



## Review

# Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates

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## ARTICLE INFO

## Article history:

Received 10 March 2010

Received in revised form 13 July 2010

Accepted 17 July 2010

Available online 27 July 2010

## Keywords:

Cold acclimation

Frost tolerance

CBF

Photosynthetic acclimation

AFP

Fructans

## ABSTRACT

We review recent progress in understanding cold and freezing stress responses in forage grass species, notably *Lolium* and *Festuca* species. The chromosomal positions of important frost tolerance and winter survival QTLs on *Festuca* and *Lolium* chromosomes 4 and 5 are most likely orthologs of QTLs on *Triticeae* chromosome 5 which correspond to a cluster of CBF-genes and the major vernalization gene. Gene expression and protein accumulation analyses after cold acclimation shed light on general responses to cold stress. These responses involve modulation of transcription levels of genes encoding proteins involved in cell signalling, cellular transport and proteins associated with the cell membrane. Also, abundance levels of proteins directly involved in photosynthesis were found to be different between genotypes of differing frost tolerance levels, stressing the importance of the link between the function of the photosynthetic apparatus under cold stress and frost tolerance levels. The significance of the ability to undergo photosynthetic acclimation and avoid photoinhibition is also evident from numerous studies in forage grasses. Other interesting candidate mechanisms for freezing tolerance in forage grasses are molecular responses to cold stress which have evolved after the divergence of temperate grasses. This includes metabolic machinery for synthesis of fructans and novel ice-binding proteins.

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## 1. Introduction

Plants in cold climates are frequently exposed to sub-zero temperatures in the autumn, winter, and spring seasons. Exposure to sub-zero temperatures requires a battery of molecular and physiological adaptations to minimize frost related injuries which potentially can be fatal. Cold acclimation (CA) is a process whereby plants in response to low but non-freezing temperatures undergo

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a range of biological changes in order to increase their frost tolerance (FT) and prepare for the winter season [1]. The process of CA encompasses biological modifications on many levels, e.g. modulation of gene expression levels [2], accumulation and degradation of proteins [3–5] changes in sugar content [6], and changes in the photosynthetic machinery [7].

A large part of the research concerning plant cold stress response and FT has been carried out using the model species, *Arabidopsis thaliana* (named herein as *Arabidopsis*) and *Oryza sativa* (rice) [1]. As a consequence, the research on low-temperature stress responses in non-model species has focussed on genetic mechanisms, which originally were identified in the model species and have been conserved between plant lineages over hundreds of millions of years. In many cases a direct inference of gene function based on homology between model dicot plants and agriculturally important species is elusive. Moreover, neither *Arabidopsis* nor rice is adapted to a perennial life in extreme winter climates. This is important because adaptation to a perennial life history in harsh winter climates must have required changes at the genetic level which cannot be studied using an annual model species. Hence if we only use model plant species to investigate the molecular basis of cold and frost stress response this might provide limited insights into the genetic mechanisms underlying these traits in important agricultural species.

The *Pooideae* grasses (temperate grasses) is a large and economically important sub-family including cereals (*Triticeae* tribe) and forage grasses (*Poaceae* tribe). Divergence of temperate grasses from the most recent common ancestor shared with rice is thought to have happened ~46–42 million years ago (Mya) [8,9]. Parallel to the origin and early evolution of the *Pooideae* group the global climate became gradually cooler [10]. As opposed to rice, which is adapted to warm and humid environments, *Pooideae* grasses radiated in cooler environments [11]. This is reflected by the present distribution of *Pooideae* species which is extremely skewed towards cooler environments [12]. Thus evolution of cold and frost stress responses, either through fine tuning of ancient abiotic stress responses or evolution of novel adaptations to cold environments must have been central for the *Pooideae* sub-family.

The evolutionary history of temperate grasses makes them an excellent model system for studying adaptations to cold and frost stress. During the last decade several research groups have focussed their research on understanding the cold and freezing stress responses in forage grass species (*Poaceae* tribe), mainly *Lolium* and *Festuca* species, and recently also *Phleum pratense* L. Species of the *Poaceae* tribe are excellent models for plant adaptations to cold environment because of their adaptation to habitats in the northernmost part of the Northern hemisphere, i.e. the circumpolar arctic region. The research on the *Poaceae* species includes mapping of quantitative trait loci (QTL), transcriptomics, proteomics, and functional gene studies, and plant physiology research of low-temperature (CA) response and FT. We do not intend for this review to serve as an elaborate review of every aspect of CA and FT but aim to summarize the progress in CA and FT research on forage grasses from the past decade. We will start by highlighting results from studies at the -omics level, and then focus on three specific mechanisms involved in CA and FT where research performed on forage grasses has made significant contributions; (1) photoinhibition, (2) ice interacting proteins, and (3) fructan synthesis.

