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# Transcriptomic analyses reveal genotype- and organ-specific molecular responses to cold stress in *Elymus nutans*

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## Abstract

*Elymus nutans* is an important alpine perennial forage of the *Pooideae* subfamily, which can survive subzero temperatures. To understand the molecular mechanisms underlying cold tolerance in *E. nutans*, we performed the transcriptional analysis by RNA-Seq in two genotypes, the tolerant Damxung (DX) and the sensitive Gannan (GN), under cold stress. The new *E. nutans* transcriptomes comprised 200 520/200 836 and 181 331/211 973 transcripts in leaves/crowns of DX and GN, respectively. More cold-stress-related genes were identified in leaves than in crowns of both genotypes throughout the whole cold stress. The most prominent functional category in leaves of both genotypes at 3 h of stress was transcriptional regulation. Brassinosteroid and jasmonic acid mediated signalling pathways played central roles in regulating downstream protective responses in DX after 24 h of cold stress. Prolonged cold stress caused more severe transcriptome responses in crowns and leaves of DX compared to GN. The most significant transcriptomic changes in both genotypes were associated with the response to abiotic stresses and the oxidation-reduction processes, implying reprogramming of the cellular metabolism as an adaptation to cold stress. This study reveals mechanisms of genotype- and organ-specific cold stress response in *E. nutans* and thus provides a basis for future breeding strategies aimed at improving the tolerance of cold-sensitive plants.

*Additional key words:* abiotic stress response, brassinosteroids, jasmonic acid, RNA-Seq.

## Introduction

A cold-tolerant perennial plant *Elymus nutans* is mainly distributed on the Qinghai-Tibet Plateau and in the Himalayas (Wang *et al.* 2006). Due to its high adaptability, excellent nutrition, high yield, and resistance to various abiotic stresses, it is frequently used for ecological restoration and the construction of artificial grasslands (Wang *et al.* 2006). One of our previous studies (performed at physiological levels) revealed that this plant has a strong tolerance to cold stress (Fu *et al.* 2016a). However, little is known about the underlying molecular mechanisms of cold tolerance in *E. nutans*.

Complex adaptive strategies have evolved in plants, enabling them to cope with cold stress, including morphological, physiological, and molecular responses (Thomashow 1999, Shi *et al.* 2015). During this adaptive process, plants accumulated osmoprotectants (Xu *et al.* 2014), altered membrane lipid composition (Calzadilla *et al.* 2016), enhanced antioxidant activities (Leyva-Pérez *et al.* 2015), and activated secondary metabolism pathways such as phenylpropanoid biosynthesis (Abeynayake *et al.* 2015). The physiological responses result mainly from changes in the expression of cold-responsive (*COR*) genes, which are controlled by diverse transcription factors. Among these, the C-repeat

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*Abbreviations*: BR - brassinosteroid; CBF - C-repeat binding factor; COG - orthologous groups; COR - cold-responsive genes; DEGs - differentially expressed genes; DX - Damxung; FEH - fructan exohydrolase; GN - Gannan; GO - gene ontology; HSPs - heat shock proteins; JA - jasmonic acid; KEGG - Kyoto encyclopedia of genes and genomes; nr - non-redundant; nt - nucleotide sequence; TFs - transcription factors; 1-SST - sucrose: sucrose-1-fructosyltransferase.
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binding factor (CBF)/dehydration responsive element provides one of the central pathways for the cold response (Chinnusamy *et al.* 2007). Phytohormones play vital roles in the adaptation to abiotic stresses by regulating a cascade of responses (Santner and Estelle 2009). Jasmonic acid (JA) is involved in the response to various stresses, such as pathogen infection, drought, and low temperature stress (Hu *et al.* 2013, Zhao *et al.* 2013). Cold induced accumulation of soluble sugars in plants has been shown to provide an osmoprotective function (Zeng *et al.* 2011). Furthermore, sugars serve as important signalling molecules modulating gene expression *via* highly complex mechanisms (Yue *et al.* 2015). Evidence suggests interactions between sugars and abscisic acid, cytokinin, and auxin signalling pathways (Gibson 2004).

Transcriptome sequencing (RNA-Seq) is an efficient approach to generate functional genomic data for non-model plants or for those with unavailable whole-genome sequencing. RNA-Seq technology has been used to explore the gene expression in response to cold stress in many important forages such as *Leymus chinensis* (Chen *et al.* 2013), *Lolium perenne* (Abeynayake *et al.* 2015), or *Brachypodium distachyon* (Li *et al.* 2012). However, the cold stress-response mechanism in *E. nutans* remains to be identified.

Here, adaptive physiological and transcriptomic mechanisms in leaves and crowns of two *E. nutans* genotypes, the wild genotype Damxung and the cultivar Gannan with contrasting cold tolerance, were investigated under cold stress. We focused on genotype- and organ-specific transcriptome responses as well as on the rearrangement of the primary and secondary metabolism in both genotypes under cold stress.

## Materials and methods

**Plants and treatment:** Seeds of *Elymus nutans* Griseb. cold tolerant genotype Damxung (DX) were collected from wild plants growing in Damxung County, Tibet China (30°28′N, 91°06′E, altitude 4678 m). Seeds of cold sensitive cv. Gannan (GN) were obtained from *Lanzhou Xinglong Grass Industry Technology Service Company* and they were planted in Gannan (33°51′N, 101°40′E, altitude 2500 m), Gansu, China.

Surface-sterilized seeds of the two *E. nutans* genotypes were cultured in a climate chamber at day/night temperatures of 25/25 °C, a 14-h photoperiod, a photon flux density of 350 μmol $m^{-2}$ $s^{-1}$, and a constant humidity of 70 %. Cold stress was performed on these samples by transferring 28-d-old seedlings (three-leaf stage) to growth chambers set to 4 °C under the other conditions described above. Leaf and crown tissues (about 1 cm in length) were taken at 0 h (control), 3 h, 24 h, and 5 d after cold treatments, and then quickly frozen in liquid nitrogen and stored at -80 °C. Experiments were performed in three biological replicates each consisting of 15 pooled leaf or crown tissues from the same 15 seedlings. Three replicates from independent plates, cultivated in parallel, were harvested per time points.

