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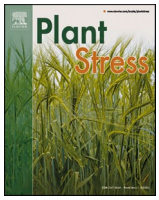

Transcriptome profiling reveals insight into the cold response of perennial ryegrass genotypes with contrasting freezing tolerance

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ABSTRACT

Low freezing tolerance threatens the survival and productivity of perennial ryegrass under northern climate. In this study, we aimed to identify transcriptional changes in plants subjected to low and freezing temperatures as well as to elucidate differences between tolerant and sensitive genotypes. Response to freezing stress was evaluated in a panel of 160 perennial ryegrass genotypes by measuring electrolyte leakage after exposure to -12 °C and -14 °C for 24 h. Two tolerant and two sensitive genotypes were selected for the transcriptome analysis. Crown tissue samples were collected at six treatments: before the start of cold acclimation (control point), at the start of acclimation, after one week of acclimation, after three weeks of acclimation, after freezing at -5 °C and freezing at -10 °C. A total of 11,125 differentially expressed genes (DEGs) were identified in the sensitive and 12,937 DEGs in the tolerant genotypes, when comparing the control vs. each of the acclimation and freezing treatments, as well as the end of acclimation vs. freezing treatments. Among the identified DEGs 3323 were unique to the sensitive genotypes, 5135 were unique to the tolerant genotypes and 7802 were shared. Genes upregulated during cold acclimation and freezing stress were linked to the MAPK signalling pathway, circadian rhythm, starch and sucrose metabolism, plant-pathogen interaction, carbon fixation, alpha-linoleic acid metabolism, carotenoid metabolism, glyoxylate and dicarboxylate metabolism pathways. Downregulated genes were linked to ATP-dependent chromatin remodelling, fatty acid elongation and DNA replication. The downregulation of fatty acid elongation and glutathione metabolism DEGs could indicate that the studied genotypes respond to cold stress in a novel or not yet well-characterized manner.

Introduction

In the Nordic-Baltic region and temperate latitudes in general, global warming can have a positive aspect for agricultural production and when coupled with proper adaptation management can encourage adoption of new forage crop species as well as increase the productivity (Kemešytė et al., 2023; Olesen et al., 2011; Wiréhn, 2018). On the other hand, climatic fluctuations and weather anomalies may expose perennial forage crops to new abiotic stress types, such as more frequent warm spells in autumn causing unstable snow cover resulting in deacclimation and reacclimation cycles (Jørgensen et al., 2010). These changes negatively affect cold acclimation of perennial forage crops leading to frost injuries or even crop loss (Dalmannsdottir et al., 2017; Kovi et al., 2016; Uleberg et al., 2014). The unfavourable autumn and winter conditions are the main factors limiting the geographical distribution of species and yield stability. However, during evolution, temperate plants have developed cold adaptation strategies when tolerance is gained during autumn via cold acclimation (also known as hardening) process temperatures (Ding et al., 2013; Thomashow, 1999). Low, non-freezing temperatures ranging from +5 °C to 0 °C for at least a 4-week period, increases the efficiency of plant acclimation leading to higher survival rate and yield (Jaškūnė et al., 2022; Rapacz et al., 2014) by inducing

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changes at physiological level via modification of cell wall and membrane fluidity as well as activation of signalling pathways (Tao et al., 1998). Acclimation process starts with the detection of cool temperatures stress followed by increased membrane rigidity, Ca^{2+} influx, initiation of signalling pathways, and the subsequent responses of ICE-CBF-COR transcriptome reprogramming as well as post-transcriptional regulation and posttranslational modification (reviewed in Juurakko et al., 2021; Sustek-Sánchez et al., 2023). When plants experience low-temperature stress, ICE1 can be released from JAZ proteins that are bound by DELLAs, leading to the activation of CBF3 expression. In turn, CBF3 triggers the expression of genes responsive for decreasing the levels of bioactive gibberellic acid (GA) and facilitates the accumulation of DELLAs. C-repeat binding factors (CBFs), also known as dehydration-responsive element-binding proteins (DREBs), are very important in acclimation process as they are responsible for activating the expression of COR genes via binding to cis-element in the promoter of COR genes (Ritonga and Chen, 2020). Another cold response pathways are abscisic acid (ABA) signalling and mitogen-activated protein kinase (MAPK) cascade pathways functioning as conveyors of stress signals from receptors to effectors initiating adaptive processes (Hossain et al., 2010; Moustafa et al., 2014). Most of the current knowledge about gene regulatory networks involved in cold tolerance comes from studies in Arabidopsis. Although many of these networks are evolutionarily conserved, there are large differences between dicots and monocots and between annual and perennial plant species. Thus, there is a need for more research on crop plants, especially perennial crops exposed to the effects of climate change.

Perennial ryegrass (*Lolium perenne* L.) is the most economically important cool-season pasture and turf grass species. It has a large biogeographical distribution, covering nearly whole Europe (Blanco-Pastor et al., 2019), New Zealand and other temperate areas (Chapman et al., 2023). Perennial ryegrass is valued for excellent forage quality, grazing tolerance, rapid establishment and good seed production. However, these superior properties are exhibited under optimal growth conditions while under drought or low temperatures it performs poorly (Aleliūnas et al., 2015; Helgadóttir et al., 2018; Jaškūnė et al., 2020). Several studies have evaluated the mechanisms involved in cold acclimation at physiological, proteomic and metabolomic levels. Perennial ryegrass accessions of contrasting freezing tolerance were studied for proline, water soluble carbohydrates (WSCs) and lipid content in crown tissue during 21 days of cold acclimation (Hoffman et al., 2010). Freezing tolerant plants accumulated more WSCs, especially sucrose, whereas proline level increased equally in both tolerant and sensitive genotypes. Similar experiments were performed using leaf tissue to evaluate the protein content and different metabolites composition of freezing tolerant and sensitive plants (Bocian et al., 2011, 2015). Freezing tolerant genotypes differed from sensitive ones by higher levels of chloroplast proteins (Bocian et al., 2011) and earlier accumulation of proline and asparagine (Bocian et al., 2015). Increased fructan content was measured in the roots and above ground biomass of a freezing tolerant genotype during cold acclimation (Abeynayake et al., 2015b). Cold acclimation followed by freezing stress induces a complex phytohormonal changes in perennial ryegrass; accumulation of trans-zeatin in the crown and root tissue was recorded during cold acclimation and exposure to freezing led to up-regulation of abscisic and jasmonic acid (Prerostova et al., 2021).

Transcriptome analysis is one of the most effective approaches to identify the genes involved in abiotic stress and to describe the regulatory pathways and mechanisms (Aleliūnas et al., 2020; Dong et al., 2020). However, studies on transcriptional level changes under cold stress in perennial ryegrass is still scarce. Ice recrystallization inhibition (IRI), dehydrin (DHN), and cold-regulated (COR) genes were upregulated, and chlorophyll-binding protein genes were downregulated during cold acclimation in a study that utilized Affymetrix Barley1 GeneChip to study differences between cold acclimated and non-acclimated perennial ryegrass plants (Zhang et al., 2017). Genes related to carbohydrate metabolism, photoperiod regulation and signal transduction were differentially expressed in the leaves of freezing tolerant and sensitive genotypes (Abeynayake et al., 2015a; Paina et al., 2014).

In the present research, we utilized perennial ryegrass genotypes with varying freezing tolerance to study transcriptome profiles, aiming for a deeper understanding of the molecular mechanisms underlying the response to cold (low positive temperature) and freezing (below zero temperature) stress. Similar to previous research, we compared transcriptomic responses before and during cold acclimation (at 7, 14, and 21 days). However, unlike prior studies, we also evaluated transcriptomic responses after exposing plants to freezing temperatures (−5 and −10 °C). This study aims to (i) identify differentially expressed genes during cold acclimation and freezing, and (ii) compare the profiles of these genes between freezing-tolerant and freezing-sensitive genotypes, thereby revealing the molecular pathways that differentiate their stress responses.

Materials and methods

Plant material and electrolyte leakage measurement

A collection of 160 perennial ryegrass genotypes was screened for freezing tolerance. The plant material was established by randomly selecting five genotypes from each of the 32 experimental populations and cultivars utilized in a Nordic/Baltic pre-breeding project (Rognli et al., 2018). Each genotype was vegetatively propagated and planted in cell packs filled with peat substrate, with 4 ramets per cell. They were kept in the greenhouse for 21 days, 16/8 h photoperiod, until they were fully established and then moved into growth chambers (Plant Master, Germany), set at 12/12 h photoperiod and 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), for cold acclimation. Acclimation was performed in two steps, starting at 5 °C for 7 days, and continuing at 2 °C for 14 days. At the end of the cold acclimation period, leaf samples of each genotype from the 4 replicates were taken and placed in 25 ml tubes, filled with 2 ml of distilled water. The tubes were randomly placed in the test tube racks and moved into the climate chamber PE 2412 UY-LX (Angelantoni Industrie, Italy) set at 2 °C. Then the temperature was reduced to −6 °C and held constant until it settled down to −6 °C in all racks. The temperature was monitored by inserting temperature probes into one test tube per rack and recording with a KD7 recorder (Lumel, Poland). After reaching the uniform temperature, it was gradually lowered at a rate of 1.2 °C h^{-1} to a target temperature and held for 24 h. The freezing test was repeated twice, with −14 °C as the target temperature of the first experiment (Exp1), while the second (Exp2) had a target temperature of −12 °C. After the freezing test, tubes with the leaves were moved to the growth chamber set to 2 °C and after 24 h, 10 ml of deionized water was added into each tube. The tubes were shaken overnight at 120 rpm and then initial conductivity (C_{ini}) was measured with YSI 3100 conductivity meter with YSI 3253 Glass Dip Cell (YSI Incorporated, USA). The total conductivity (C_{tot}) was measured after autoclaving the samples at 120 °C for 15 min. Electrolyte leakage was estimated as EL = $(C_{ini})/(C_{tot})$ x 100. Freezing tolerance was defined as percentage of EL at the targeted temperatures of −14 °C and −12 °C.