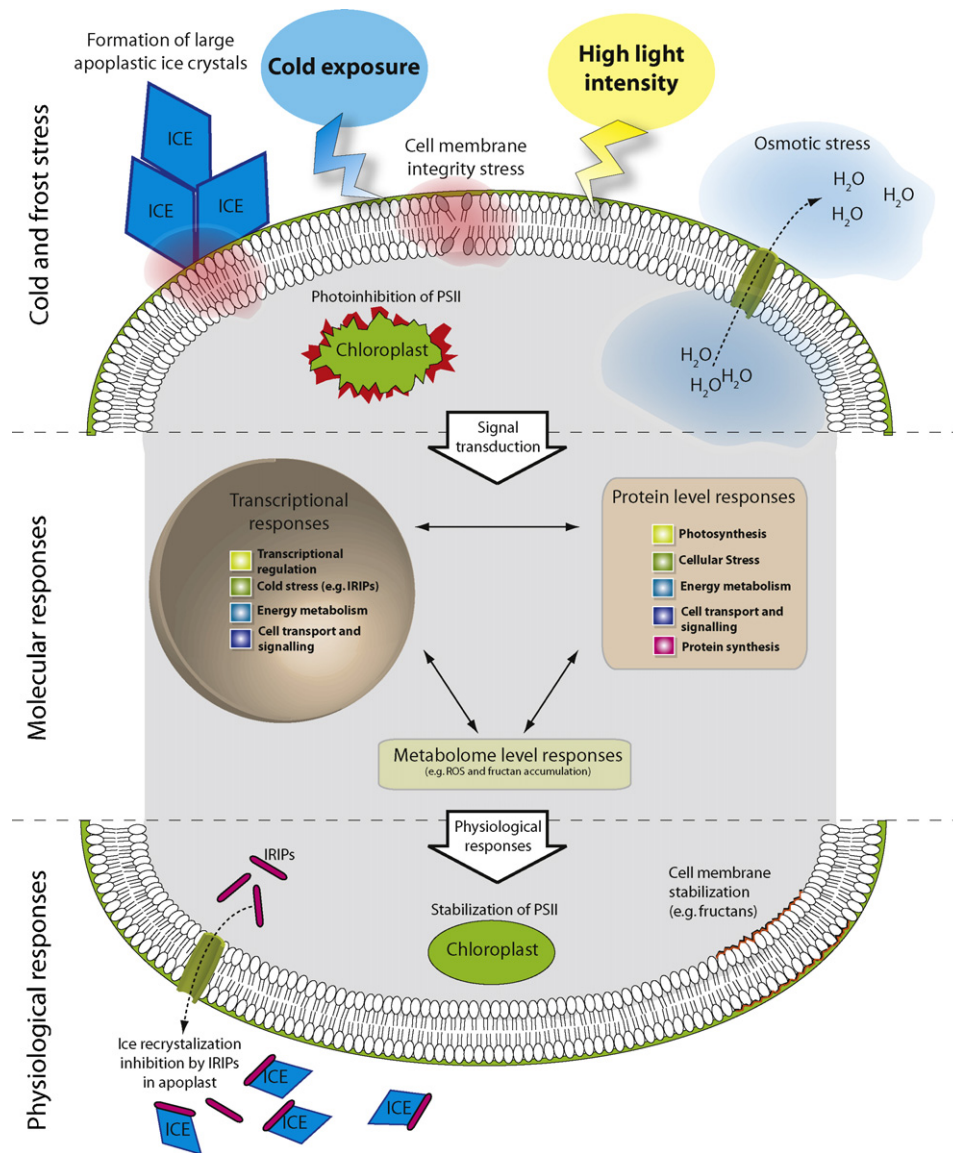
## 2. QTL mapping and genomics

Winter survival (WS) is a very complex trait determined by combinations of frost, desiccation, water logging, ice-encasement, anoxia, and snow cover. However, FT is the single component that generally explains most of the variation in WS [13]. There are few reports of Quantitative Trait Locus (QTL) mapping of FT and WS in forage grass species. Mapping of frost and drought tolerance QTLs