**Morphological and physiological measurements:** Three seedlings were randomly chosen from each group. Plant heights and fresh mass were immediately measured. Relative water content (RWC) in leaves and crowns was measured according to Barrs and Weatherley (1962). Malondialdehyde (MDA) content was measured using 10 % (m/v) trichloroacetic acid (Dhindsa *et al.* 1981). The content of $H_2O_2$ was measured according to the method described by Shi *et al.* (2013). Proline content was measured according to Bates *et al.* (1973).

**RNA extraction and cDNA library construction:** Total RNA was extracted from leaf and crown samples of two genotypes using *Trizol* reagent (*Takara*, Otsu, Japan). The sequencing library construction and sequencing were performed in the Beijing Genomics Institute (BGI), Shenzhen, China, with *Illumina HiSeq 4000* (*Illumina*, San Diego, CA, USA). In total, 48 libraries with a 100 bp single-end reads were constructed and sequencing the purified libraries was carried out on a *HiSeq 4000*.

***De novo* assembly and functional annotation:** Raw reads were filtered to remove low quality reads (more than 20 % Q ≤ 10 bases), adaptor-containing reads, and reads containing more than 5 % ambiguous bases (N) and then the clean reads were obtained (Cock *et al.* 2010). *Trinity v. 2.0.6* (https://trinitysoftwarecenter.com/) software was used for *de novo* transcriptome assembly, then *Tgicl* (ftp://occams.dfci.harvard.edu/pub/bio/tgi/software/tgicl/) was used to obtain non-redundant sequences (Grabherr *et al.* 2011). *De novo* transcriptomes were assembled for DX and GN genotypes separately (minimum contig length of 200). Sequencing data were deposited to the NCBI sequence read archive (SRA) database (accession numbers: SRP074469 and SRP090720).

All of the assembly sequences were used for *BLASTx* (*v.2.2.23*) searches with annotation against the *NCBI Nr* database (data: 2015.12) (ftp://ftp.ncbi.nih.gov/blast/db/). Unigene sequences were also aligned and annotated by *BLAST* searches to other databases in the following order: *Nt* (ftp://ftp.ncbi.nih.gov/blast/db/), *KOG* (ftp://ftp.ncbi.nih.gov/pub/COG/KOG/), *Swiss-Prot* (http://www.uniprot.org/), *KEGG* (http://www.genome.jp/kegg/), and *GO* (http://www.geneontology.org/). The unigenes were divided into singletons and clusters (similarity > 80 %) using *BLASTx*. Sequence orientations were determined in

accordance to the best hit in the database (at an e-value threshold of 1.0 e-5).

**Differentially expressed genes analysis:** Gene expressions for each sample were calculated by fragment per kilobase of exon model per million mapped reads (FPKM). Differentially expressed genes (DEGs) between cold-treated and control samples from both genotypes using *NOIseq* (http://genome.cshlp.org/content/early/2011/09/07/gr.124321.111) A |log2(foldchange)| ≥ 2 and $P \geq 0.8$ was set as the threshold for DEGs (Tarazona *et al.* 2011). For intrinsic transcriptome differences in both genotypes, differential gene expression was analyzed relative to GN genotype. Cluster analyses of the identified DEGs were performed using the complete linkage hierarchical analysis of *TIGR MeV 4.2* (http://www.tm4.org.) software. The *GO* and *KEGG* pathway enrichment analyses were performed using *Phyper of R* (https://www.r-project.org/) sofware. *GO* terms and *KEGG* pathways with false discovery rate (FDR) corrected *P*-value ≤ 0.05 were considered significantly enriched.

**Validation of RNA-seq data by real-time qPCR:** The same RNA samples for the RNA-seq analysis were used for real-time qPCR). The primer pairs used in real-time qPCR reactions are listed in Table 1 Suppl. The relative abundances of transcripts were calculated relative to the reference genes according to Livak and Schmittgen (2001). Each PCR experiment was performed in three biological replicates.

**Statistical analysis:** Differences in physiological responses in both genotypes were examined by one-way *ANOVA*. Means were separated using Duncan's test at $P < 0.05$. Statistical analysis was run by the *SPSS 17.0* software (*SPSS Inc*., Chicago, IL, USA).

## Results

Cold stress significantly reduced plant height and fresh mass of GN, but not of DX (Fig. 1*A,B*). Concurrent with growth inhibition, a lower RWC was detected in leaves and crowns of GN compared to DX exposed to cold treatment for 24 h and 5 d (Fig. 1*C*). MDA and $H_2O_2$ content increased in leaves and crowns of both plants under cold stress ($P < 0.05$), while a smaller increase was observed in DX (Fig. 1*D,E*). DX accumulated 26.92 % and 43.1 % higher proline content than GN in leaves exposed to cold treatment for 24 h and 5 d, while values were 47.76 % and 47.89 % higher in crowns under identical conditions (Fig. 1*F*).

For leaf and crown samples from both genotypes, approximately 65 and 67 Mb clean reads (clean reads ratio > 97 %), containing 6.5 Gb clean bases were obtained. These high-quality reads in crowns of DX and GN were *de novo* assembled *via Trinity*, resulting in 200 836 and 211 973 transcripts with an average length of 1 097 and 1 078 bp and an N50 length of 1 697 and 1 671 bp, respectively. Numbers of transcripts in leaves of both genotypes have been reported in our previous study (Fu *et al.* 2016b). Length distributions of transcripts in leaves and crowns of both genotypes are provided in Fig. 1 Suppl.

