Transcriptome analysis of *Sonneratia caseolaris* seedlings under chilling stress

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Sonneratia caseolaris is a native mangrove species found in China. It is fast growing and highly adaptable for mangrove afforestation, but suffered great damage by chilling event once introduced to high latitude area. To understand the response mechanisms under chilling stress, physiological and transcriptomic analyses were conducted. The relative electrolyte conductivity, malondialdehyde (MDA) content, soluble sugar content and soluble protein content increased significantly under chilling stress. This indicated that *S. caseolaris* suffered great damage and increased the levels of osmoprotectants in response to the chilling stress. Gene expression comparison analysis of *S. caseolaris* leaves after 6 h of chilling stress was performed at the transcriptional scale using RNA-Seq. A total of 168,473 unigenes and 3,706 differentially expressed genes (DEGs) were identified. GO and KEGG enrichment analyses showed that the DEGs were mainly involved in carbohydrate metabolism, antioxidant enzyme, plant hormone signal transduction, and transcription factors (TFs). Twelve genes associated with carbohydrate metabolism, antioxidant enzyme, phytohormones and TFs were selected for qRT-PCR verification, and they indicated that the transcriptome data were reliable. Our work provided a comprehensive review of the chilling response of *S. caseolaris* at both physiological and transcriptomic levels, which will prove useful for further studies on stress-responses in mangrove plants

Transcriptome Analysis of *Sonneratia caseolaris* Seedlings under Chilling Stress

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Abstract

Sonneratia caseolaris is a mangrove species native to China. It is fast growing and highly adaptable for mangrove afforestation, but injured by cold temperatures when grown at high latitudes. To understand the response mechanisms under chilling stress, we conducted physiological and transcriptomic analyses. The relative electrolyte conductivity, malondialdehyde (MDA) content, soluble sugar content and soluble protein content increased significantly under chilling stress. This indicated that *S. caseolaris* suffered damage and increased the levels of osmoprotectants in response to the chilling stress. We performed gene expression comparison analysis of *S. caseolaris* leaves after 6 h of chilling stress at the transcriptional scale using RNA-

Seq. A total of 168,473 unigenes and 3,706 differentially expressed genes (DEGs) were identified. GO and KEGG enrichment analyses showed that the DEGs were mainly involved in carbohydrate metabolism, antioxidant enzyme, plant hormone signal transduction, and transcription factors (TFs). Sixteen genes associated with carbohydrate metabolism, antioxidant enzyme, phytohormones and TFs were selected for qRT-PCR verification, and they indicated that the transcriptome data were reliable. This study reviewed the chilling response of *S. caseolaris* at both physiological and transcriptomic levels, and the results increase our understanding of stress-responses in mangrove plants.

Keywords: *Sonneratia caseolaris*; chilling stress; RNA-Seq; Differentially expressed genes

Introduction

The growth, development, and geographical distribution of plants are limited by temperatures in their habitat (Jin *et al*., 2013). Low temperature stress can be divided into chilling stress (<10°C) and freezing stress (<0°C) (Zhou *et al*., 2011). Plants have evolved a series of response mechanisms to adapt to low-temperature environments. These include regulation of gene expression and physiological and biochemical process changes that enhance cold resistance (Thomshow, 1999; Sung, *et al*., 2003), These changes have been studied in model plants like *Arabidopsis thaliana* and some cereal crops ((Buti *et al*., 2018; Zhang *et al*., 2004; Roman *et al*., 2012). Response mechanisms enhancing cold resistance mainly include signaling cascade, modification of the membrane lipid composition, accumulation of osmoprotectants, and increased activity of antioxidant enzymes to scavenge reactive oxygen species (ROS) (Theocharis *et al*. 2012; Nievola *et al*. 2017; Nakashima *et al*., 2009). In addition, phytohormones such as auxin, abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), salicylic acid(SA), gibberellin(GA), cytokinin(CK), and brassinosteroid (BR) and their signal transduction related genes also involved in regulating the gene expression in response to low-temperature stress (Peleg *et al*., 2011). Exogenous ABA improved the cold tolerance of *Brassica napus* (He, *et al*., 2019). Some members of WRKYs and MYBs transcription factors (TFs) regulated gene expression to participate in plant

cold resistance (An, *et al*., 2020; Marè, *et al*., 2004).