using regrowth tests, and WS QTLs based on field survival, in the 'B14/16 × HF2/7' full-sib family of meadow fescue (*Festuca pratensis* Huds.) was reported by Alm [14] and Alm et al. unpublished results. Major QTLs for FT/WS were located on chromosomes 1F, 2F, 5F and 6F, and for drought tolerance traits on chromosomes 1F, 3F, 4F, and 5F. In many cases the QTLs co-located with genes involved in abiotic stress tolerance, e.g. orthologs of barley *Dhn* (dehydrin) genes. QTLs for several stress tolerance traits mapped to the same regions on *Festuca* chromosomes 1F, 4F and 5F. Frost and drought both induce cell dehydration which induces many stress-responsive genes via the DREB1A/CBF-transcription factors [1,15] (Fig. 1). Co-location of WS QTLs with drought tolerance QTLs indicates that dehydration is the major factor behind these QTLs, while co-location with FT QTLs indicate frost tolerance as the main factor. WS QTLs not co-located with any component stress factors are most probably caused by genes affecting seasonal adaptation, e.g. photoperiodic sensitivity. Coincidence of QTLs provided thus information about the roles of frost and dehydration in the genetic control of WS, and illustrates the advantage of mapping several stress tolerance traits in the same family. Two FT/WS QTLs on chromosome 5F most likely correspond to *Fr-A1* [16] and *Fr-A2* [17] on wheat homoeologous group 5A, while a small QTL for FT on chromosome 4F, located at the position of *FpVRN1*, an ortholog of the wheat *VRN1* gene [18], most likely is a pleiotropic effects of vernalization and/or a photoperiodic genes as in wheat [19,20].

A single QTL for electrical conductivity (i.e. an indirect measurement for FT) was detected on linkage group (LG) 4 in the p150/112 mapping population of perennial ryegrass (*Lolium perenne*) [21]. Comparison with QTLs on *Triticeae* homoeologous chromosomes 4 was rather inconclusive due to lack of common markers, but the authors discussed whether the location corresponded to the vernalization/FT (*VRN1/Fr1*) region on homoeologous chromosomes 5 of wheat [16]. No QTL for WS was detected in this family. Identification of QTLs controlling FT and WS in an annual (*Lolium multiflorum*) × perennial (*L. perenne*) ryegrass interspecific hybrid population was reported by Xiong et al. [22]. They measured FT by ion leakage and electrical conductivity following natural cold acclimation in the field in two consecutive years. The two methods gave very similar results. WS was recorded by visual scoring in the field in two springs. Two QTLs associated with WS on linkage groups 4 and 5, and one QTL for FT on LG 5 were consistently detected over several years and different maps (male and female). They proposed that the QTL for WS on LG 4 could correspond to the QTL for electrical conductivity reported by Yamada et al. [21] and might be caused by the action of an ortholog of the vernalization gene of wheat and barley. The other consistent and overlapping QTLs detected for WS and FT on LG 5 by Xiong et al. [22] were proposed to be orthologous to the QTL for FT detected in a homoeologous position in wheat and barley chromosomes 5, similar to the situation in *Festuca*.

Twenty CBF-genes have been identified in barley (*Hordeum vulgare*), of which 11 are found in two tight tandem clusters on the long arm of chromosome 5H in the same region as the *Fr-H2* frost resistance locus [23,24]. An orthologous genomic region in *Triticum monococcum* contains similar CBF gene clusters located at the *Fr-A<sup>m</sup>2* frost resistance QTL [17,25]. In *Lolium perenne* five *LpCBF* genes were identified of which four mapped tightly on LG5 in a position orthologous to the CBF-clusters in wheat and barley [26]. One CBF gene (*FpCBF6*), which is induced and has peak expression 2 h after start of cold acclimation, has been identified in meadow fescue [Alm et al., unpublished results]. Based on phylogenetic analysis, *FpCBF6* was placed in the subclade CBF3-3a [27] together with *OsDREB1A/CBF3*, *HvCBF6*, *TaCBF6*, *LpCBF3* and *FaDREB1A*. It maps on meadow fescue chromosome 5F and co-locate within the major FT/WS QTL that most likely is orthologous to the *Fr-H2* and *Fr-A<sup>m</sup>2* frost resistance QTLs in barley and wheat, respectively [Alm et al., unpublished results].



**Fig. 1.** Summary model of molecular mechanisms important for frost tolerance in perennial grasses. The figure does not depict all mechanisms involved in frost tolerance but those discussed in this review. (1) *Top panel:* cold temperatures in combination with high light intensity result in various stresses and physiological responses. Ice formation in apoplast can lead to direct physical stress on the cell membrane but also osmotic stress due to incorporation of water molecules into growing ice crystals. High light intensity combined with low temperature during autumn and early winter trigger photoinhibition of the chloroplast. (2) *Middle panel:* molecular responses to cold stress during autumn and early winter encompass large scale modulations of gene expression levels and abundance of proteins responsible for further transcriptional regulation and signalling (e.g. CBFs), energy metabolism and modification of the photosynthetic machinery, and proteins or enzymes that directly or indirectly help stabilize cell membrane integrity (e.g. IRIPs and fructosyl transferases). (3) *Lower panel:* known and presumed physiological responses to avoid cellular destruction during cold and freezing stress, and secure survival of perennial grasses through the winter.