The assembled sequences were used to search in public databases, including *NCBI Nr*, *Nt*, *Swiss-Prot*, *COG*, *GO*, *KEGG*, and *Interpro*. In total, 164 827/177 438 (82.20/88.35 %) transcripts in leaves/crowns of DX were matched to a sequence in at least one of the above-mentioned databases, while for GN, matching transcripts were 150 545/186 446 (83.02/87.96 %). The distribution of species of the top *BLASTx* hits against the *Nr* database for the *E. nutnas* transcriptome shows that the transcripts had the maximal similarity with sequences from *Hordeum vulgare subsp. vulgare* and *Aegilops tauschi*, followed by *Triticum urartu* and *Brachypodium distachyon* (Fig. 2 Suppl.).

A total of 62 654 (31.29 %) and 55 886 (30.82 %) transcripts in leaves and 38 008 (28.92 %) and 38 733 (28.27 %) transcripts in crowns of DX and GN were successfully annotated with *GO* terms and classified into 57 functional groups, including 23 groups in biological process, 17 in molecular function, and 17 in cellular components (Fig. 3 Suppl.). *KEGG* annotated 82 192 (40.99 %) and 76 364 (42.11 %) transcripts in leaves as well as 102 103 (50.84 %) and 105 927 (49.97 %) in crowns of DX and GN, respectively. These were classified into five main biochemical pathways (Fig. 4 Suppl.) and most of them were metabolic pathways.

The Venn diagram shown in Fig. 5 Suppl. summarizes the effects of cold treatment on both genotypes, revealing that leaves expressed more cold-stress-related DEGs than crowns at each phase of the cold treatment (5 436 *vs*. 2 234 in DX, and 4 323 *vs*. 2 270 in GN). A total of 4 074 genes in leaves and 2 211 in crowns were differentially expressed during cold-stress. Short-term cold stress induced less transcriptome changes in crowns and leaves of both genotypes, prolonged cold stress, however, caused more pronounced transcriptome responses (Fig. 5 Suppl.). In leaves, the total number of DEGs detected at 3 h, 24 h, and 5 d of cold stress were 1 842/1 580, 1 742/2 225, and 3 639/2 078 in DX/GN, respectively. In crowns, there were 363/471 DEGs at 3 h, 1 033/1 522 at 24 h, and 1 369/914 after 5 d in DX/GN, respectively.

In total, 5 600 DEGs were detected in at least one sample of the leaves and crowns of both genotypes over the cold-stress, compared to their respective controls.

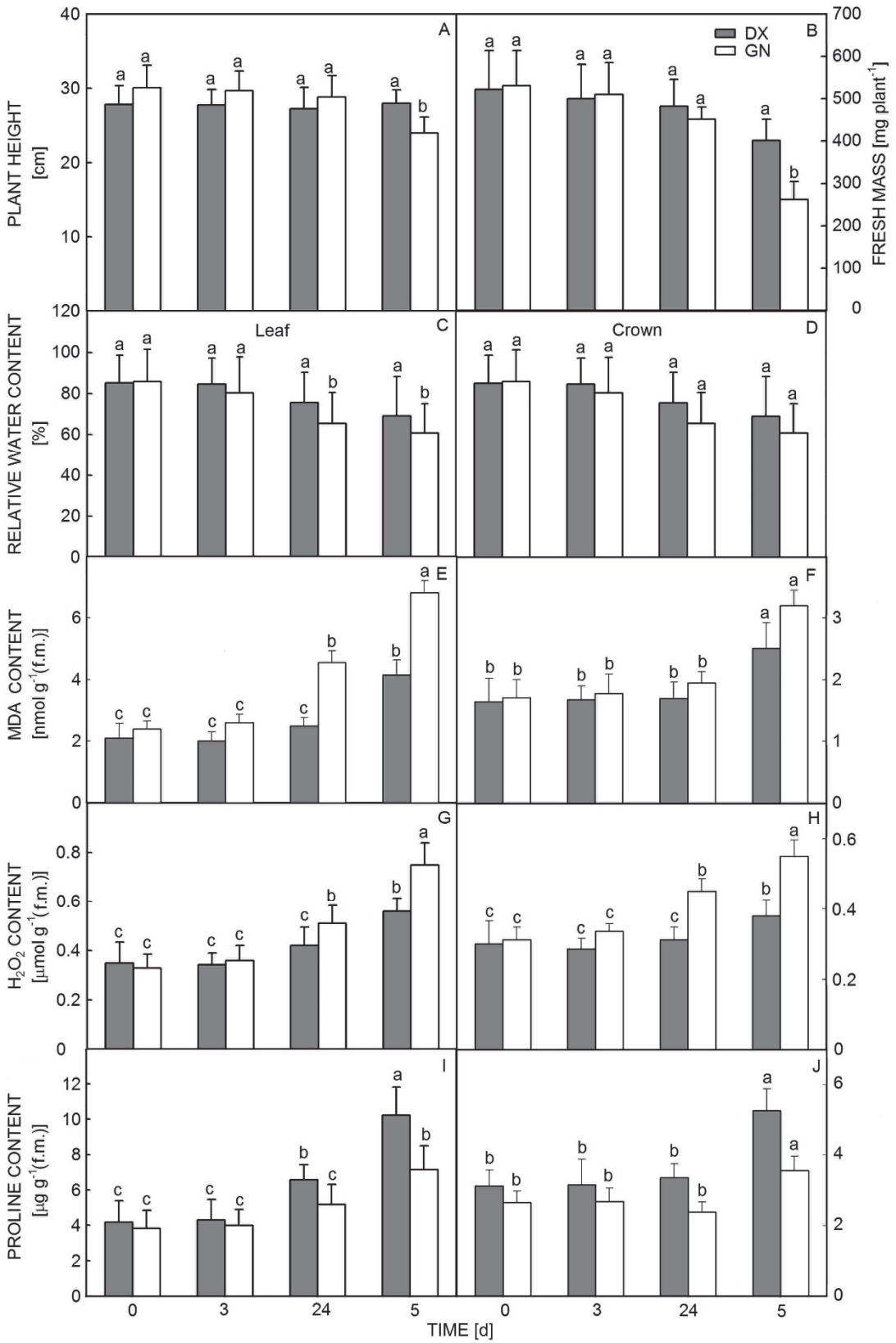


Fig. 1. Effect of cold stress on plant height (*A*), fresh mass (*B*), relative water content (*C*,*D*), malondialdehyde (MDA) content (*E*,*F*), $H_2O_2$ content (*G*), and proline content (*I*, *J*) in the leaves and crowns of DX and GN genotypes. Means ± SE, $n = 3$, different letters indicate significant differences at the 0.05 level according to Duncan test.