Mangroves are halophytic temperature sensitive shrubs or trees that grow in the intertidal areas of tropical and subtropical coasts. They include about 70 species from 20 plant families (Duke, 1992). Mangroves are important in carbon balancing, protecting coastlines and beaches from floods and storms, reducing soil erosion, and providing habitats and shelters for animals. They also provide food, wood, and medical compounds for humans (Duke, 1992; Tomlinson, 1999; Schmitt and Duke, 2015). Large areas of mangroves, however, have been converted to agriculture, aquaculture, and industrial use (Duke *et al*., 2007; Robertsen and Alongl, 1992; Wang and Wang, 2007). There are approximately 22,684 ha of mangroves in China, which is 44% of the area existing in the 1950s (survey by State Forestry Administration in 2001). Since 2001, the government of China has focused on mangrove protection and restoration using conservation measures and large-scale afforestation in southeast China. The mangrove area in 2019 had increased to 29,000 ha. *Sonneratia caseolaris* is a mangrove species that is naturally distributed in the Hainan islands. Because of its rapid growth and adaptability, it was introduced to more northern and colder areas in China, such as Shenzhen and Leizhou in Guangdong Province, Qinzhou and Fangchenggang in Guangxin Province in 1994, where it grows well (Wang *et al.,* 2002; Zan *et al*., 2003). *S. caseolaris* suffered substantial chilling injury in 2008, and there was heavy defoliation in all growing areas. All of the trees in Guangxi Province died. There was also significant defoliation of trees in native locations of Wenchang. Young seedlings were the most susceptible growth stage and suffered the great injury (Chen *et al.,* 2017). Mangrove species with greater tolerance to low temperature are needed for mangrove restoration. Understanding the molecular mechanism of *S. caseolaris* tolerance to chilling stress will be of great value in restoration efforts.

Next-generation sequencing (NGS) is an efficient and inexpensive method for collecting transcriptome data. It can be used for species that have not been sequenced. Based on RNA-Seq platforms, genome-scale transcriptome analyses have been used to identify the mechanism of stress response without whole-genome sequence information in different species (He *et al*., 2015; Kumar *et al*., 2015). Transcriptome analysis has been used to study adaptive mechanism of

mangroves, such as *Acanthus ilicifolius*, *Avicennia offcinalis*, *Bruguiera gymnorhiza*, *Aegiceras corniculatum* and *Kandelia obovata* (Fang *et al*., 2016, Krishnamurthy *et al.,* 2017, Su *et al*., 2019, Yamanaka *et al*., 2009; Yang *et al*., 2015a; Yang *et al.,*2015b). The chilling stress mechanisms of *S. caseolaris*, however, remains unknown.

To understand the mechanisms of *S. caseolaris* under chilling stress, we first measured the physiological changes in *S. caseolaris* under chilling stress, Then, we used RNA-seq for comparative analyses of *S. caseolaris* leaves between a control group (0 h, CK) and a chilling treated group (4°C 6 h, CT). The reliability of the transcriptome results was verified by real-time quantitative polymerase chain reaction (qRT-PCR). The data improved our understanding of the chilling response mechanism in *S. caseolaris* and identified potential cold tolerance gene targets in this, and other mangrove species.

MATERIALS AND METHODS

Plant materials and treatments

We collected ripe fruits of *S. caseolaris* in Hainan Dongzhaigang National Nature Reserve (19° 57′ 23″ N, 110° 30′ 10″ E) in China. The fruit was soaked in distilled water for 7 d and then individually planted in pots with a mud and sand (1:1) mixture as support. Growth chamber conditions were 30/28°C (day/night) and a 14:10 h (L: D) photoperiod, 400 μmol m^{-2} s^{-1}photon flux density, and 90% constant humidity. The germinated seeds were cultured in a growth chamber for 5 weeks, at which time the seedlings were about 12 cm high with 12 leaves. We performed the chilling stress treatment on these seedlings in a growth chamber, by setting the temperature to 4°C and leaving the other conditions unchanged. The second leaf from the top of the seedlings were collected as samples from 0 h (CK) 3 h, 6 h, 12 h to 24 h separately, and then frozen in liquid nitrogen and stored at -80°C. Each treatment had three biological replicates.

Physiological response assays of chilling-treated seedlings

We measured relative electrolyte conductivity following Wang *et al*. (2017). The

malondialdehyde (MDA) content was measured by the method of Yao *et al* (2011). The soluble sugar content was measured by the method of Wang *et al*. (2013). The soluble protein content was measured by using the Bradford method (Bradford *et al.,* 1976). Three biological replicates were measured at each time point.

RNA extraction

Based on the physiological results, we used the seedlings without chilling as the control group (CK_1, CK_2, CK_3) and seedlings under chilling stress for 6 h were set as the treatment group (CT_1, CT_2, CT_3) for RNA-seq. Total RNA of leaf samples was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. RNA purity was checked by T6 Spectrophotometer (Puxi, Beijing, China). RNA integrity and concentration were measured by the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

cDNA library construction and transcriptome sequencing

A 3 μg amount of total RNA of each sample was enriched using magnetic beads with Oligo (dT) and then fragmented into short fragments ~200 bp by an interrupting reagent. The first strand cDNA was synthesized by a random hexamer primer, and the second strand of cDNAs was synthesized using the buffer, dNTPs, DNA polymerase I, and RNase H. After the double-stranded cDNA was generated and processed with end-repair, A-tailed, and adapters ligation, we constructed a cDNA library by PCR amplification. The cDNA library was then sequenced using the BGISEQ-500 platform with paired-end reads of 100 bp, by the Beijing Genomic Institution (www.genomics.org.cn, BGI, Shenzhen, China). All raw reads data from this study were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive database under the accession number PRJNA668053.

