimation in perennial ryegrass [28**]. Previous studies have shown the up-regulation of *1-SST* and *6-SFT* promoter-driven reporter genes by a R2R3 MYB transcription factor from wheat [40], suggesting that MYB transcription factors might play an important role in coordinating synthesis and accumulation of fructans. Fructans, which are water-soluble fructosyl polysaccharides, accumulate in the vacuoles and in storage tissues of temperate grasses during cold acclimation. Fructans are long-term reserve carbohydrates, which also functions as osmolytes and cryoprotectants, enabling plants to survive the winter period [41,42].

As deacclimation progresses, the changes in the transcriptome that occur in *A. thaliana* during acclimation disappear [33**]. Cell wall related genes such as the arabinogalactan protein gene and the photosynthesis related gene encoding D2 subunits of the photosystem II complex, which are suppressed during cold acclimation, were revived once deacclimation proceeded. Zuther *et al.* [43**] characterized the responses of natural accessions of *A. thaliana* that vary widely in their freezing tolerance, to deacclimation conditions. This study showed that the transcript levels of selected cold-induced genes declined sharply over three days in all accessions after transfer of cold acclimated plants to ambient temperatures, while freezing tolerance only declined in freezing tolerant accessions. Some correlations between freezing tolerance and the expression levels of *COR* genes, as well as many correlations among transcript and soluble sugar levels, that were highly significant in cold acclimated plants, were lost during deacclimation [43**], while other correlations persisted. For example,

transcript levels of *COR6.6* and *COR15A* were no longer correlated with LT50 after 3 d of deacclimation, while transcript levels of *COR47* and *COR78* remained correlated. A strongly reduced expression of the cold induced genes *COR15A*, *COR15B*, *COR47* and *GoIS3* during deacclimation was also reported for Col-0 from microarray hybridization experiments [44]. All three dehydrins investigated in blueberry [45], and two investigated each in canola and wheat [32**] showed a strong reduction in their abundance during deacclimation. However, in two grass species only one of the two detected dehydrins showed this behavior, while the other clearly induced during deacclimation [46]. In addition, the content of cold induced ice recrystallization inhibition proteins (IRIPs) decreased strongly in perennial grasses during deacclimation [27*,47]. In a study of a set of seven cold-induced genes during a prolonged cold acclimation–deacclimation–reacclimation cycle in *F. pratensis*, *CR7* was the only gene whose expression was significantly upregulated and correlated with freezing tolerance after reacclimation, suggesting a particular role of this gene in reacclimation [27*]. Byun *et al.* [33**] showed that the genes that were differentially expressed between *A. thaliana* accessions exclusively during reacclimation were Dof-type zinc finger domain-containing protein, lipid transfer protein 3 (LTP3), protein phosphatase 2C, photosystem I reaction center subunit II, and DREB2B.

Proteomic and metabolic responses to low temperature

Strong gene expression, resulting in abundant mRNA, does not mean that the corresponding protein is also abundant in the plant cells during freezing. Hence, proteomics (study of the proteome), is important because proteins represent the actual functional molecules in the plant cells. Recent studies [48*] on proteome analysis of freezing tolerance in red clover (*T. pratense*) found strong increases of stress response proteins and carbohydrate and energy metabolism proteins in response to cold acclimation. Several key proteins like KS-dehydrins, cold responsive vegetative storage proteins (VSP), and a cold acclimation specific (CAS) protein were upregulated (up to 30-fold abundance) in response to cold acclimation and seems to be of major importance for the acquisition of freezing tolerance in red clover. Proteins like alpha-tubulin, sucrose synthase, methionine synthase down regulated in response to cold acclimation [48*].

A close relationship between the accumulation of dehydrins and the acquisition of freezing tolerance has been demonstrated in white clover (*Trifolium repens*), annual bluegrass (*Poa annua*), and creeping bentgrass (*Agrostis stolonifera*) [46,49]. In alfalfa, the presence of specific dehydrin variants has been linked to superior freezing tolerance levels [50]. Multiple roles has been attributed to dehydrins, including membrane and protein stabilization in freeze desiccated cells, cryoprotection and ROS

scavenging [51]. The accumulation of cold acclimation specific (CAS) transcripts and proteins has been previously related to freezing tolerance in alfalfa (*Medicago sativa*) [52]. Several metabolites are known to contribute to freezing tolerance, including amino acids, soluble sugars, polyamines and polyols [53]. Among them, particular focus has recently been directed towards understanding the multifunctional role of soluble sugars in enhancing cold tolerance [54]. Fructans, soluble fructosyl polysaccharides, are storage carbohydrates in a large number of higher plants. Fructans accumulate in perennial grasses as a long-term reserve carbohydrate to survive the winter period [41,42]. Hisano *et al.* [41] overexpressed two wheat fructosyltransferase genes encoding sucrose–fructan 6-fructosyltransferase (6-SFT) and sucrose–sucrose 1-fructosyltransferase (1-SST) in perennial ryegrass (*L. perenne*) and showed that this lead to increased accumulation of fructans and improved freezing tolerance at the cellular level. Recent studies on fructan metabolism during cold acclimation in perennial ryegrass [28**] indicate that accumulation of high-DP fructan in roots is an adaptive trait for plant recovery during cold. Also, fructan accumulation levels were found to be higher in synthetic wheat lines with high freezing tolerance, compared to the low freezing tolerance lines [30]. Correlations between freezing tolerance and the content of fructose, glucose and sucrose that were highly significant in cold acclimated plants were lost during deacclimation process [43**].

Conclusions

Freezing tolerance is an extremely important and complex trait with huge economic impact in overwintering crop plants. Molecular and physiological responses during cold acclimation, and their correlation with freezing tolerance, have been studied quite extensively in many crop plant species and in the model *A. thaliana*. In general, low temperature alter gene expression of a very large number of genes. Some transcriptional responses are common in most species, for example induced expression of *COR*-genes, *CBF*-genes and dehydrins. On the contrary, rather little research has been performed to understand these responses during deacclimation and reacclimation. It seems clear that the ability to reacclimate varies between species and genotypes, and are influenced by environmental and developmental factors. Temperate grasses and legumes are economically very important crop species, and in view of the need to adapt varieties to a more unstable winter climate, more research need to be performed in these species to understand deacclimation resistance and reacclimation capacity. It is challenging to elucidate generic responses due to environmental and genotypic effects, and their interactions. Plant developmental stages, tissue type, and various aspects of the light conditions need to be considered in much more detail when designing experiments, and for critical evaluation of cold acclimation, deacclimation and reacclimation responses affecting freezing tolerance.

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This study demonstrated the capacity for substantial cold acclimation in red clover. They found stress-response proteins (including vegetative storage proteins, dehydrins, and ROS scavenging enzymes), and carbohydrate and energy metabolism proteins increased the most in response to cold acclimation.