Hierarchical clustering revealed that the DEGs clustered separately in leaves and crowns of DX and GN, indicating changes in genotype and tissue specific gene expression under cold stress (Fig. 6 Suppl.).

The cold-induced DEG profiles detected *via* RNA-Seq were confirmed for 23 selected genes using real-time qPCR analysis (Fig. 7 Suppl.). Gene expression profiles identified *via* RNA-Seq exhibited a high degree of similarity ($R^2$ = 0.7267) to those obtained from real-time qPCR analysis, thus confirming reliability and robustness of the RNA-Seq data.

To investigate the intrinsic variation in cold tolerance of DX and GN genotypes, gene expressions under control conditions were analyzed; 918/661 and 688/527 genes were up/down-regulated in leaves and crowns of DX compared to GN. GO enrichment analysis of the DEGs enabled identification of most up-regulated genes in DX leaves that were involved in stress-stimulated transcription regulation, oxidation-reduction processes, lipid and sugar metabolism; however, genes encoding dehydrin, particularly *DHN2*, *DHN3*, *DHN4*, cold-shock protein (*CS120*), and cold-regulated 413 plasma membrane protein (*COR413PM1*) in crowns were significantly enriched. Moreover, the majority of the down-regulated genes were related to phenylpropanoid metabolism.

The largest numbers of DEGs were genotype-specific for each stage of cold stress (Fig. 2). A total of 372/462 and 357/104 genes were exclusively up- or down-regulated in DX/GN leaves (and 91/140 and 127/103 in crowns) after 3 h of cold stress (Fig. 2*A*). Several *GO* terms associated with the transcription regulation were conserved among the DEGs detected in leaves of both genotypes at short-term cold stress. Further analysis revealed that these genes encode CBFs, NAC, HSF, and the auxin-responsive protein (Fig. 3). Maximally enriched

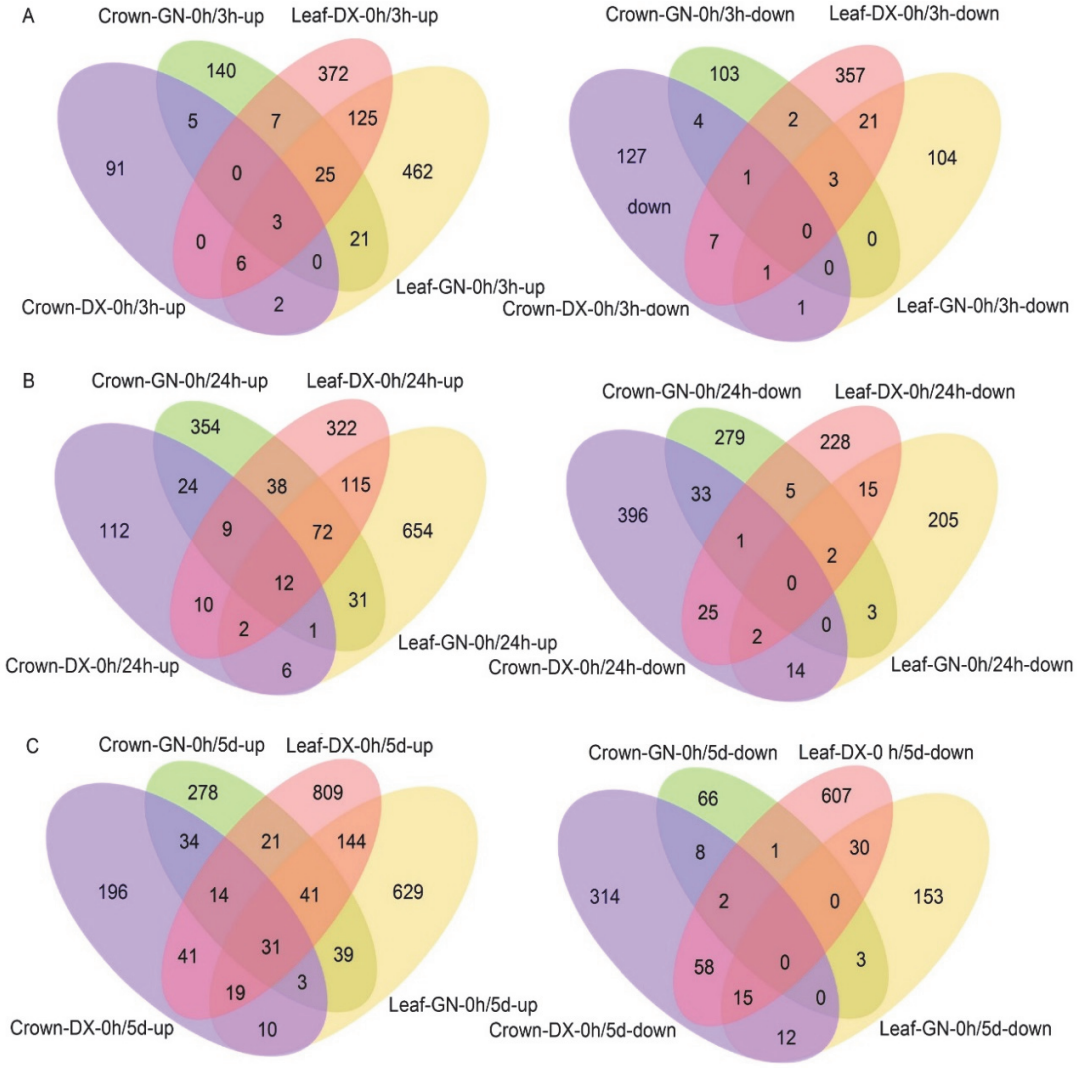


Fig. 2. Comparative analysis of the numbers of DEGs of cold-up-regulated and cold-down-regulated genes found exclusively in the leaves and crowns of DX and GN genotypes under cold stress lasting for 3 h (*A*), 24 h (*B*),and 5 d (*C*).