The coincident location of several of the QTLs in *Festuca* and *Lolium* with QTLs and genes in *Triticeae* species indicate the action of structural or regulatory genes that are conserved across evolutionarily distant species [28]. In this respect CBF-transcription factors and dehydrin genes regulating the expression of cold and drought regulated genes, and the vernalization response genes appear to play decisive roles. The major structural difference between the *Festuca/Lolium* and *Triticeae* homoeologous chromosomes 4 and 5 [29,30] is especially interesting in comparative genomics of frost tolerance. The major vernalization gene *VRN1* and the FT QTLs/CBF-gene clusters are both located on homoeologous chromosomes 5 in *Triticeae*, while in *Festuca/Lolium* they are on different chromosomes, i.e. (*VRN1*) on chromosome 4 and (FT QTLs/CBF-genes) on chromosome 5. This makes it feasible to separate the indirect effects of the major vernalization gene on CA and FT from the effects of

genes on chromosome 5 directly involved in developing frost tolerance.

### 3. Transcriptomics of cold acclimation and frost tolerance

Comprehensive research on transcriptional modulation during CA has been carried out in *Arabidopsis* using different microarray technologies and different statistical criteria. These studies have estimated the number of *Arabidopsis* genes being regulated by CA to be in the order of 2–13% [31–34]. Recently, CA transcriptional responses have also been investigated in cold tolerant Poideae species such as winter and spring wheat (*Triticum aestivum*) cultivars [35,36], barley (*H. vulgare*) [37], *L. perenne* [38], and *F. pratensis* [39]. Only two of these studies [36,37] surveyed the entire genome and the results showed that Poideae grasses have a comparable

number (~9–12%) of cold regulated genes to that of *Arabidopsis*. However, these two studies were carried out on cereal species (wheat and barley) hence we can only assume that this global picture of the amount of transcriptional changes during CA is similar in forage grasses. The overall picture of the type of transcriptional changes occurring during CA in Pooideae grasses is also very similar to what has been found in *Arabidopsis* (see above mentioned references). Genes involved in signaling, regulation of transcription, cellular transport, cell membrane, and genes with putative protective roles during freezing-related stress are up-regulated, while genes involved in metabolism, respiration and photosynthesis are down-regulated [35–39].

More interestingly is therefore what we have learned from studying CA transcriptional responses in Pooideae grasses that is different or novel compared to the *Arabidopsis* studies. One aspect of CA transcriptional response which cannot be studied in *Arabidopsis* is the difference in cold regulated genes between over-wintering perennial crown tissue and the annual leaf tissue. In wheat strikingly little overlap was observed (1–10%) between genes which underwent transcriptional changes in leaf compared to crown tissue during CA [36]. A similar trend is also evident in forage grasses. For example, genes involved in photosynthesis is common in CA-EST libraries from leaves [38] but almost absent in crown tissue [39]. This does not imply that CA processes in leaves are not important for winter survival of perennial tissues, in fact leaves are very important for production of CA-associated metabolites such as sugars; it simply means that understanding biologically relevant transcriptional responses to cold stress in agriculturally important Pooideae grasses is difficult if the focus is on annual non-grass model plants only. Another important lesson from studying CA transcriptomes of Pooideae grasses is the abundance of Pooideae-specific cold regulated genes. This includes for example genes encoding novel ice interacting proteins and novel enzymes involved in sugar metabolism [37–39] (treated in greater detail in the last part of this review).

In *Arabidopsis* the genetic basis for differences in frost tolerance has been investigated by studying variation in transcriptional response during CA using inbred mutant lines [33]. This type of experiment is unfortunately not feasible in Pooideae forage grasses due to their self-incompatibility and outbreeding nature. However in Pooideae cereals, where inbreeding is possible, such experiments have been carried out. chloroplast development pathway mutants, Svensson et al. demonstrated that only 11% of all cold responsive genes maintained normal regulation during CA in plants with non-functioning chloroplasts [37]. This emphasizes the importance of photosynthesis-related molecular mechanisms during CA and acquirement of frost tolerance in Pooideae grasses.

An alternative to experiments with inbred mutant genotypes is to study genetic differences between genotypes selected for divergent frost tolerance, and this has been carried out in forage grasses. By analyzing contrasting gene expression patterns after long-term CA (19 days) between high frost tolerant (HFT) and low-frost tolerant (LFT) genotypes of *F. pratensis*, Rudi et al. [39] identified potential candidate genes underlying the differences in frost tolerance. Surprisingly only 7 (1.3%) of the genes studied were >2-fold differentially expressed between the HFT and LFT genotypes. This result could imply that the difference in FT levels is not causally linked to large global variation in transcriptional responses during CA but instead determined by either (i) transcriptional differences in a few important genes or (ii) post-transcriptional and post-translational mechanisms. However we must stress that Rudi et al. [39] studied transcription differences after 19 days of CA; hence variation in early transient transcriptional regulation would not be detected in this study.