RNA sampling, extraction and sequencing for transcriptome analysis

For the transcriptome analysis, a set of four perennial ryegrass genotypes, consisting of two tolerant and two sensitive were selected based on EL estimation results. Each selected genotype was vegetatively propagated into 20 replicates, consisting of 2–3 ramets, planted in cell packs filled with peat substrate. The established plants were cold acclimated for 21 days as described above. Before exposure to freezing temperatures, the plants were removed from the soil, the roots were washed and trimmed to 5 cm. Each plant was placed into 50 ml test tubes with 5 ml of water and left for adaptation in the freezing chamber

for 10 h at 1 °C. Afterwards the temperature was lowered to - 5 °C at the rate - 1 °C per hour, kept at - 5 °C for 3 h, then the temperature was lowered to - 10 °C. Crown tissue samples were collected at six times points in 3 replicates (Fig. 1) and immediately frozen in liquid nitrogen. T2, T3, T5 and T6 samplings were made one hour after reaching the target temperature. T1 was made a day prior to the start of cold acclimation, and T4 was made right before moving plants to the freezing chamber. First four samplings (T1-T4) were made at 11 a.m. RNA extraction along with DNA digestion was carried out using QIAGEN's RNeasy Plant Mini Kit and RNase-free DNase, according to the manufacturer guidelines. Library preparation and sequencing (20 million paired reads/sample) were outsourced to Novogene Co Ltd (Cambridge, UK).

Alignment, abundance estimation and functional annotation

To obtain high quality reads, the raw reads were inspected using FastQC (0.11.9) (Ward et al., 2020) followed by trimming and adapter removal using fastp (0.23.2) (Chen et al., 2018) with options –length_required 100, –cut_window_size 4, –cut_mean_quality 15. The cleaned reads were then aligned to the reference genome of perennial ryegrass (Ensembl release 59) using hisat2 (2.2.1). The binary alignment/map (bam) files generated from hisat2 were used to extract a transcript level counts matrix with featureCounts from subread (2.0.6) (Liao et al., 2014) by providing the gene transfer format (gtf) file (ensembl release 59). The resulting counts matrix was used for differential expression analysis. For functional annotation, coding regions of the genes (nucleotide sequences) extracted from the genome using gffread (0.12.7) were blasted against protein sequences of perennial ryegrass downloaded from NCBI using diamond (v2.0.15.153) (Buchfink et al., 2021) with options –ultra-sensitive and –evalue 0.00001.

Differential gene expression analysis

The function filterByExpr from the edgeR package was used to filter out genes with low expression. Principal component analysis based on genes retained after filtering was performed using dudi.pca function from R package ade4 (Dray and Dufour, 2007). The quasi-likelihood approach (Lund et al., 2012) was employed to identify differentially expressed genes. Genes involved in cold acclimation were identified by performing contrasts between T1 vs T2, T1 vs T3 and T1 vs T4 followed by contrasts between T1 vs T5, T1 vs T6, T4 vs T5, T4 vs T6, and T5 vs T6 to identify genes involved in freezing stress responses. The above-mentioned contrasts were performed separately in sensitive and tolerant genotypes (e.g. Sen T1 vs Sen T2, Tol T1 vs Tol T2). Furthermore, contrasts were performed at respective treatments between freezing tolerant and sensitive genotypes to identify differences in gene expression between genotypes (e.g. Sen T2 vs Tol T2, Sen T4 vs Tol T4). Only genes with p.adj <0.05 and log fold change $\geq$ log2(1.5) were considered as differentially expressed. To identify the genes responsible for differences in cold acclimation and freezing tolerance between sensitive and tolerant genotypes, DEGs from the direct comparisons between genotypes were retained only if they were differentially expressed in at least one of the treatment contrasts (T1 vs T2, T1 vs T3, T1 vs T4, T1 vs T5, T1 vs T6, T4 vs T5, T4 vs T6, and T5 vs T6) in both sensitive and tolerant genotypes. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on sets of upregulated and downregulated genes were performed by enrichGO and enrichKEGG functions from R package clusterProfiler (4.10.1) (Wu et al., 2021).

Results

Freezing tolerance assessment

A substantial variation for freezing tolerance parameters was observed among the tested genotypes especially at the target temperature −12 °C (Exp 1). The mean electrolyte leakage (EL%) at −14 °C (Exp2) was higher than that of EL% at −12 °C ($p < 0.0001$, Student's *t*-test). The variation between repeats was very high (Fig. 2), and the correlation between the results of Exp1 and Exp2 was very weak ($r = 0.20$, $p < 0.05$). Selection of the two most freezing tolerant (low EL%) and sensitive (high EL%) genotypes for the subsequent transcriptome analysis was carried out by identifying those genotypes, which were consistently placed in the first quartile and in the fourth quartile in both Exp1 (Figs. 2A, 3A) and Exp2 (Figs. 2B, 3A). The results obtained from the EL experiments corresponded with the clustering observed when performing the principal component analysis based on the DEGs of the sampled plants.

Alignment, functional annotation, and explanatory data analysis

The alignment rate was > 72 % for all samples in the study with a mean alignment rate of ~ 78 %. Homology-based search of coding regions of the genes against the protein sequences of perennial ryegrass retrieved from NCBI's database annotated 35,926 (92 %) genes. After filtering genes with low counts, a total number of 28,535 genes were retained for differential expression analysis and the mean library size was around ~ 17.5 million reads per sample. Principal component analysis (PCA) based on all the genes retained after filtering ordered treatments T1 to T6 from left to right along PC1 (~31 % variation) and

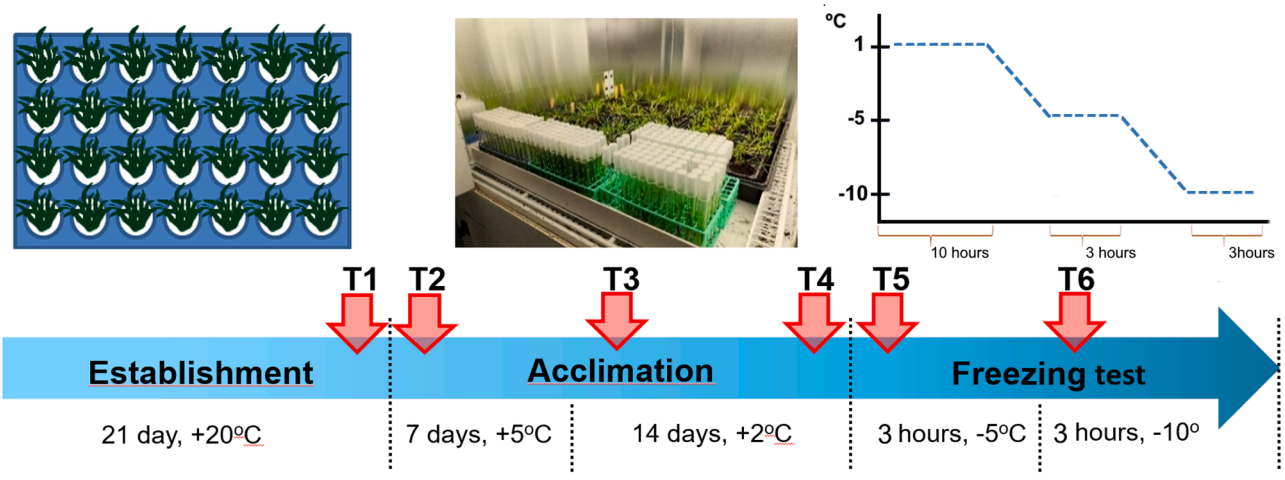

Fig. 1. The design of the cold acclimation and freezing tolerance experiment with indicated time points (red arrows) of RNA sampling, where T1 is control, T2 – beginning of acclimation at +5 °C, T3 – beginning of acclimation at +2 °C, T4 – end of acclimation, T5 – freezing at −5 °C and T6 – freezing at −10 °C.

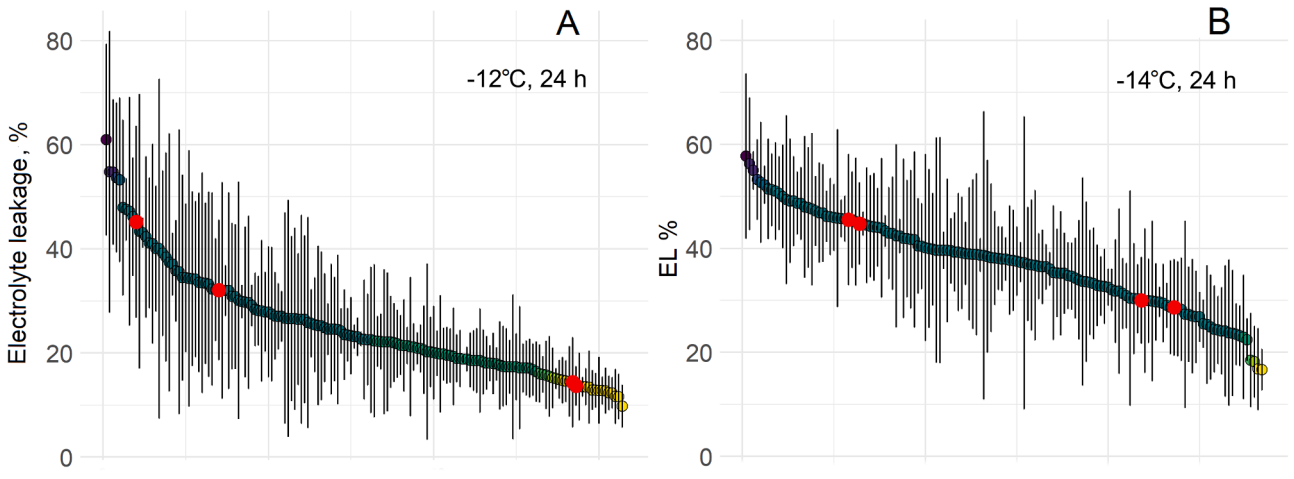

Fig. 2. Electrolyte leakage (EL) variation among perennial ryegrass genotypes (n = 160) assessed for freezing tolerance at −12 °C (A) and −14 °C (B). Error bars represent standard deviation (SD). The genotypes selected for the transcriptome analysis are indicated in red.