Fig. 3. Functional categorization of DEGs in leaves and crowns of DX and GN genotypes based on *GO* enrichment analysis. Different colours in the heat map represent different significance levels (corrected *P*-values) of the over-representation: *blue* < 0.05, *pink* < 0.01, *red* < 0.001.

categories of genes in DX crowns were specifically up-regulated in response to stress, those that encode dehydrin in particular. Notably, many genes were down-regulated in leaves and crowns of GN after 3 h of cold stress, including those that respond to stress, hydrogen peroxide catabolic process, brassinosteroid (BR) mediated signalling pathway, and cellular response to BR stimulus. Furthermore, most over-represented *GO* categories of β-fructofuranosidase, fructosyltransferase, and sucrose 1F-fructosyltransferase activity were up-regulated in DX leaves after 3 h of cold exposure, and these fructan biosynthesis-related genes showed a continuous response in both leaves and crowns of DX during cold stress.

The highest number of enriched GO terms was detected in leaves of both genotypes associated with the response to abiotic stress after 24 h of cold stress, including mostly genes encoding HSPs and dehydrin (Fig. 3). Other specifically up-regulated genes in DX leaves included coenzyme M biosynthetic/metabolic process as well as BR/JA mediated signalling pathway. Interestingly, numerous photosynthesis-related genes were induced in DX crowns following 24 h and 5 d of cold stress which is consistent with the previous studies in rice and barley (Kwasniewski *et al*. 2016, Wang *et al*. 2016). (Fig. 3). In contrast to DX, the over-dominant categories for down-regulated genes in GN crowns

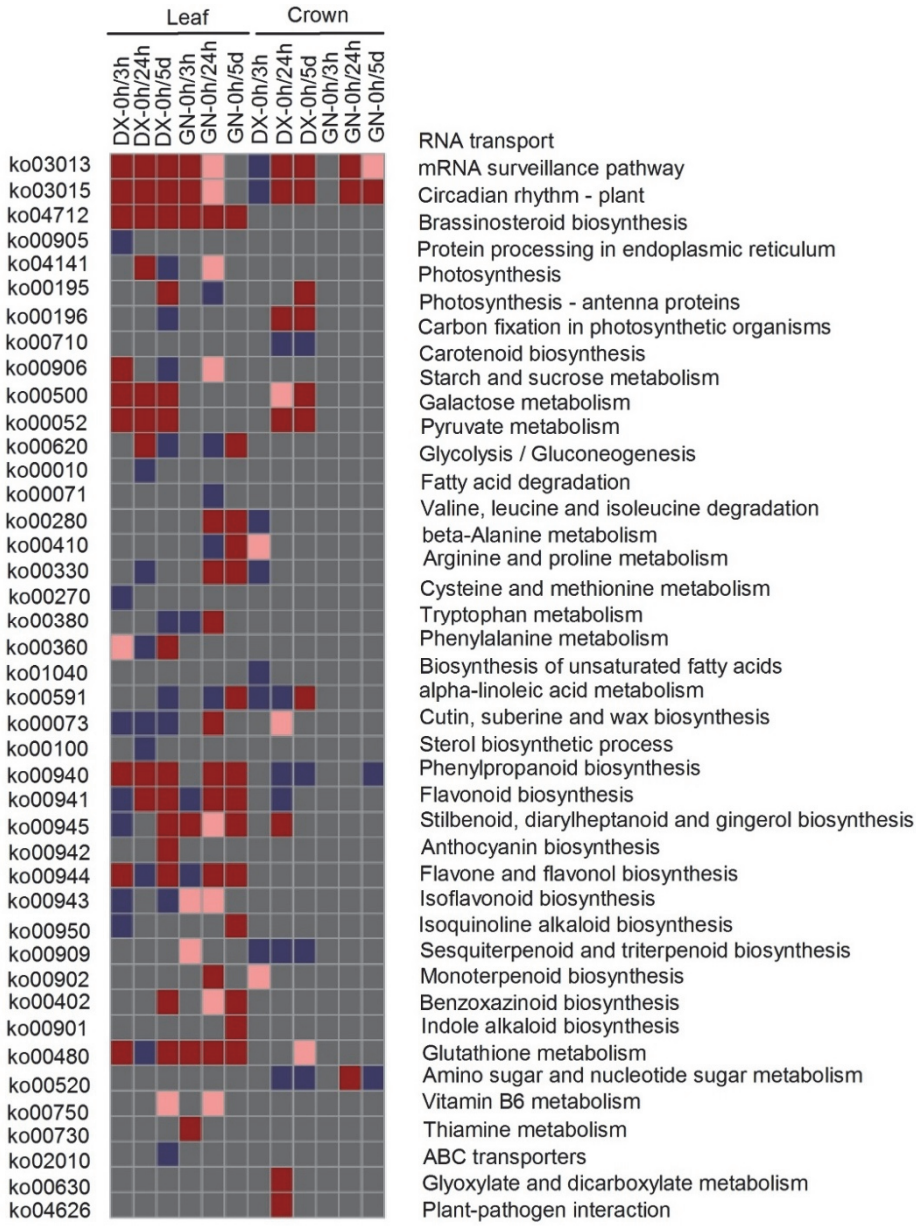


Fig. 4. Major pathways of DEGs in leaves and crowns of DX and GN genotypes based on *KEGG* enrichment analysis. Different colours in the heat map represent different significance levels (Q-values) of the over-representation: *blue* < 0.05, *pink* < 0.01, *red* < 0.001.

included protein complex subunit organization, BR mediated signalling pathway, anatomical structure development, and auxin-activated signalling pathway.