Three out of the seven genes found to differ in expression between high- and low-frost tolerant genotypes had significant

blast results to a tumor-suppressor protein, a phosphate/phosphate translocator, and a protein disulfide isomerase [39]. Based only on homology these three genes are putatively involved in regulation of translation, oxidative protein folding and metabolic regulation, respectively. There are evidence from other studies to support the involvement of the tumor-repressor homolog and the phosphate/phosphate translocator homolog in freezing stress. Recently, the tumor-suppressor protein homolog has been cloned in *Caragana jubata*, a temperate plant that grows under extreme cold and at higher altitudes in Himalaya, and this gene was shown to be up-regulated after low-temperature treatments [40]. Further, sucrose flux through sucrose biosynthesis has been shown to modify development of freezing tolerance in *Arabidopsis* [41]. Because phosphate/phosphate translocators play a vital role in the sucrose biosynthesis pathway, increased expression of this gene would be expected to influence sucrose metabolism and therefore possibly also frost tolerance. This assumes of course that the same or a similar molecular mechanism is conserved in monocot grasses.

#### 4. Frost tolerance and proteomics

Even though there is a hierarchical, and to some extent deterministic, relationship between the transcriptome and the proteome response, gene expression levels and protein levels are in many cases not strictly correlated (e.g. [42]). Hence, genome-wide transcription analyses do not offer the complete picture of plant molecular responses during CA and cold stress. It is therefore important to complement gene expression studies with studies of proteomic changes under CA and investigate how changes in protein abundance during CA correlate with differences in FT.

Many CA-proteomic studies have been carried out in *Arabidopsis* [43–46] and rice [47–51]. Even though rice belongs to the grass family the results from the studies in rice are only partly transferable to Pooideae grasses since rice cannot cold acclimate and develop tolerance to frost. Some investigations have been conducted on cereal species with CA-ability but these studies are mainly restricted to studying a few genes, e.g. *Wsc120* in *T. aestivum* [52] and *Cor14b* in *H. vulgare* [7,53]. Only one comprehensive study of proteome responses to CA has been carried out in Pooideae forage grass [4]. In this study *F. pratensis* genotypes with distinct levels of FT were selected for comparative analyses of leaf protein accumulation before and after 2, 8, 26 h, and 3, 5, 7, 14 and 21 days of CA. High-throughput two-dimensional electrophoresis (2-DE) in combination with electrospray ionization mass spectrometry was used to study how the proteome of the different genotypes changed in response to CA. Comparisons between HFT and LFT plants revealed a total of 41 (5.1%) proteins which showed a minimum of 1.5-fold difference in abundance during the CA process. The largest differences in protein abundance (28.1%) appeared relatively early, most often on the 2nd day of CA. At the time point when maximal levels of FT and maximal difference in FT between HFT and LFT individuals were reached (the 21st day of CA), 10 out of the 41 proteins (24%) had >1.5 fold differences in abundance between HFT and LFT.

The majority of differentially accumulated *F. pratensis* proteins were proteins which are directly involved in photosynthesis, stressing the importance of the link between the function of the photosynthetic apparatus under cold stress and FT levels (Fig. 1). Several of the *F. pratensis* proteins identified had not been reported before to possibly be involved in the development of CA and FT, even in model species. This group includes chloroplast-localized Ptr ToxA binding protein 1, globulin 2, 50S ribosomal protein L10 from chloroplasts, 30S ribosomal protein S10 from chloroplasts, ADP (adenosine diphosphate) – glucose pyrophosphatase, and ADP-ribosylation factor 1 [4]. A direct comparison of the results obtained for *F. pratensis* with those described by others, e.g. for *Arabidopsis*

[43–46] is unfortunately not possible due to different methodological approaches of the studies. In the case of *F. pratensis* the protein abundance of HFT and LFT plants were compared before CA and at eight different time points of CA while in the *Arabidopsis* studies the abundance of particular proteins of non-acclimated and cold acclimated plants was mainly analyzed (e.g. [43]). Also an extract of total leaf proteins from *F. pratensis* was used, whereas in *Arabidopsis* protein extracts derived from different cell compartments, including the nucleus [44], plasma membranes [46] and chloroplasts [45], were applied.

Kosmala et al. [4] reported that degradation of the oxygen-evolving enhancer protein 1 was observed during CA of *F. pratensis*. Studies in rice have shown degradation of the Rubisco large subunit, Rubisco activase, sedoheptulose-1, 7-bisphosphatase, PS II oxygen-evolving complex protein 2, ATP (adenosine triphosphate) synthase alpha chain, and ATP synthase CF1 beta chain during low-temperature treatment. It is well known that the ability of cells to adapt to new environmental conditions requires a rapid reconstruction of existing regulatory pathways. Thus, protein degradation is one of the most essential components of plant responses to environmental stimuli [54].