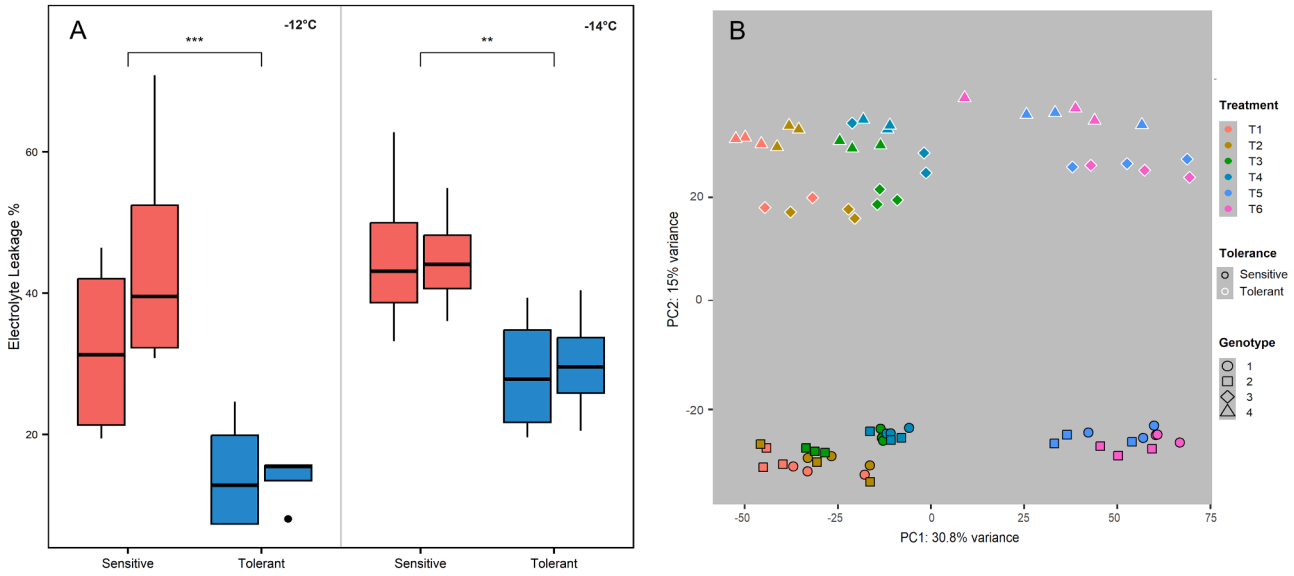

Fig. 3. Electrolyte leakage (EL) and principal component analysis (PCA) of differentially expressed genes of perennial ryegrass genotypes with contrasting freezing tolerance. (A) Variation of EL among freeze sensitive (red) and tolerant (blue) assessed after 24 h at −12 °C and −14 °C. (B) shows the clustering of freeze tolerant and sensitive genotypes along principal component axes 1 and 2.

PC2 explaining 15 % of variation separated the tolerant and sensitive genotypes (Fig. 3B). The first two principal components indicate that experimental factors are the primary source of variation, thus validating the experimental setup.

DEGs during cold acclimation and freezing stress

A total of 11,125 differentially expressed genes (DEGs) were identified in the sensitive and 12,937 DEGs in the tolerant genotype (Fig. 4), when comparing a control (T1) versus each acclimation (T2, T3, T4) and freezing (T5, T6) treatments, as well as the end of acclimation (T4) versus freezing treatments (T5 and T6). Among the identified DEGs 3323 were unique to the sensitive genotype (sensitive specific cold-responsive genes), 5135 were unique to the tolerant genotype (tolerant specific cold-responsive genes) and 7802 were shared between both genotypes (core cold-responsive genes). There were no differentially expressed genes identified at contrast T5 vs T6 neither in the tolerant nor the sensitive genotype. The tolerant genotype had more upregulated and downregulated DEGs compared to the sensitive genotype during cold acclimation (T1 vs T2, T1 vs T3, T1 vs T4) and at the onset of freezing temperatures (T1 vs T5) (Fig. 4B). The number of DEGs was approximately identical at contrasts T4 vs T5, compared to T4 vs T6 in the tolerant genotype, while it was higher at contrast T4 vs T6 compared to T4 vs T5 in the sensitive genotype (Fig. 4B). Several genes encoding proteins and transcription factors (TFs) were differentially expressed in both genotypes and are known to be involved in cold acclimation and freezing tolerance, such as DREB/CBF, cold-shock proteins, late embryogenesis abundant proteins (LEA), heat shock proteins (HSP) and others described in Table 1. KEGG pathway analysis of DEGs revealed that genes upregulated during cold acclimation and freezing stress are linked to the MAPK signalling pathway, circadian rhythm, starch and sucrose metabolism, plant-pathogen interaction, carbon fixation in photosynthetic organisms, alpha-linoleic acid metabolism, carotenoid metabolism, glyoxylate and dicarboxylate metabolism pathways while downregulated genes were linked to ATP-dependent chromatin remodelling, fatty acid elongation and DNA replication (Figs. 5 and 6).

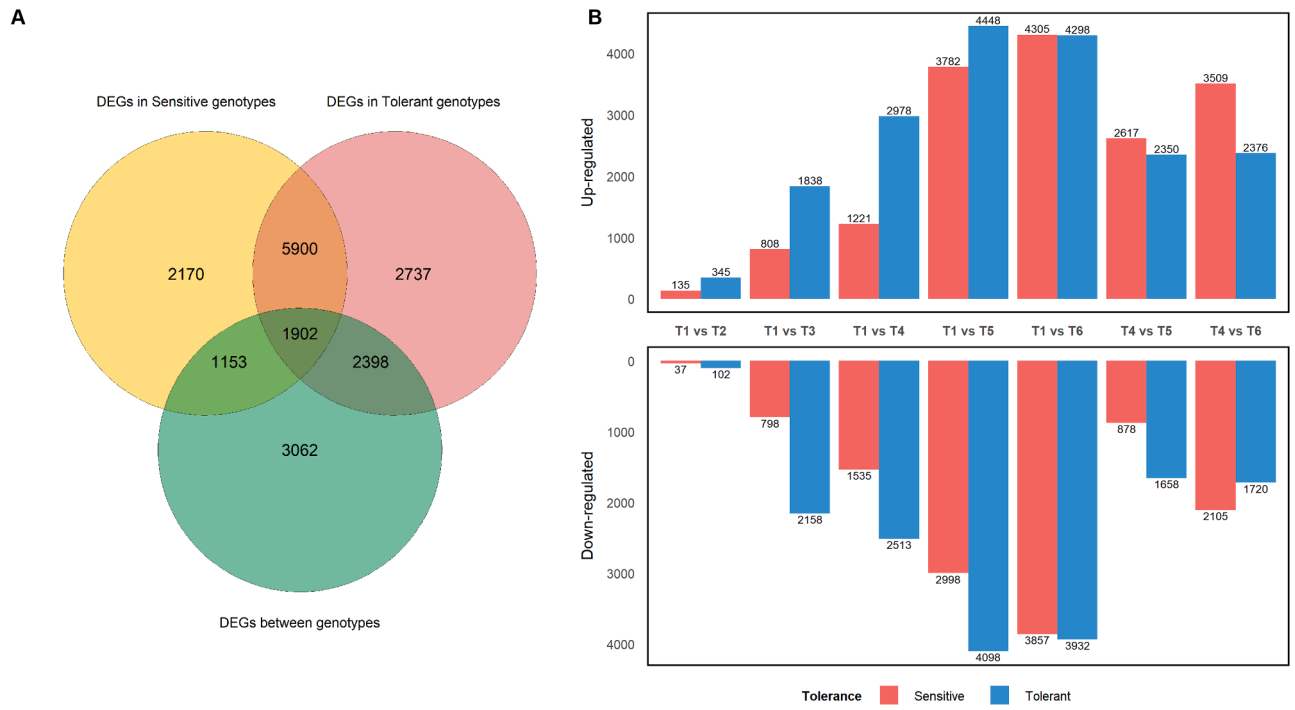

Fig. 4. Differentially expressed genes (DEGs) in the freeze tolerant and sensitive genotypes, where contrasts between genotypes are shown in Figure A, while B) shows number of upregulated and downregulated genes between treatment contrasts in genotypes.