Expression of 1 926 genes was specifically affected *via* extended cold stress in DX, while 1 126 were specifically regulated in GN (Fig. 2*C*). The most up-regulated genes in DX crowns were connected with photosynthesis, while in GN crowns, most of these genes were involved in DNA integration and RNA-dependent DNA replication (Fig. 3). Genes related to abiotic responses were enriched in crowns of both genotypes. Further analysis revealed that dehydrin and chitinase may be important regulators of cold resistance in both genotypes after 5 d of cold stress. Additionally, a pronounced down-regulation of linoleate 13S-lipoxygenase activity was only observed in GN crowns. The majority of genes that were specifically affected by 5 d of cold stress in leaves of both genotypes were associated with response to abiotic stresses and oxidation-reduction processes. Furthermore, most genes involved in transmembrane transport and response to karrikin were up-regulated in DX leaves.

Notably, 311 (16.9 %)/43 (11.8 %) specifically expressed genes in leaves/crowns of DX at the short-term cold stress remained significantly altered at 5 d of cold stress (Fig. 8 Suppl.). In crowns, 11 functionally unidentified genes with high expression abundance (> 5-fold) may be critical for DX adaptation to short-term and long-term cold stress. Additionally, a gene annotated as acyl-[acyl-carrier-protein] desaturase involved in biosynthesis of unsaturated fatty acids was up-regulated in DX crowns during cold. Except for numerous *CORs*, sucrose: sucrose-1-fructosyltransferase (*1-SST*), and most phenylpropanoid biosynthesis-related genes that were induced during the whole cold stress, three TF-related genes were differently regulated in DX leaves. Nevertheless, only dehydration-responsive element-binding protein 1H (*DREB1H*) showed a steady up-regulation over 5 d of cold treatment (Table 2 Suppl.).

The majority of enriched genes persistently regulated in GN leaves were connected with the response to abiotic stresses. Predominantly, up-regulated genes in this group were annotated as *COR* genes. Over the course of cold stress exposure, these genes were moderately induced. Three two-component response regulator proteins associated with the regulation of circadian rhythm were down-regulated in GN leaves. The down-regulation of MADS-box TF (*MADS7*) coordinated with 9 highly down-regulated (< 6 fold) unidentified functional genes in crowns might contribute to the sensitivity of GN (Table 3 Suppl.).

*KEGG* analysis identified a total of 42 major pathways, and most genes of metabolic processes show genotype specificity (Fig. 4). For both genotypes, **t**he highest number of genes involved in RNA transport and mRNA surveillance pathway were observed in short-term pathway were in short-term cold-stress-treated leaves and crowns, while prolonged cold stress revealed the largest number of genes connected with metabolic pathways and biosynthesis of secondary metabolites. Phenylpropanoid, flavonoid, stilbenoid, diarylheptanoid, and gingerol biosyntheses were major secondary metabolic pathways in both genotypes. Most genes of phenylpropanoid biosynthesis were up-regulated in leaves and crowns of both genotypes with high genotype and tissue specificity. In DX, the induced *DHN2*, *Rab15*, and several unidentified functional proteins of this process in crowns, as well as cytochrome P450 involved in stilbenoid, diarylheptanoid, and gingerol biosynthesis in leaves all play important roles in the regulation of cold tolerance. Flavonoid biosynthesis presented differential response patterns to cold stress between genotypes, and more genes were induced in DX leaves. *CHS1*, *CYP75B2*, and *CYP93A3* were induced in DX leaves under cold stress; however, they were down-regulated in GN leaves. Protein processing in endoplasmic reticulum in leaves and amino sugar and nucleotide sugar metabolism in crowns in both genotypes were significantly enriched during long-term cold stress (Fig. 4). Most of the genes within these pathways encoded heat shock proteins (HSP), chitinase, and extensin. In addition, the up-regulation of cutin, suberine, and wax biosynthesis and α-linolenic acid metabolism in DX leaves and crowns may contribute to an improved adaptation to cold stress. Four metabolic pathways were induced in DX only, including biosynthesis of unsaturated fatty acids, BR biosynthesis, sterol biosynthesis, and glycolysis/gluconeogenesis. Interestingly, photosynthesis related pathways were enriched in DX crowns, in which many chlorophyll *a-b* binding proteins and photosystem I reaction center proteins were up-regulated.

## Discussion

The two *E. nutans* genotypes, the cold tolerant DX and cold sensitive GN, were used in this study. As observed in previous studies, accumulation of $H_2O_2$ and MDA in crowns and leaves of both plants under cold stress indicates that oxidative stress occurred (Xu *et al.* 2014, Gao *et al.* 2016); however, lower accumulation was observed in DX. Compared to DX, cold-treated GN seedlings suffered from a more pronounced growth inhibition as well as lower RWC, particularly after 5 d of cold treatment. Proline can protect the integrity of ROS scavengers and it is furthermore an important osmolyte, thus increasing stress tolerance (Xiu *et al.* 2016). DX accumulated more proline and reduced water loss, which may contribute to the cold tolerance of DX.

The most important short-term cold response in leaves and crowns of both genotypes is the specific up-regulation of genes that encode proteins related to abiotic stress responses such as CBF-related TFs, dehydrin, HSPs, glutathione S-transferase, chitinase, 1-SST, and β-fructo-furanosidase. These expressed changes in abiotic stress genes are accompanied by the up-regulation of JA and BR mediated signalling pathway, as well as lipoxygenase and cytochrome $P_{450}$ (*CYP90B1*) in DX leaves during 24 h of cold stress. In contrast, JA and BR mediated signalling pathways were repressed in GN plants. These results suggest that activation of JA and BR signalling plays a central role in regulating the downstream protective responses in DX, which may contribute to a tolerance of DX. This is consistent with our previous study that revealed progressive accumulation of JA content in DX under cold exposure (Fu *et al.* 2016a). Similarly, JA and BR have been reported to trigger plant defence mechanisms when exposed to various abiotic and biotic stresses (Yu *et al.* 2012, Zhao *et al.* 2015). JA enhances freezing tolerance of *Arabidopsis* via positive regulation of the inducer of CBF expression-C-repeat binding factor/DRE binding factor 1 (ICE-CBF/DREB1) pathway (Hu *et al.* 2013).