In another recent study Kosmala et al. [5] analyzed the protease activity after 3 weeks of CA in two *F. pratensis* genotypes with high and low  $T_{EL50}$  values ( $T_{EL50}$  = temperature of 50% survival determined by electrical conductivity). A difference in the protease activity of HFT and LFT genotypes appeared after 2 h of cold acclimation and the activity of the proteases was significantly higher in the LFT genotype than the HFT genotype at almost all the studied time points during 21 days of CA. After 3 weeks of CA the protease activities increased 3-fold in LFT and 2-fold in the HFT plant compared to the activities detected before CA [5]. However, at this time point it would be difficult to determine which of the identified proteolytic activities actually was associated with cold acclimation and differentially expressed FT among the *F. pratensis* genotypes.

The proteomic approach presented herein for *F. pratensis* should be further extended to cover a wider part of the proteome. Furthermore, integration of plant responses to cold and frost stress on the transcriptome, proteome, and metabolome levels, similar to that described for *Arabidopsis* (e.g. [55–57]) is necessary to fully understand the mechanisms underlying genetic differences in FT among genotypes of forage grass species.

## 5. Photoinhibition avoidance in cold and freezing tolerance

Photoinhibition is the process whereby light energy absorbed in the photosynthetic light processes exceeds energy demand of the dark processes which leads to PSII over-reduction and subsequent inhibition of the photosynthetic capacity (Fig. 1). This can result not only in destruction of the photosynthetic apparatus but also in damage of whole cells due to production of reactive oxygen species accompanying PSII over-reduction [58]. Under low temperatures in winter and spring, the photosynthetic fixation of CO<sub>2</sub> is very limited, a condition which can cause photoinhibition even under relatively low irradiance [59]. The tolerance to this cold-induced photoinhibition seems to be closely related to freezing tolerance, a relationship which is partially due to common mechanisms of acclimation to both stresses as demonstrated in winter wheat and rye (*Secale cereale* L.) [60]. PSII over-reduction may also act as one of the signals triggering gene expression of cold regulated genes involved in CA as shown in rye [61] (Fig. 1). Reduction of PSII capacity must be carefully controlled by the plant to avoid processes that destroy photosystems and cells due to the overproduction of reactive oxygen species under photoinhibition [62–64]. As a consequence photosynthetic acclimation to photoinhibitory conditions are also induced under cold [60].

Two main strategies of acclimation of the photosynthetic apparatus are found in higher plants during cold hardening [60,65]. The first strategy, called the photochemical mechanism, depends on increasing the energy demand by increasing carbon assimilation and carbon metabolism [60]. This strategy is observed especially in over-wintering herbaceous plants which are not able to accumulate sufficient amounts of photoassimilates during the summer. Accumulation of high amounts of photoassimilates in autumn and early winter acts as an energy source for the cold acclimation process [60,66]. The second strategy relies on an intensification of protective non-photochemical mechanisms that harmlessly dissipate excess excitation energy as heat. The non-photochemical mechanism is the most important in equilibrating between absorption and utilization of light energy [63] and has been shown to be important also in herbaceous plants when low temperatures generate a strong photoinhibitory pressure [67].

Perennial forage grasses are very interesting model systems for studying mechanisms of photosynthetic acclimation to cold. Firstly, acclimation of the photosynthetic apparatus to high light-low temperature conditions is essential for perennial forage grasses to develop winter hardiness and FT. Laboratory experiments and field experiment over multiple winters has showed that the capacity for photosynthetic acclimation was correlated with genotypic differences in WS [68]. Furthermore, genotypes with impaired photosynthetic acclimation were shown to be unable to undergo proper CA and increase their freezing tolerance [68]. However the correlation between winter hardiness (or freezing tolerance) and the ability of photosynthetic acclimation was ~0.7, indicating that in the case of some plants, freezing tolerance and tolerance to cold-induced photoinhibition are at least partially independent mechanisms [68]. Secondly, there seems to be a link between FT levels and the type of photosynthetic acclimation which grasses undergo. Studies on androgenic genotypes of allotetraploid *Festulolium* cultivars (*F. pratensis* (4×) × *L. multiflorum* (4×)) demonstrated that plants with higher winter hardiness in the field, and higher FT in the laboratory were also more tolerant to cold-induced photoinactivation of PSII. The higher tolerance to photoinhibition was mainly due to increased non-photochemical dissipation of excess energy, however in one of the androgenic genotypes reduced non-photochemical dissipation was compensated by an increased electron transport (i.e. photochemical mechanism) [68]. Further studies have shown that perennial *F. pratensis* genotypes with high FT has increased dissipation capacity compared to biennial *L. multiflorum* genotypes in which only increased quenching of photochemical energy was observed [69].