DEGs responsible for differences in freezing tolerance between tolerant and sensitive genotypes

Comparative transcriptomic analysis between sensitive (baseline) and tolerant genotypes identified 4720 (2408 up, 2312 down), 3708 (1902 up, 1806 down), 3921 (1745 up, 2176 down), 4187 (2793 up, 1394 down), 3727 (1900 up, 1827 down) and 4057 (2149 up, 1908 down) differentially expressed genes at T1, T2, T3, T4, T5, and T6 respectively (Supplementary file 1). To identify the genes responsible for the differences in freezing tolerant and sensitive genotypes, DEGs from between genotype contrasts were filtered out based on the core cold-responsive genes (7802 genes) (Fig. 4). After filtering, 926, 644, 752, 640, 682, and 810 genes were retained at contrast T1, T2, T3, T4, T5, and T6 respectively (Supplementary file 2). Further inspection of the filtered gene lists revealed several genes, known for their roles in cold and abiotic stress responses in plants, were highly differentially expressed between the genotypes during cold stress (Fig. 6, Table 1, Supplementary Table 1, Supplementary Fig. 1). Genes coding for cold shock protein CS120-like, cold-regulated 413 protein, dehydrin DHN3-like, HSP, HSF A-2a-like, glycine-rich cell wall structural protein 1, CBL-interacting protein kinase 21-like, LEA 1 subgroup, bHLH 6-like TF, SCREAM 2 TF, MADS-box TF 14-like, MADS-box TF 50-like, common-plant regulatory factor 1-like, b-ZIP TF 23-like, hydrophobic protein OSR8-like and cysteine-rich receptor-like protein kinase 10 are among the few DEGs with elevated expression in tolerant genotypes. In sensitive genotypes, genes encoding DREB 1B-like, NDR1/HIN1-like protein, 36.4 kDa proline-rich protein-like, sucrose 1-fructosyltransferase-like, MYB77-like TF, wall-associated receptor kinase 1-like, calcium-dependent protein kinase (CDPK) 13-like, CDPK 26-like, calcium binding protein KIC-like were observed to have higher expression. Interestingly, a gene coding for cold-responsive protein kinase (CRPK) 1-like is downregulated with the onset of cold in tolerant genotypes but its expression remained stable (not significantly DE) in sensitive genotypes. GO analysis of filtered DEGs sets identified GO terms lipid oxidation (GO:0,034,440) and dioxygenase activity (GO:0,051,213) were enriched among genes with higher expression in sensitive genotypes at T5 and T6 (Supplementary Fig. 2), while GO terms; response to water (GO:0,009,415) and response to acid chemical (GO:0,001,101) were enriched in genes with higher expression in tolerant genotypes at T4.

Discussion

Perennial ryegrass in Europe spans from the Iberian Peninsula to Scandinavia (Blanco-Pastor et al., 2019) and thus is subjected to an array of climatic stressors and their combinations. Though warmer winters have led to less winter damage of forage crops in the Baltic region (Kemešytė et al., 2020, 2023), low freezing tolerance is still an important limiting factor for perennial ryegrass cultivation. Better understanding of the molecular mechanism behind perennial ryegrass cold acclimation and freezing stress response remains an important research field from both a practical and fundamental point of view.

Transcriptomic profile during cold acclimation

To better understand the different pathways that were enriched at certain treatments, the DEGs at basal level (T1) were compared to those of the rest of treatments. The analysis revealed that tolerant genotypes consistently exhibited a higher number of differentially expressed genes (DEGs) during cold acclimation (T1 vs T2, T1 vs T3 and T1 vs T4) than sensitive genotypes (Fig. 4B). In the comparison between T1 and T2 (beginning of cold acclimation), both the sensitive and tolerant genotypes upregulated genes involved in the MAPK signalling pathway. The MAPK pathway plays a crucial role in signal transduction during stress responses and is involved in the calcium signalling response to cold acclimation in different plant species as shown by Jagodzik et al. (2018) and Guo et al. (2018). The genes involved in the arginine and proline metabolism were enriched in the sensitive genotypes, while genes related to the circadian rhythm were enriched in tolerant genotypes. The involvement of the circadian rhythm pathway aligns with findings by Jang et al. (2024), indicating its role in regulating cold-responsive genes like DREB1. Proline is known as an osmoprotectant accumulating during cold acclimation and can be used for energy production under stress conditions (Liang et al., 2013). In L. *perenne*, a previous study observed higher levels of proline in the crown tissue of freezing tolerant plants

Table 1
Genes identified as differentially expressed between the sensitive and tolerant genotypes during cold acclimation and freezing stress.

Gene ID	Description	Role	Higher expression in	DE in contrasts	Ref.
KYUSg_chr2.31927	bHLH 6-like transcription factor (TF)	MYC2 has been shown to be involved in chilling resistance. It positively regulates the expression of CBFs.	Tolerant	T3, T4, T5, T6	10.3389/fpls.2022.868874
KYUSg_chr2.47834	36.4 kDa proline-rich protein-like	EARLI1 can improve membrane or cell wall stability under cold stress.	Tolerant	T3, T4, T5, T6	10.1111/ j.1365–3040.2004.01198.x
KYUSg_chr2.47869	36.4 kDa proline-rich protein-like	EARLI1 can improve membrane or cell wall stability under cold stress.	Sensitive	T1, T2, T3, T4, T5, T6	10.1111/ j.1365–3040.2004.01198.x
KYUSg_chr2.50031	Calcium-dependent protein kinase 13-like	CDPK13 positively regulates cold response in rice.	Sensitive	T3, T4, T5, T6	10.1007/ s00438–007–0220–6
KYUSg_chr3.34	sucrose:sucrose 1-fructosyltransferase-like	1-SST is downregulated in cold tolerant wheat cultivars.	Sensitive	T5, T6	10.1271/bbb.66.2297
KYUSg_chr3.13649	LEA protein 14-A-like, subgroup 2	Proteins belonging to the group II LEA proteins can provide protection against cold in different plant species.	Tolerant	T3, T4	10.1007/ s10725–015–0113–3 10.3390/biom11111662
KYUSg_chr3.42386	SCREAM2-like TF	ICE2 positively regulates cold tolerance.	Tolerant	T1, T2, T3, T4, T5, T6	10.1016/j.gene.2008.10.016
KYUSg_chr4.1396	cold-shock protein CS120-like (dehydrin)	CS120 is involved in the COR signaling pathway.	Tolerant	T2, T3, T4, T5, T6	10.1371/journal. pone.0249975
KYUSg_chr4.1433	cold-shock protein CS120-like (dehydrin)	CS120 is involved in the COR signaling pathway.	Tolerant	T4, T5	10.1371/journal. pone.0249975
KYUSg_chr4.51811	MADS-box TF 50-like	MADS50 is a floral regulator that could be involved in vernalization.	Tolerant	T4, T5, T6	10.1111/ j.1365–313X.2004.02082.x
KYUSg_chr4.8841	cold-regulated 413 protein	COR413 genes positively regulate cold response.	Tolerant	T3, T4	10.1104/pp.102.015255
KYUSg_chr4.9742	MADS-box TF 14-like	MADS14 is involved in the transition between apical and inflorescence meristems.	Tolerant	T1, T2, T3, T4, T5, T6	10.1105/tpc.112.097105
KYUSg_chr5.18506	36.4 kDa proline-rich protein-like	EARLI1 can improve membrane or cell wall stability under cold stress.	Sensitive	T1, T2, T3, T4, T5, T6	10.1111/ j.1365–3040.2004.01198.x
KYUSg_chr5.19513	LEA protein, subgroup 1	Proteins of this subgroup have been shown to be involved in drought stress response.	Tolerant	T3, T4, T5	10.1186/ s12863–017–0596–1
KYUSg_chr5.33969	abscisic acid 8′-hydroxylase 3-like	CYP707A3 regulates degradation of ABA under drought stress response.	Tolerant	T2, T3, T4, T5, T6	10.1111/ j.1365–313X.2006.02683.x
KYUSg_chr5.39012	DREB 1B-like	DREB1B positively regulates cold stress response.	Sensitive	T5, T6	10.1007/ s12041–012–0201–3
KYUSg_chr5.39032	DREB 1B-like	DREB1B positively regulates cold stress response.	Sensitive	T5	10.1007/ s12041–012–0201–3
KYUSg_chr5.42448	hydrophobic protein OSR8-like	The over expression of RCI2 has been shown to provide cold stress tolerance.	Tolerant	T1, T2, T3, T4, T5, T6	10.1007/ s00425–015–2386–1
KYUSg_chr6.14911	glycine-rich cell wall structural protein 1	LpGRP1 is related to cold tolerance after cold acclimation.	Tolerant	T2, T3, T4, T5, T6	10.1007/ s00438–005–0095–3
KYUSg_chr6.3990	Dehydrin DHN3-like	High levels of DHN3 have been shown to provide drought stress tolerance.	Tolerant	T4, T6	10.4161/psb.6.10.17088
KYUSg_chr7.20811	cold-responsive protein kinase 1-like	CRPK1 promotes the degradation of CBF proteins by phosphorylating 14–3–3 proteins.	Sensitive	T5, T6	10.1016/j. molcel.2017.02.016
KYUSg_chr7.28634	beta-1,2-xylosyltransferase XYXT1-like	XYXT1 is involved in the response to cold.	Tolerant	T1, T2, T3, T4, T5, T6	10.1093/pcp/pcy003 10.1016/j. plantsci.2015.03.022
KYUSg_chr7.40936	sucrose:sucrose 1-fructosyltransferase-like	1-SST is downregulated in cold tolerant wheat cultivars.	Tolerant	T1, T2, T3, T4, T5, T6	10.1271/bbb.66.2297
KYUSg_scaffold_6468.345	Calcium-dependent protein kinase 26-like	CPK26 has an unknown role.	Sensitive	T1, T2, T3, T5, T6	10.1016/j. tplants.2012.08.008

after 14 days of cold acclimation but no statistically significant differences after the cold acclimation had finished (after 21 days) (Hoffman et al., 2010). Similarly, other researchers showed that the leaf tissue of freezing tolerant L. *perenne* plants exhibited a higher concentration of proline throughout the cold acclimation process than non-freezing tolerant plants (Bocian et al., 2015). It is therefore surprising that proline metabolism genes were enriched in the sensitive genotypes but not in the tolerant ones. During the last stages of cold acclimation (T3 and T4 treatments) the genes upregulated in tolerant genotypes have been demonstrated to belong to circadian rhythm, starch and sucrose metabolism, ribosome biogenesis, and inositol phosphate metabolism pathways. Similar responses to cold have been reported in *Arabidopsis thaliana* and *Brassica napus* (Cheong et al., 2021; Yan et al., 2022). In these same stages of acclimation (T3 and T4), both the sensitive and tolerant genotypes downregulated genes involved in fatty acid elongation. It has been observed that under cold stress, the roots of maize plants display opposite responses, since they upregulate genes involved in fatty acids synthesis and elongation (Guo et al., 2018). Moreover, in tolerant genotypes, genes involved in glutathione metabolism were enriched among DEGs at T1 vs T3 and T1 vs T4. The role of glutathione in cold acclimation relates to its antioxidant ability to control the excess of ROS molecules resultant from the stress response, and to its capability to post-transcriptionally modify proteins involved in cold acclimation (Dorion et al., 2021). The downregulation of fatty acid elongation and glutathione metabolism DEGs could indicate different responses of perennial ryegrass plants towards cold acclimation than the one reported for other monocots like maize.