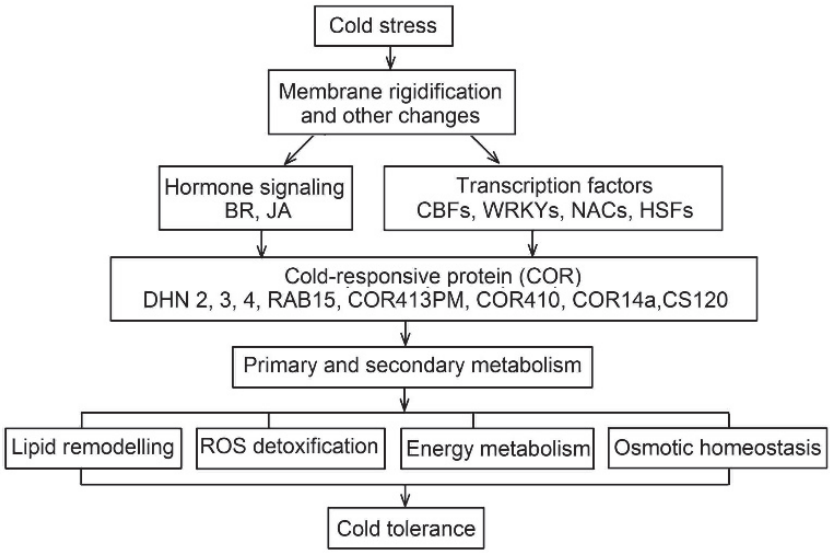


Fig. 5. Hypothetical model summarizing the cold response occurred in *E. nutans* under cold stress. BR - brassinosteroids; CS - cold shock protein; JA - jasmonic acid; CBFs - C-repeat-binding factors; DHN - dehydrin; HSFs - heat stress transcription factor; NAC - NAM, ATAF1/2, and CUC; PM - plasma membrane; RAB -responsive to abscisic acid; ROS - reactive oxygen species, WRKY - WRKYGQK (a highly conserved heptapeptide).

The over-expressed CBFs/DREBs positively correlated with cold tolerance and activated the expression of downstream CORs *via* specific binding to the dehydration responsive element/C-repeat (DRE/CRT) *cis*-acting element in their promotors (Chan *et al.* 2015). In this study, the largest number of cold-inducible TFs belonged to the APETALA2/ethylene-responsive element binding factor (AP2/EREBP) family, which has been shown to be among the best-characterized CBFs/DREBs in the cold regulatory pathway. In DX, we found three TFs that were induced in a sustained manner during cold stress. However, only *DREB1H* showed a steady up-regulation during the entire cold-stress, suggesting that this gene might play a positive role in mediating cold-tolerance in DX. Three genes annotated as two-component response regulators related to the circadian rhythm were down-regulated in GN leaves when subjected to early and extended cold exposure, indicating that the circadian rhythm was disturbed in GN under cold stress. Similar results were obtained for the cold-sensitive *Lolium perenne* when exposed to cold stress (Abeynayake *et al.* 2015). Additionally, the other up-regulated TFs at the short-term cold stress in both genotypes (such as WRKY, NAC, and HSF), have been reported to participate in various cold stress responses (Calzadilla *et al.* 2016). Particularly, WRKY TFs regulate a range of stress-responsive gene expressions *via* a JA-dependent pathway (Diallo *et al.* 2014). HSF TFs have been shown to regulate HSPs expression (Sun *et al.* 2015). Most HSPs serve as molecular chaperones and prevent protein unfolding and aggregation, thus maintaining cellular protein homeostasis and ultimately protecting organisms from damage (Wang *et al.* 2004). In this study, several HSFs were over-expressed in a genotype- and organ-specific manner. Consequently, numerous specific HSPs were up-regulated in leaves and crowns of DX during long-term cold stress. Thus, we speculate that different TFs in both genotypes may partially explain the different transcriptome alterations induced by cold stress.

LEA proteins function as antioxidants and membrane protein stabilizers during cold stress (Tunnacliffe and

Wise 2007). Several dehydrin-related genes were exclusively over-accumulated in DX leaves and crowns. Additionally, *LEA14-A*, *COR413PM*, *COR410*, and *CS120* showed increased transcriptional abundance in DX leaves compared to GN under both control and cold stress conditions, indicating less cellular membrane damage and thus enhancing cold tolerance in DX. Our data support results of Gao *et al.* (2016), who reported for sheep grass that cold-tolerant transgenic plants revealed higher expressions of numerous CORs. Furthermore, 11 highly induced but unidentified functional proteins in crowns may play a vital role in the enhanced cold-tolerance of DX.

Prolonged cold stress induced more drastic changes in oxidation and reduction processes and photosynthesis-related pathways in leaves and crowns of both genotypes. Cellular redox homeostasis is maintained by a complex antioxidant defence system, including antioxidants and antioxidant enzymes like superoxide dismutase, catalase, peroxidase (POD), and glutathione S-transferase (GST) (Foyer and Noctor 2009). DX accumulated lower MDA and $H_2O_2$, in consequence of up-regulated of antioxidant enzyme-related genes, mainly those encoding POD and GST, which implies that DX possesses an improved ROS detoxification capacity in response to cold stress.