Experiments with *F. pratensis* × *L. multiflorum* hybrids illustrate that transfer of the non-photochemical mechanism from *Festuca* to *Lolium* results in increasing frost tolerance [68,69]. More detailed studies of the genomic composition of *Festuca* × *Lolium* hybrids have given some indications of which chromosomal regions that harbour the underlying genes. Hybrid genotypes with a *L. multiflorum* genetic background and a chromosome 4 introgression from *F. pratensis* were more frost tolerant than pure *Lolium* genotypes [69] and this is probably due to changes in the non-photochemical mechanism of photosynthetic acclimation. In a chromosome substitution population in which each *L. perenne* chromosome has been replaced by its *F. pratensis* homologue [70], the chromosome 4-substitution line has both higher expression of non-photochemical mechanism of the photosynthetic acclimation and the highest freezing tolerance [69].

Some evidence also exists for the importance of adaptation to photoinhibition under low temperatures in natural populations of *Poa* species. Photosynthetic acclimation to cold conditions is thought to play a role in the altitudinal range distribution of *Festuca* species in Babia Gora Mountain in Poland. In Babia Gora *Festuca rubra* distribution is limited to lower altitude habitats compared

to species of mountainous fescues (*Festuca versicolor* and *Festuca supina*) which have more efficient non-photochemical mechanisms of photosynthetic acclimation to low temperatures than *F. rubra* [71].

## 6. *Pooideae* lineage specific genes: ice interacting proteins and fructan metabolism

Several gene families, or sub-classes of gene families, are known to be lineage specific for the *Pooideae* grasses, for example sub-clades of the CBF-transcription factor family [72], the fructosyl transferase (FST) gene family [73], and one *Pooideae*-specific ice re-crystallization inhibition protein coding gene family [9]. It has been speculated if these *Pooideae*-specific gene family expansions have been important for adaptation of a common *Pooideae* ancestor to cold climates and subsequently ecological expansion of *Pooideae* into cooler habitats [74]. In the following sections we will focus on IRIP and FTS genes and their importance for FT in *Pooideae* grasses. Since relatively little research has been done on CBFs in forage grasses we recommend a recent review on grass CBFs in cereals [75] for further discussion on this topic.

### 6.1. Ice interacting proteins

Anti-freeze proteins (AFP), i.e. proteins that modify the water freezing process, have evolved independently in many organisms [76] and are thought to be an important adaptation to a life in frost-exposed environments. AFPs can be classified into two groups according to their function; (1) AFPs that affect the temperature at which water freezes (thermal hysteresis), and (2) AFPs that are ice re-crystallization inhibition proteins (IRIP). Only the latter type is thought to be important in plant AFP action [77]. Ice re-crystallization is the process where larger ice crystals grow at the expense of smaller crystals. However, when IRIPs are present under freezing conditions ice re-crystallization is inhibited, forcing new small crystals to form rather than an increase in the crystal size. It is thought that controlled ice crystal growth provides protection from physical damage caused by ice formation in the apoplastic space (Fig. 1).

The first report of ice-binding proteins in plants came from experiments with rye (*S. cereale*) [78]. Subsequently many plant AFPs of different evolutionary origin have been discovered [79,80]. In forage grasses the first report of an ice interacting protein were in a study by Sidebottom et al. [80]. The authors reported the discovery of a cold-induced mRNA transcript encoding a partial ice re-crystallization inhibition protein (IRIP) isolated from *L. perenne* (LpAFP, AJ277399). Several IRIP homologs have subsequently been isolated from forage grass species and it is now clear that they belong to a *Pooideae*-specific multi-gene family [9,81–83]. Protein modelling and functional protein studies have shown that NxVxG/NxVxxG repeat motifs forms a beta-roll with a beta-sheet ice-binding face that can bind to ice and is responsible for ice re-crystallization inhibitory action *in vitro* [84,85]. The grass IRIP genes encode a signal peptide that targets the proteins to the apoplast [9], and apoplastic export of IRIPs have been shown to happen *in vivo* in transgenic *Arabidopsis* plants [82].

Interestingly the ice interacting domain of IRIPs is not homologous to proteins in rice, *Arabidopsis*, or any other plant outside the *Pooideae* sub-family but have probably evolved through repeat-motif expansion events after the *Pooideae* divergence [9]. Synthetic AFPs have been shown to enhance FT, but the importance of IRIPs in determining FT in forage grasses have yet to be demonstrated. However, recently *Arabidopsis* plants expressing *L. perenne* IRIP genes were shown to display improved cell membrane stability during freezing and increased FT [86]. This strongly supports the notion

that IRIP genes are important for survival in frost-exposed environments.