Transcriptomic profile during freezing

The comparison between the basal (T1) and freezing stress (T5 and T6) conditions uncover similar responses of the genotypes to freezing temperatures. Both genotypes, sensitive and tolerant, have upregulated genes involved in carbon, sucrose and starch metabolism. Sensitive genotypes have upregulated galactose metabolism genes both in T5 and T6, and during the last stages of cold acclimation (T3 and T4), while in tolerant genotypes the upregulation of this pathway is only present at the lowest freezing temperature (T6). In the sensitive genotypes, the

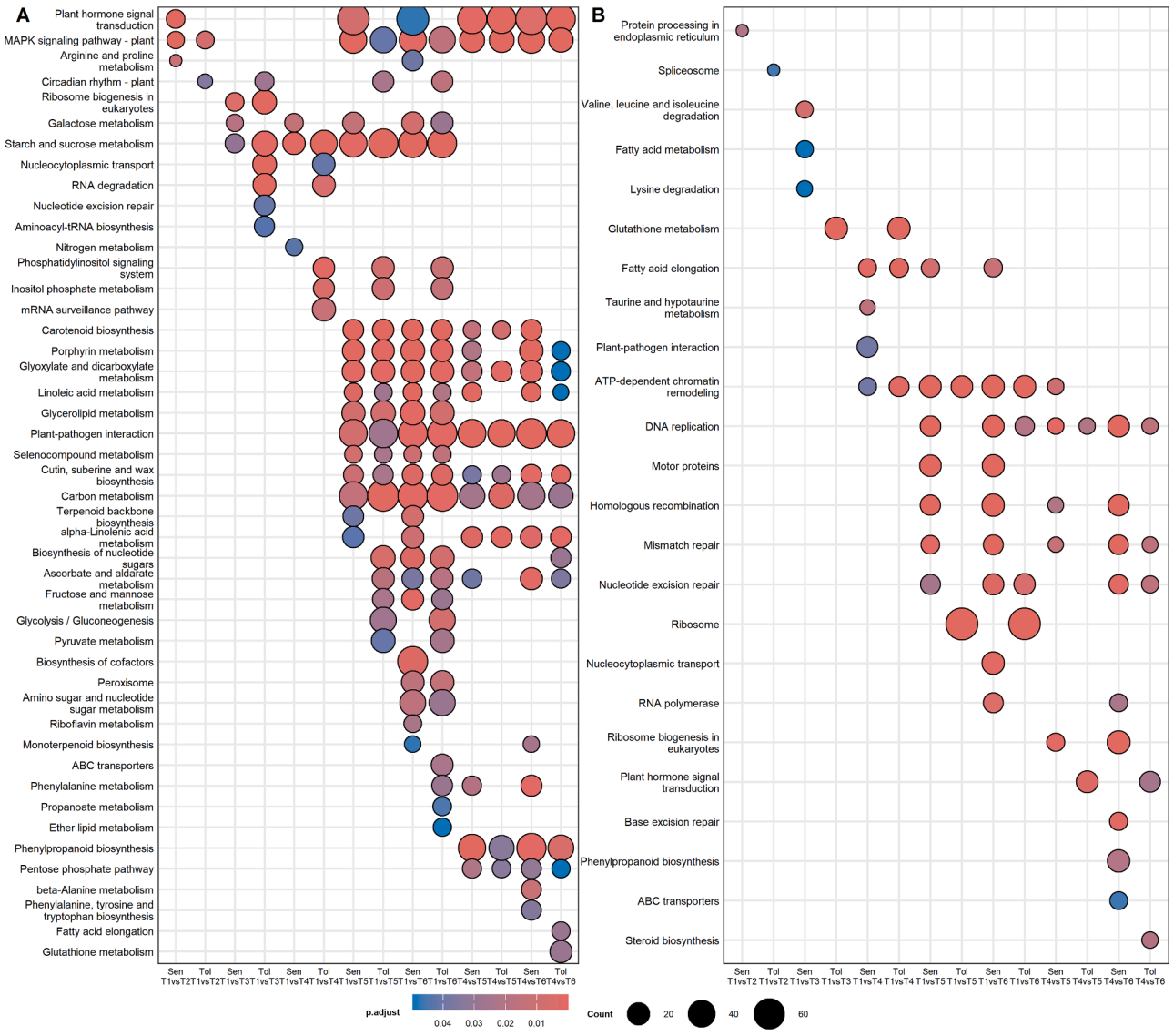

Fig. 5. Enriched KEGG pathways among upregulated genes (A) and downregulated genes (B) during cold acclimation and freezing stress in genotypes with contrasting freezing tolerance, where Sen is the sensitive and Tol is the tolerant perennial ryegrass genotypes. T1 to T6 indicate the treatment, where T1 is control, T2 – beginning of acclimation at +5 °C, T3 – beginning of acclimation at +2 °C, T4 – end of acclimation, T5 – freezing at −5 °C and T6 – freezing at −10 °C.

downregulation of genes involved in cell division and replication, namely the motor protein, ATP-dependent chromatin remodelling, DNA replication and homologous recombination pathways (Kostyrko et al., 2015; Titus and Wadsworth, 2012) is more noticeable in the sensitive genotypes than in tolerant plants. The ATP-dependent chromatin remodelling (in T5 and T6), the DNA replication pathway (in T6) and the ribosome (T5 and T6) pathways are downregulated in the tolerant plants suggesting a decreased cell division and protein synthesis activity in the tolerant genotypes in response to freezing. Additionally, the tolerant genotypes present upregulation of DEGs related to the circadian rhythm in response to freezing stress, similarly to how they behave at the early and mid-stage of cold acclimation. These findings show that tolerant genotypes cease their growth under low-temperature stress during acclimation, which may not be the case with sensitive genotypes. And the opposite, sensitive genotypes have an inadequate cold acclimation process that could undermine their ability to cope with freezing temperatures. In addition, the fact that the tolerant genotypes downregulated DEGs related to pathways previously associated to cold acclimation and freezing tolerance in other plant species, such as the fatty acid elongation and glutathione metabolism pathways, suggests that the studied genotypes of *L. perenne* cope with cold stress in a different, novel or not well-characterized manner.

Functional role of genes identified as differentially expressed between tolerant and sensitive genotypes

Comparative transcriptomic analysis has identified several genes which might be responsible for the differences between the sensitive and tolerant genotypes under low temperature and freezing stress (Fig. 6 and Table 1). The genes coding for ICE2, cold shock protein CS120-like, dehydrin DHN3-like, heat shock protein (HSP) HSF A-2a-like, glutathione S-transferase 1-like, LEA proteins and glycine-rich cell wall structural protein 1 were observed to have significantly higher expression in the tolerant genotypes. These genes are known to play key roles, in the ICE-CBF-COR signalling pathway, protecting cellular structures during stress, enhancing antioxidant capacity, and stabilizing proteins and membranes during freezing stress (Fursova et al., 2009; Hundertmark and Hincha, 2008; Karlson and Imai, 2003; Sustek-Sánchez et al., 2023). In the current study, the tolerant genotypes had significantly lower electrolyte leakages (Fig. 3A) compared to the sensitive