The photosynthetic apparatus is a major source of ROS formation (Triantaphylidès and Havaux 2009). Photosynthesis was inhibited in both plants after exposure to continuous cold stress; however, higher photosynthetic capacity was observed in DX. This was confirmed by our previous study showing that higher variavle to maximum fluorescence ratio ($F_v/F_m$) was detected in DX leaves (Fu *et al.* 2016b). The higher photosynthetic capacity in DX leaves was attributed to up-regulation of photosystem II reaction center photosystem II subunit 28 (PSB28) protein and chlorophyll *a-b* binding protein in combination with the down-regulation of photosystem II 10 kDa polypeptide and PS II extrinsic subunits **(**PsbQ-like) protein. Reduction in photosynthesis in GN leaves was partially due to the down-regulation of PSB28, the inactivation of ribulose bisphosphate carboxylase small chain protein (RBCS), and decreases in chlorophyll biosynthesis. Interestingly, many photosynthesis-related genes, including chlorophyll *a-b* binding protein, photosystem I reaction center proteins, and *RBCS* were up-regulated in DX crowns. This is consistent with the previous studies in rice and barley under drought and salt stress (Kwasniewski *et al.* 2016, Wang *et al.* 2016). These up-regulated photo-synthesis-related genes in DX crowns may play a protective role, rather than a role in photosynthesis.

Reduced photosynthesis regularly alters soluble sugar content under cold stress. In DX leaves, most genes involved in starch and sucrose metabolism pathways were up-regulated within 24 h of cold stress, while most were inhibited in leaves and increased in crowns during prolonged cold stress. This could reflect an existing sink-source relationship between crowns and leaves. In leaves and crowns of DX, most genes encoding 1-SST involved in fructan biosynthesis were up-regulated; however, fructan exohydrolases (FEHs), which are related to fructan degradation were down-regulated. This result is in agreement with a report on cold-treated *Lolium perenne*, with higher fructan content in the tolerant genotype (Abeynayake *et al.* 2015). Additionally, cold induced genes encode trehalose biosynthesis enzymes in leaves and crowns of DX. The accumulated soluble sugars not only act as important osmoprotectants, but play a central role as ROS scavengers, and as signalling molecules modulating expression of numerous genes in response to diverse stresses (Keunen *et al.* 2013).

Photosynthesis rate declines under stress and cellular energy is particularly provided *via* glycolysis and TCA cycle (Fernie *et al.* 2004). Up-regulation of glycolysis/ gluconeogenesis pathways in DX leaves is essential to avoid energy starvation, caused by reduced photo-synthesis after prolonged cold stress. Fatty acid and amino acid breakdown are critical to maintain energy status during stress conditions. In GN leaves, fatty acid degradation as well as valine, leucine, and isoleucine degradation were up-regulated during long-term cold stress, while the down-regulation of valine, leucine and isoleucine degradation and increased proline biosynthesis were observed in DX plants. The valine, leucine, isoleucine, and proline are known to play positive roles in responses to diverse abiotic stresses (Joshi *et al.* 2010).

The accumulation of unsaturated fatty acids and sterol can enhance cold-tolerance by maintaining membrane fluidity when subjected to cold stress (Leyva-Pérez *et al.* 2015). The up-regulation of α-linolenic acid metabolism in leaves and crowns of DX and down-regulation in GN, suggests that α-linolenic acid metabolism may contribute to the improved cold tolerance of DX. Enhanced α-linolenic acid metabolism has also been observed in the subnival alpine plant *Chorispora bungeana* (Zhao *et al.* 2012). Additionally, increased expression of genes, associated with lipid transfer protein and cutin, suberine, and wax biosynthesis detected in DX leaves, further confirmed that DX possesses a more efficient mechanism to rearrange lipid metabolism, thus alleviating cellular damage under cold stress.

Cold stress affected plant growth and development in both genotypes *via* adjustment of cell wall composition. In DX leaves, the up-regulation of extensin involved in cell wall loosening and the down-regulation of xyloglucan endotransglucosylase/hydrolase protein (XTH), which is associated with cell wall-strengthening indicates that the DX leaf is kept in a growth-ready state under cold stress. This is supported by an increased plant height in DX after 5 d of cold stress. Similar results were observed for *Brachipodium distachyon* exposed to drought stress (Verelst *et al.* 2013). Beyond cell wall loosening, cell expansion requires the co-ordinated

uptake of water. Enhanced aquaporin accumulation in DX leaves during extended cold stress might allow maintenance of water uptake and, combined with increased cell wall loosening, ensure continuous cell elongation, resulting in diminished growth inhibition in DX under cold stress. Gibberellins (GAs) are associated with the promotion of cell expansion *via* cell wall loosening (Verelst *et al.* 2013). In this study, enhanced expression of $GA_{20}$-oxidases, together with the up-regulation of genes that encode cellulose biosynthesis involved in cell wall biogenesis, may cause reduced plant height in GN when subjected to cold stress. This may result from the increased expression of GA oxidases, leading to GA inactivation and reducing GA content, in turn stabilizing DELLA proteins, thus inhibiting leaf growth (Dubois *et al.* 2013).

Phenylpropanoid metabolism produces a series of secondary metabolites that are involved in protective mechanisms against abiotic stresses (Vogt 2010). Flavonoid accumulation protects plants since they act as antioxidants, thus preventing cellular oxidative damage, and they are also likely osmoregulators maintaining water homeostasis (Yonekurasakakibara *et al.* 2014). Most enzymes participating in phenylpropanoid and flavonoid biosyntheses in DX leaves and crowns were induced during cold exposure. The up-regulation of genes that are involved in these pathways may help to adjust homeostasis and scavenge ROS, which contributes to improved cold tolerance in DX.

In conclusion, this study provides a comprehensive analysis of the physiological and transcriptomic responses to cold stress in both leaves and crowns of two *E. nutans* genotypes with different cold-tolerance. Cold-induced transcriptomic responses of both *E. nutans* were largely genotype- and organ-specific. Based on the physiological and transcriptomic analysis, a model for depicting the events involved in cold stress in *E. nutans* could be summarized in Fig. 5. Complex relationships between hormone signalling and TFs cooperatively regulate cellular responses to cold stress in both genotypes. The enhanced cold tolerance of DX could be associated with numerous specific CORs and unidentified functional proteins induced by cold. These in turn further activate physiological defence responses, including the adjustment of membrane lipid remodelling, enhancement of ROS detoxification ability, modifications of cell wall architecture, and maintenance of cellular osmotic and energy metabolic homeostasis. The present study provides significant cues for further studies of the molecular mechanisms underlying cold stress responses in plants.

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