### 6.2. Fructan synthesis

Fructans are soluble fructosyloligosaccharides derived from sucrose, which are used for carbohydrate storage in some (~15%) flowering plants, including the cereals and forage grasses [87]. It has long been recognized that temperate grasses accumulate fructans in response to seasonal drop in temperature in the field and under exposure to cold temperatures in the laboratory [65,73,88–92]. Yet historically, the direct functional significance of fructan synthesis under low-temperature stress and the role of fructan synthesis in adaptation to cold climates have been controversial [93]. Research on forage grasses from the past decade have provided evidence that fructan molecules themselves have a direct effect on freezing and low-temperature stress tolerance [94,95]. The question today is not *if* but *how* fructans help increase the FT.

The study of fructan biosynthesis under cold temperatures has been important to understand the role of fructans in cold stress. Fructan sugars are synthesised by four fructosyl transferase (FST) enzymes [94]. The initial step in fructan synthesis is the conversion of two sucrose molecules to a fructan molecule by the 1-Sucrose-sucrose-fructosyltransferases (1-SST). Further polymerization are then carried out by other FSTs, i.e. Fructan:fructan 1-fructosyltransferases (1-FFT), Fructan:fructan 6G-fructosyltransferases (6G-FFT), and Sucrose:fructan 6-fructosyltransferases (6-SFT), resulting in five possible types of fructan sugars [see [94] for review]. Several studies on cereals and forage grasses have demonstrated that FST gene expression levels are regulated by exposure to low temperatures [89,96]. However, the most elaborate study on expression of FST genes under CA was done by Hisano et al. [73] in *L. perenne*. The authors investigated the expression patterns of multiple FST genes during the CA process and found that it was consistent with the proposed model of fructan synthesis pathway. Expression levels of genes involved in the initial steps of fructan synthesis (1-SST and 6G-FFT) increased shortly after transferring the plants to cold conditions, while genes encoding enzymes that elongate the fructan chains (putative 6-SFT) increased their expression levels throughout the cold acclimation process. The correlation between FT gene expression and fructan sugar accumulation under CA has been demonstrated through several experiments [73,97]. Tamura et al. [98] reported that the PpFT1 gene from timothy (*P. pratense*) is a novel gene with unique enzymatic properties that differ from previously cloned plant 6-SFTs, and is involved in the synthesis of highly polymerized levans in timothy. Accumulation of large amounts of carbohydrates as fructans with high degree of polymerization by *PpFT1* and other enzymes might be involved in the superior winter hardiness of timothy.

The direct functional role of fructans on cold stress tolerance is thought to be linked to increased cell membrane stabilization (Fig. 1). Fructans have been shown to interact with model cell membranes [99,100] and change their stability under freezing [101]. A pioneering study using transgenic plants have provided evidence that fructans stabilize cell membranes *in vivo* under freezing. Hisano et al. [6] made transgenic *L. perenne* plants that overexpressed wheat 1-SST and 6-SFT genes and showed that increased fructan synthesis reduced electrolyte leakage (i.e. reduced cell membrane damage) during freezing. Similar results have later been replicated in transgenic tobacco expressing a lettuce 1-SST gene [102]. Two additional studies using transgenic plants with increased fructan content have showed increased freezing tolerance in tobacco [103] and increased chilling tolerance in rice [104], however, these studies did not measure cell membrane damage.

## 7. Concluding remarks

Perennial forage grasses are essential elements of sustainable and multifunctional farming systems providing both feed for ruminants and ecosystem services, e.g. carbon sequestration, soil formation and protection, nutrient cycling, and aesthetic landscape values. This is especially the case in high-latitude and high-altitude temperate regions where perennial grasses make up a very large part of the agricultural land. Climate changes are predicted to give more unpredictable and unstable winter climates with more cycles of freezing-thawing and less long-lasting snow cover. These climates pose new combinations of winter stresses, and in many cases increasing stress, for perennial plants in many regions.

In this review we have summarized present knowledge on important molecular mechanisms underlying cold and freezing stress survival in perennial forage grasses (Fig. 1). Even though the last decade of research have disclosed important molecular systems involved in winter survival and adaptation to cool environments we have still a long way to go to understand frost tolerance at the whole plant level. Important topics to address in future research include: how does plants sense temperature changes – are there one universal or several different thermosensor systems? We need to understand more about the detailed role of carbohydrate composition and turnover at the whole plant level during different types of winter climates; how do plants regulate respiration during winter, and how is photosynthetic activity controlled at low light intensity during warmer periods of the winter at higher latitudes, or at low light intensity under snow cover? We also lack understanding of the reversion from a cold acclimated state to a growth state, the de-hardening process, and how this is regulated at the molecular level. Repeated cycles of hardening/de-hardening/re-hardening during winters are likely outcomes of the predicted climate changes affecting the northern hemisphere. A better understanding of these processes is vital in order to predict and mitigate the effects of climate change and develop forage grass cultivars with superior combinations of winter survival, dry matter production and nutritive quality.

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