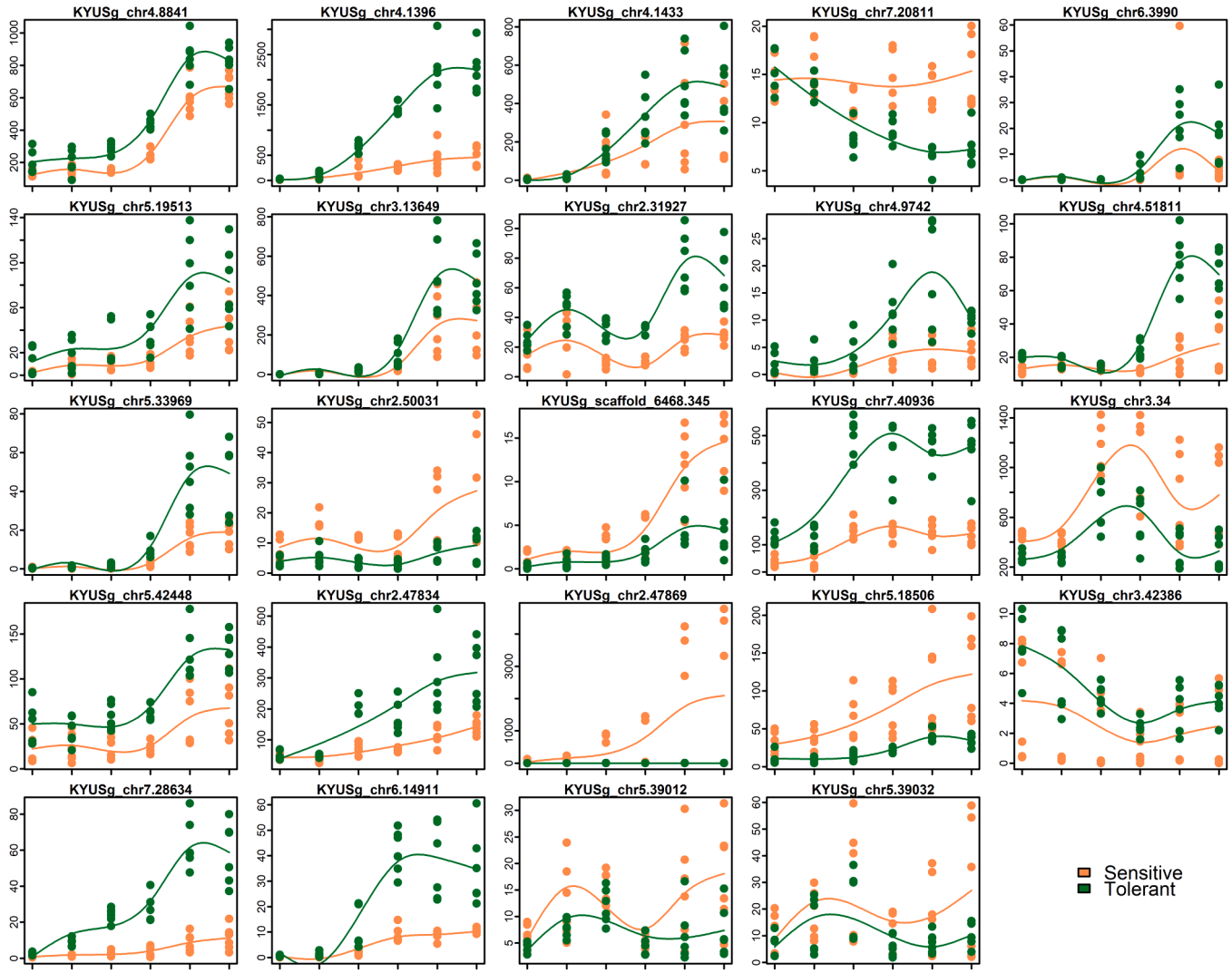

Fig. 6. Some of the genes identified as differentially expressed between the sensitive and tolerant genotypes during cold acclimation and freezing stress. Ticks on the x axis denote treatments (T1 to T6 from left to right) and y axis denotes trimmed mean of M values (TMM) normalized counts per million (CPM).

genotypes, reflecting better cellular integrity under stress conditions. This phenotypic resilience correlates with the robust expression of the aforementioned genes in tolerant genotypes. In contrast, genes coding for DREB 1B-like, beta-fructofuranosidase, and calcium-dependent protein kinase (CDPK) were expressed at a higher level in sensitive genotypes highlighting the contrasting strategies employed by tolerant and sensitive genotypes in response to cold stress. Previous studies have identified DREB1 as a regulator of cold stress responses in Arabidopsis and rice (Liu et al., 1998). When comparing transgenic lines of Arabidopsis with impaired expression of DREB1B with wild-type plants, researchers observed that the survivability of the plants was affected by cold acclimation but that their constitutive freezing tolerance was independent of the DREB1B expression (Novillo et al., 2007). This could be the reason why in our study we observe a higher expression of DREB1B in sensitive genotypes at the beginning of cold acclimation (T2, Fig. 6) than in the tolerant ones, even though they are less resilient towards freezing conditions in terms of their electrolyte leakage. Moreover, genes associated with lipid oxidation, such as acyl-coenzyme A oxidase 4, lipoxygenase 2.3, chloroplastic-like and linoleate 9S-lipoxygenase 3 which upregulate with the onset of freezing temperatures in both genotypes appear to have significant higher expression in sensitive genotypes at T5 and T6 compared to tolerant genotypes (Supp Fig. 3). Over expression of lipoxygenases is linked with increased lipid peroxidation (Lim et al., 2015). Lipid oxidation, while serving as a signalling mechanism can also damage membranes (Alché, 2019; Niki et al., 1991) suggesting us that higher electrolyte leakage in sensitive genotypes could be due to membrane damage as a result of lipid peroxidation. At the beginning of cold acclimation (T2) and at –5 °C (T5), an elevated level of expression was observed of transcription factor MYC2, which is a positive regulator of the ICE-CBF-COR signaling pathway and thus related to freezing tolerance (Song et al., 2022). Higher expression of EARLI1, a lipid transfer protein improving membrane stability under low temperature conditions (Bubier and Schläppi, 2004), was observed in the tolerant genotypes during cold acclimation (T3 and T4) and freezing (T5 and T6), while the sensitive ones expressed it at all stages. Presumably, it may be related to the flowering pattern of sensitive genotypes, as EARLI1 has been reported to respond toward vernalization (Wilkosz and Schläppi, 2000). The gene CDPK13 had elevated expression in both tolerant and sensitive genotypes under freezing temperatures (T5 and T6). Similar responses were shown in a cold tolerant phenotype of rice (Abbasi et al., 2004) and transgenic rice plants overexpressing CDPK13 with improved cold tolerance (Komatsu et al., 2007). However, in our study the sensitive genotypes also had a high expression of CDPK13, suggesting that it may not be related to the different electrolyte leakage observed between the tolerant and sensitive genotypes. 1-SST fructosyltransferase is involved in fructan metabolism.

Its role in cold tolerance was described in perennial ryegrass by Abeynayake et al. (2015a, b) and it was also observed in our studied genotypes. One transcript identified to be translated into 1-SST was differentially expressed in the sensitive plants (KYUSg_chr3.34), while a different transcript had an elevated expression in the tolerant genotypes (KYUSg_chr7.40936). In freezing tolerant wheat plants, the expression levels of this gene have been reported to be downregulated under freezing conditions, suggesting the shift from the synthesis of fructans to simpler sugars (Kawakami and Yoshida, 2002). In both tolerant and sensitive genotypes, the transcript identified as 1-SST was highly expressed at the late stages of cold acclimation (T3 and T4), but the expression decreased with the onset of freezing temperature (T5). Interestingly, expression was higher in the sensitive genotypes than in the tolerant, suggesting they behave similarly to what was reported in winter wheat. The COR413 genes encode both plasma and thylakoid membrane-related proteins involved in cold stress response as part of the ICE-CBF-COR signalling pathway (Breton et al., 2003). Expression of COR413 has been shown to increase in response to cold in *Arabidopsis* which in turn translates into an increased expression of other COR and CBF genes such as COR15 and CBF2 (Hu et al., 2021; Hwarari et al., 2022). This gene had a high expression in the tolerant genotypes during T3 and T4 suggesting its involvement in the cold acclimation of these L. *perenne* genotypes. In the tolerant genotypes, the GRP1 gene had an elevated expression level in all treatments (T2, T3, T4, T5 and T6). This gene has been suggested to be involved in the acquisition of freezing tolerance in L. *perenne* after cold acclimation (Shinozuka et al., 2006) and our results corroborate these findings. Similar results have also been reported in transgenic Arabidopsis plants encoding different GRP genes (including a rice homolog of GRP1) after cold acclimation treatments (Kim et al., 2010). Finally, the CRPK1 gene was also identified to have differential expression between genotypes. CRPK1 has been suggested to be a negative regulator of freezing tolerance and is involved in the degradation of CBF proteins by phosphorylating 14–3–3 proteins that interact with them in the nucleus (Liu et al., 2017). In this study, the expression levels of CRPK1 remained stable throughout the different treatments in the sensitive genotypes, while its expression levels progressively diminished in the tolerant genotypes after the cold acclimation began and continued to decrease in the following treatments. This expression pattern suggests that the sensitive genotypes might have an impaired cold acclimation process, which does not allow the plants to inhibit the degradation of the CBF proteins to achieve freezing tolerance.

Conclusions

The aim of the transcriptomic analysis was to connect the phenotypic response (electrolyte leakage) of freezing tolerant and sensitive perennial ryegrass genotypes with gene expression. The results obtained provide insight into the contrasting response during cold acclimation and freezing stress of the studied genotypes. The phenotypic differences in electrolyte leakage between the tolerant and sensitive genotypes could be attributed to the differences in expression of cold shock protein CS120-like, dehydrin DHN3-like, heat shock protein A-2a-like, LEA proteins, glycine-rich cell wall structural protein 1 and genes associated with lipid oxidation. The sensitive genotypes had in general fewer DEGs compared to tolerant genotypes during cold acclimation and the beginning of freezing stress. The findings imply that sensitive genotypes respond to cold and freezing stress slower resulting into impaired cold acclimation which finally turns into their inability to cope with freezing temperatures. Further investigations should be conducted with the DEGs identified in the study, to better elucidate the pathways responsible for providing freezing tolerance in perennial ryegrass.

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CRediT authorship contribution statement

Akhil Reddy Pashapu: Writing – original draft, Visualization, Formal analysis. **Gražina Statkevičiūtė:** Writing – original draft, Visualization, Investigation, Formal analysis. **Ferenz Sustek-Sánchez:** Writing – review & editing, Writing – original draft. **Mallikarjuna Rao Kovi:** Writing – review & editing, Conceptualization. **Odd Arne Rognli:** Writing – review & editing, Supervision, Conceptualization. **Cecilia Sarmiento:** Writing – review & editing, Writing – original draft. **Nils Rostoks:** Writing – review & editing, Funding acquisition. **Kristina Jaškūnė:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The RNA-seq data generated in this study is available at EMBL-EBI under the accession number E-MTAB-14223.

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Supplementary materials

Appendix. Supplementary materials: The Supplementary Material includes File 1, File 2 and File 3 (containing Supplementary Table 1, Supplementary Fig. 1, Supplementary Fig. 2 and Supplementary Fig. 3).

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100598.

References

Abbasi, F., Onodera, H., Toki, S., Tanaka, H., Komatsu, S., 2004. OsCDPK13, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. Plant Mol. Biol. 55 (4), 541–552. https://doi.org/10.1007/S11103-004-1178-Y/METRICS.

Abeynayake, S.W., Byrne, S., Nagy, I., Jonavičiene, K., Etzerodt, T.P.T.P., Boelt, B., Asp, T., 2015a. Changes in Lolium perenne transcriptome during cold acclimation in two genotypes adapted to different climatic conditions. BMC Plant Biol. 15 (1), 250. https://doi.org/10.1186/s12870-015-0643-x.

Abeynayake, S.W., Etzerodt, T.P., Jonavičienė, K., Byrne, S., Asp, T., Boelt, B., 2015b. Fructan metabolism and changes in fructan composition during cold acclimation in perennial ryegrass. Front. Plant Sci. 6, 329. https://doi.org/10.3389/fpls.2015.00329.

Alché, J., de, D., 2019. A concise appraisal of lipid oxidation and lipoxidation in higher plants. Redox. Biol. 23, 101136 https://doi.org/10.1016/J.REDOX.2019.101136.

Aleliūnas, A., Jaškūnė, K., Statkevičiūtė, G., Vaitkevičiūtė, G., Brazauskas, G., Armonienė, R., 2020. Transcriptome changes triggered by a short-term low temperature stress in winter wheat. Zemdirbyste 107 (4), 329–336. https://doi.org/10.13080/z-a.2020.107.042.

Aleliūnas, A., Jonavičienė, K., Statkevičiūtė, G., Vaitiekūnaitė, D., Kemešytė, V., Lübberstedt, T., Brazauskas, G., 2015. Association of single nucleotide polymorphisms in LpIRI1 gene with freezing tolerance traits in perennial ryegrass. Euphytica 204 (3), 523–534. https://doi.org/10.1007/s10681-014-1330-y.

Blanco-Pastor, J.L., Manel, S., Barre, P., Roschanski, A.M., Willner, E., Dehmer, K.J., Hegarty, M., Muylle, H., Ruttink, T., Roldán-Ruiz, I., Ledauphin, T., Escobar-Gutiérrez, A., Sampoux, J.P., 2019. Pleistocene climate changes, and not agricultural spread, accounts for range expansion and admixture in the dominant grassland species Lolium perenne L. J. Biogeogr. 46 (7), 1451–1465. https://doi.org/10.1111/jbi.13587.

Bocian, A., Kosmala, A., Rapacz, M., Jurczyk, B., Marczak, Ł., Zwierzykowski, Z., 2011. Differences in leaf proteome response to cold acclimation between Lolium perenne plants with distinct levels of frost tolerance. J. Plant Physiol. 168 (11), 1271–1279. https://doi.org/10.1016/j.jplph.2011.01.029.

Bocian, A., Zwierzykowski, Z., Rapacz, M., Koczyk, G., Ciesiołka, D., Kosmala, A., 2015. Metabolite profiling during cold acclimation of Lolium perenne genotypes distinct in the level of frost tolerance. J. Appl. Genet. 56, 439–449. https://doi.org/10.1007/s13353-015-0293-6.

Breton, G., Danyluk, J., Frenette Charron, J.B., Sarhan, F., 2003. Expression profiling and bioinformatic analyses of a novel stress-regulated multispanning transmembrane protein family from cereals and arabidopsis. Plant Physiol. 132 (1), 64–74. https://doi.org/10.1104/PP.102.015255.

Bubier, J., Schläppi, M., 2004. Cold induction of EARLI1, a putative Arabidopsis lipid transfer protein, is light and calcium dependent. Plant Cell Environ. 27 (7), 929–936. https://doi.org/10.1111/J.1365-3040.2004.01198.X.

Buchfink, B., Reuter, K., Drost, H.G., 2021. Sensitive protein alignments at tree-of-life scale using DIAMOND. Nat. Methods 18, 366–368. https://doi.org/10.1038/s41592-021-01101-x.

Chapman, D.F., Wims, C.M., Ludemann, C.I., Kuhn-Sherlock, B., 2023. The contribution of perennial ryegrass (Lolium perenne L.) breeding to whole pasture productivity under dairy cattle grazing in New Zealand. 1. Variation in yield, nutritive value and persistence-related traits. Grass Forage Sci. 78 (1), 64–84. https://doi.org/10.1111/GFS.12590.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34 (17), i884–i890. Bioinformatics, 34(17), i884–i890.

Cheong, B.E., Beine-Golovchuk, O., Gorka, M., Ho, W.W.H., Martinez-Seidel, F., Firmino, A.A.P., Skirycz, A., Roessner, U., Kopka, J., 2021. Arabidopsis REI-LIKE proteins activate ribosome biogenesis during cold acclimation. Sci. Rep. 11 (1), 1–25. https://doi.org/10.1038/s41598-021-81610-z, 2021 11:1.

Dalmannsdottir, S., Jørgensen, M., Rapacz, M., Østrem, L., Larsen, A., Rødven, R., Rognli, O.A., 2017. Cold acclimation in warmer extended autumns impairs freezing tolerance of perennial ryegrass (Lolium perenne) and timothy (Phleum pratense). Physiol. Plant 160, 266–281. https://doi.org/10.1111/ppl.12548.

Ding, Y., Liu, N., Virlouvet, L., Riethoven, J.J., Fromm, M., Avramova, Z., Goh, C., Nam, H., Park, Y., Sung, D., Kaplan, F., Lee, K., Guy, C., Baldwin, I., Schmelz, E., Conrath, U., Beckers, G., Flors, V., Garcia-Agustin, P., Prat, S., 2013. Four distinct types of dehydration stress memory genes in Arabidopsis thaliana. BMC Plant Biol. 13 (1), 229. https://doi.org/10.1186/1471-2229-13-229.

Dong, W., Ma, X., Jiang, H., Zhao, C., Ma, H., 2020. Physiological and transcriptome analysis of Poa pratensis var. anceps cv. Qinghai in response to cold stress. BMC Plant Biol. 20, 362. https://doi.org/10.1186/s12870-020-02559-1.

Dorion, S., Ouellet, J.C., Rivoal, J., 2021. Glutathione metabolism in plants under stress: beyond reactive oxygen species detoxification. Metabolites 11, 641. https://doi.org/10.3390/METABO11090641, 2021Page11(9), 641.

Dray, S., Dufour, A.B., 2007. The ade4 package: implementing the duality diagram for ecologists. J. Stat. Softw. 22 (4), 1–20.

Fursova, O.V., Pogorelko, G.V., Tarasov, V.A., 2009. Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in Arabidopsis thaliana. Gene 429 (1–2), 98–103. https://doi.org/10.1016/J.GENE.2008.10.016.

Guo, X., Liu, D., Chong, K., 2018. Cold signaling in plants: insights into mechanisms and regulation. J. Integr. Plant Biol. 60 (9), 745–756. https://doi.org/10.1111/JIPB.12706.

Helgadóttir, Á., Aavola, R., Isolahti, M., Marum, P., Persson, C., Aleliūnas, A., Brazauskas, G., Krisjánsdóttir, T.A., Asp, T., Rognli, O.A., 2018. Adaptability and phenotypic stability of Lolium perenne L. cultivars of diverse origin grown at the margin of the species distribution. J. Agron. Crop. Sci. 204 (5), 493–504. https://doi.org/10.1111/jac.12273.

Hoffman, L., DaCosta, M., Ebdon, J.S., Watkins, E., 2010. Physiological changes during cold acclimation of perennial ryegrass accessions differing in freeze tolerance. Crop Sci. 50 (3), 1037–1047. https://doi.org/10.2135/cropsci2009.06.0293.

Hossain, M.A., Cho, J.Il, Han, M., Ahn, C.H., Jeon, J.S., An, G., Park, P.B, 2010. The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. J. Plant Physiol. 167 (17), 1512–1520. https://doi.org/10.1016/J.JPLPH.2010.05.008.

Hu, X., Liu, J., Liu, E., Qiao, K., Gong, S., Wang, J., Zhou, A., Zhang, J., 2021. Arabidopsis cold-regulated plasma membrane protein Cor413pm1 is a regulator of ABA response. Biochem. Biophys. Res. Commun. 561, 88–92. https://doi.org/10.1016/J.BBRC.2021.05.032.

Hundertmark, M., Hincha, D.K., 2008. LEA (Late Embryogenesis Abundant) proteins and their encoding genes in Arabidopsis thaliana. BMC Genomics 9 (1), 1–22. https://doi.org/10.1186/1471-2164-9-118/FIGURES/5.

Hwarari, D., Guan, Y., Ahmad, B., Movahedi, A., Min, T., Hao, Z., Lu, Y., Chen, J., Yang, L., 2022. ICE-CBF-COR signaling cascade and its regulation in plants responding to cold stress. Int. J. Mol. Sci. 23 (3), 1549. https://doi.org/10.3390/IJMS23031549, 2022Vol. 23, Page 1549.

Jagodzik, P., Tajdel-Zielinska, M., Ciesla, A., Marczak, M., Ludwikow, A., 2018. Mitogen-activated protein kinase cascades in plant hormone signaling. Front. Plant Sci. 9, 410346 https://doi.org/10.3389/FPLS.2018.01387/BIBTEX.

Jang, J., Lee, S., Kim, J.Il, Lee, S., Kim, J.A, 2024. The roles of circadian clock genes in plant temperature stress responses. Int. J. Mol. Sci. 25 (2), 918. https://doi.org/10.3390/IJMS25020918, 2024, Vol. 25, Page 918.

Jaškūnė, K., Aleliūnas, A., Statkevičiūtė, G., Kemešytė, V., Studer, B., Yates, S., 2020. Genome-wide association study to identify candidate loci for biomass formation under water deficit in perennial ryegrass. Front. Plant Sci. 11, 570204 https://doi.org/10.3389/fpls.2020.570204. December.

Jaškūnė, K., Armonienė, R., Liatukas, Ž., Statkevičiūtė, G., Cesevičienė, J., Brazauskas, G., 2022. Relationship between freezing tolerance and leaf growth during acclimation in winter wheat. Agronomy 12 (4), 859. https://doi.org/10.3390/agronomy12040859.

Jørgensen, M., Østrem, L., Höglind, M., 2010. De-hardening in contrasting cultivars of timothy and perennial ryegrass during winter and spring. Grass Forage Sci. 65 (1), 38–48. https://doi.org/10.1111/j.1365-2494.2009.00718.x.

Juurakko, C.L., diCenzo, G.C., Walker, V.K., 2021. Cold acclimation and prospects for cold-resilient crops. Plant Stress 2, 100028. https://doi.org/10.1016/J.STRESS.2021.100028.

Karlson, D., Imai, R., 2003. Conservation of the cold shock domain protein family in plants. Plant Physiol. 131 (1), 12–15. https://doi.org/10.1104/PP.014472.

Kawakami, A., Yoshida, M., 2002. Molecular characterization of sucrose:sucrose 1-fructosyltransferase and sucrose:fructan 6-fructosyltransferase associated with fructan accumulation in winter wheat during cold hardening. Biosci. Biotechnol. Biochem. 66 (11), 2297–2305. https://doi.org/10.1271/BBB.66.2297.

Kemešytė, V., Jaškūnė, K., Statkevičiūtė, G., 2020. Festulolium field performance under fluctuating growing conditions in Lithuania. Biol. Plant. 64, 821–827. https://doi.org/10.32615/bp.2020.165.

Kemešytė, V., Statkevičiūtė, G., Norkevičienė, E., Jaškūnė, K., 2023. Italian ryegrass as a forage crop for the baltics: opportunities and challenges in light of climate change. Plants. https://doi.org/10.3390/plants12223841.

Komatsu, S., Yang, G., Khan, M., Onodera, H., Toki, S., Yamaguchi, M., 2007. Over-expression of calcium-dependent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants. Mol. Genet. Genom. 277 (6), 713–723. https://doi.org/10.1007/S00438-007-0220-6/TABLES/1.

Kostyrko, K., Bosshard, S., Urban, Z., Mermod, N., 2015. A role for homologous recombination proteins in cell cycle regulation. Cell Cycle 14 (17), 2853–2861. https://doi.org/10.1080/15384101.2015.1049784.

Kovi, M.R., Ergon, Å., Rognli, O.A., 2016. Freezing tolerance revisited—Effects of variable temperatures on gene regulation in temperate grasses and legumes. Curr. Opin. Plant Biol. 33, 140–146. https://doi.org/10.1016/j.pbi.2016.07.006.

Liang, X., Zhang, L., Natarajan, S.K., & Becker, D.F. (2013). Proline mechanisms of stress survival. Home.Liebertpub.Com/Ars, 19(9), 998–1011. 10.1089/ARS.2012.5074.

Liao, Y., Smyth, G.K., Shi, W., 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30 (7), 923–930. https://doi.org/10.1093/bioinformatics/btt656.

Lim, C.W., Han, S.W., Hwang, I.S., Kim, D.S., Hwang, B.K., Lee, S.C., 2015. The pepper Lipoxygenase CaLOX1 plays a role in osmotic, drought and high salinity stress response. Plant Cell Physiol. 56 (5), 930–942. https://doi.org/10.1093/pcp/pcv020.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K., 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10 (8), 1391–1406. https://doi.org/10.1105/TPC.10.8.1391.

Liu, Z., Jia, Y., Ding, Y., Shi, Y., Li, Z., Guo, Y., Gong, Z., Yang, S., 2017. Plasma membrane CRPK1-mediated phosphorylation of 14-3-3 proteins induces their nuclear import to fine-tune CBF signaling during cold response. Mol. Cell 66 (1), 117–128. https://doi.org/10.1016/J.MOLCEL.2017.02.016 e5.

Lund, S.P., Nettleton, D., McCarthy, D.J., Smyth, G.K., 2012. Detecting differential expression in RNA-sequence data using quasi-likelihood with shrunken dispersion estimates. Stat. Appl. Genet. Mol. Biol. 11 (5) https://doi.org/10.1515/1544-6115.1826. Oct 22.

Moustafa, K., AbuQamar, S., Jarrar, M., Al-Rajab, A.J., Trémouillaux-Guiller, J., 2014. MAPK cascades and major abiotic stresses. Plant Cell Rep. 33, 1217–1225. https://doi.org/10.1007/s00299-014-1629-0.

Niki, E., Yamamoto, Y., Komuro, E., Sato, K., 1991. Membrane damage due to lipid oxidation. Am. J. Clin. Nutr. 53 (1), 201S–205S. https://doi.org/10.1093/AJCN/53.1.201S.

Novillo, F., Medina, J., Salinas, J., 2007. Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. Proc. Natl. Acad. Sci. U. S. A. 104 (52), 21002–21007. https://doi.org/10.1073/PNAS.0705639105/SUPPL_FILE/INDEX.HTML.

Olesen, J.E., Trnka, M., Kersebaum, K.C., Skjelvåg, A.O., Seguin, B., Peltonen-Sainio, P., Rossi, F., Kozyra, J., Micale, F., 2011. Impacts and adaptation of European crop production systems to climate change. Eur. J. Agron. 34 (2), 96–112. https://doi.org/10.1016/J.EJA.2010.11.003.

Paina, C., Byrne, S.L., Domnisoru, C., Asp, T., 2014. Vernalization mediated changes in the Lolium perenne transcriptome. PLoS One 9 (9), e107365. https://doi.org/10.1371/journal.pone.0107365.

Prerostova, S., Zupkova, B., Petrik, I., Simura, J., Nasinec, I., Kopecky, D., Knirsch, V., Gaudinova, A., Novak, O., Vankova, R., 2021. Hormonal responses associated with acclimation to freezing stress in Lolium perenne. Environ. Exp. Bot. 182, 104295 https://doi.org/10.1016/J.ENVEXPBOT.2020.104295.

Rapacz, M., Ergon, Å., Höglind, M., Jørgensen, M., Jurczyk, B., Østrem, L., Rognli, O.A., Tronsmo, A.M., 2014. Overwintering of herbaceous plants in a changing climate. Still more questions than answers. Plant Sci. 225, 34–44. https://doi.org/10.1016/j.plantsci.2014.05.009.

Ritonga, F.N., Chen, S., 2020. Plants physiological and molecular mechanism involved in cold stress tolerance in plants. Plants 9, 560. https://doi.org/10.3390/plants9050560.

Rognli, O.A., Aavola, R., Aleliūnas, A., Asp, T., Brazauskas, G., Gylstrøm, K.H., Helgadottir, A., Isolahti, M., Kovi, M.K., Marum, P., Kristjánsdóttir, T.A., Larsen, A. S., Paina, C., Persson, C., Rancāne, S., 2018. Utilization of genebank accessions to improve northern adaptation of perennial ryegrass (Lolium perenne L. G.

Brazauskas, G. Statkevičiūtė, & K. Jonavičienė (Eds.). Breeding Grasses and Protein Crops in the Era of Genomics. Springer, pp. 3–8.

Song, C., Cao, Y., Dai, J., Li, G., Manzoor, M.A., Chen, C., Deng, H., 2022. The multifaceted roles of MYC2 in plants: toward transcriptional reprogramming and stress tolerance by jasmonate signaling. Front. Plant Sci. 13, 868874 https://doi.org/10.3389/FPLS.2022.868874/BIBTEX.

Sustek-Sánchez, F., Rognli, O.A., Rostoks, N., Sõmera, M., Jaškūnė, K., Kovi, M.R., Statkevičiūtė, G., Sarmiento, C., 2023. Improving abiotic stress tolerance of forage grasses – prospects of using genome editing. In: Frontiers in Plant Science, 14. Frontiers Media S.A. https://doi.org/10.3389/fpls.2023.1127532.

Tao, D.L., Öquist, G., Wingsle, G., 1998. Active oxygen scavengers during cold acclimation of scots pine seedlings in relation to freezing tolerance. Cryobiology 37 (1), 38–45. https://doi.org/10.1006/CRYO.1998.2096.

Thomashow, M.F., 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1), 571–599. https://doi.org/10.1146/annurev.arplant.50.1.571.

Titus, J., & Wadsworth, P. (2012). Mitotic spindle assembly: the role of motor proteins. Encyclopedia of Life Sciences. 10.1002/9780470015902.A0022519.

Uleberg, E., Hanssen-Bauer, I., van Oort, B., Dalmannsdottir, S., 2014. Impact of climate change on agriculture in Northern Norway and potential strategies for adaptation. Clim. Change 122 (1–2), 27–39. https://doi.org/10.1007/s10584-013-0983-1.

Ward, C.M., To, T.H., Pederson, S.M., 2020. ngsReports: a Bioconductor package for managing FastQC reports and other NGS related log files. Bioinformatics 36 (8), 2587–2588.

Wilkosz, R., Schläppi, M., 2000. A gene expression screen identifies EARLII as a novel vernalization-responsive gene in Arabidopsis thaliana. Plant Mol. Biol. 44 (6), 777–787. https://doi.org/10.1023/A:1026536724779/METRICS.

Wiréhn, L., 2018. Nordic agriculture under climate change: a systematic review of challenges, opportunities and adaptation strategies for crop production. Land Use Policy 77, 63–74. https://doi.org/10.1016/j.landusepol.2018.04.059.

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., Fu, X., Liu, S., Bo, X., Yu, G., 2021. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. Innovation 2 (3), 100141. https://doi.org/10.1016/J.XINN.2021.100141.

Yan, L., Zeng, L., Raza, A., Lv, Y., Ding, X., Cheng, Y., Zou, X., 2022. Inositol improves cold tolerance through inhibiting CBL1 and increasing Ca2+ influx in rapeseed (Brassica napus L.). Front. Plant Sci. 13, 775692 https://doi.org/10.3389/FPLS.2022.775692/BIBTEX.

Zhang, C., Fei, S.Z., Liu, P., Ji, T., Peng, J., Frei, U., Hannapel, D.J., 2017. Transcriptome changes in response to cold acclimation in perennial ryegrass as revealed by a cross-species microarray analysis. Crop Sci. 57, 179–191. https://doi.org/10.2135/cropsci2016.04.0252, 57: S-179-S-191.