Transcripts assembly and functional gene annotation

The raw data were filtered with SOAPnuke (v1.5.2) (Li *et al.,* 2008). We discarded the low quality reads such as those with adapter sequences, ambiguous bases 'N' is more than 5%, with more than 20% Q<10 bases. The remaining clean data were used for *de novo* assembly with the Trinity program (v2.0.6) (Grabherr *et al.,* 2011) and then the unigene sequences were aligned with the following databases: NR, NT, SwissProt, KEGG, KOG, Pfam and GO by BLAST software (version 2.2.26).

Differential expression analyses

The expression level of each transcript was measured by RSEM (v1.2.12), and the expression level of a single gene was expressed as the value of FPKM (fragments per kilobase of transcript per million mapped reads) method (Li *et al.,* 2011). For the sake of determining the differentially expressed genes (DEGs), we performed differential expression analysis by the DESeq2 (Anders and Huber, 2010). A P-value $\leqslant 0.05$ and an expression level $|\log2(\text{ratio})| > 2$ were regarded as significant differential expression. Gene ontology (GO) enrichment analysis of DEGs was conducted using the GOseq R package, GO terms with $p < 0.05$ were regarded as significantly enriched in DEGs (Young *et al.,* 2010). We analyzed the KEGG pathway functional enrichment of DEGs using the R package. Significant enrichment was defined as when the FDR terms were < 0.001.

Quantitative real-time PCR analysis

We selected 12 DEGs to verify the reliability of transcriptome sequencing through qRT-PCR. The specific primers of qRT-PCR were designed by Primer3 software (http://primer3.ut.ee/) and are listed in Table S1. The qRT-PCR analyses were conducted using a LightCycler 480 RT-PCR system (Roche Applied Science, Mannheim, Germany) under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 20 s. Three replicates of each cDNA sample were performed for qRT-PCR analysis. We calculated the relative expression of each gene by the 2^{-DDCt} method (Livak *et al*., 2001) and used the actins gene of *S. caseolaris* as a

reference.

RESULTS

Physiological responses of *S. apetala* seedlings under chilling stress

After 3 h exposure to 4°C, the leaves at the top of *S. caseolaris* seedlings began to show a slight curling, and after 6 h, the leaves in the top showed evident curling. After 12 h of chilling stress treatment, part of leaves had softened and exhibited loss of strength. After 24 h of 4°C chilling stress, most of leaves had withered (Figure 1). Electrolyte leakage, MDA content, soluble sugar content and soluble protein content increased significantly (Figure 2). The electrolyte leakage increased from 6.54 ± 0.46% (0 h) to 28.56 ± 5.32% (24 h); the MDA content increased from 2.15 ± 0.79 μmol/g (0 h) to 14.01 ± 1.58 μmol/g (24 h); the soluble sugar contents increased from 2.79 ± 0.76 μmol/g (0 h) to 7.87 ± 1.67 μmol/g (24 h); and the soluble protein content increased from 6.00 ± 0.84 μmol/g (0 h) to 33.83 ± 2.27 μmol/g (24 h). All of these parameters showed a linear increase. When exposed to chilling stress for 6 h, these parameters increased 118.68%, 299.98%, 160.67% and 56.15% compared with the control group. On the basis of these results, we selected the leaf samples exposed to 0 h and 6 h of chilling stress for RNA-seq to investigate the changes in genome-wide gene expression mechanism under chilling stress. The 0 h time the control (CK) and 6 h was regarded as the treatment group (CT), with three biological replicates.

Sequencing and *de novo* assembly of transcriptome

In total, we obtained 39.33 Gb clean bases from 6 cDNA libraries, and we obtained a total of 262.21 M clean reads from 280.46 M raw reads (Table S2). The clean reads were *de novo* assembled by Trinity software and they yielded 168,473 unigenes. All unigenes were longer than 200 bp and ranged from 297 to 16,287 bp. The 200-300 bp unigenes accounted for the largest proportion (13,159, 12.26%) (Figure S1A). The mean unigene length was 1,762 bp. The N50 and N90 lengths were 2,849 bp and 937 bp respectively, and the GC content was 43.94%.

189

190 **Gene annotation and functional classification**

191 To predict the putative function of the assembled unigenes, we used BLAST to compare seven
192 databases including NR, NT, SwissProt, KEGG, KOG, Pfam and GO. The E-value cut-off was 10^{-5}.
193 A total of 125,360 (74.41%) unigenes were predicted to be homologous with known genes from
194 the databases listed earlier (Table S3). Among them, 116,619 (69.22%), 85,761 (50.90%), 90,660
195 (53.81%), 94,737 (56.23%), 100,382 (59.58%), 96,869 (57.50%) and 92,876 (55.13%) unigenes
196 were found in the seven databases. A total of 48,858 (29.00%) were found in all seven databases.
197 The NR database had the highest proportion of annotations, with 69.22% unigenes being
198 annotated. In the NR database, the unigene sequences exhibited the most similar BLASTx matches
199 to gene sequences from *Punica granatum* (84,763, 72.69%), followed by *Eucalyptus grandis*
200 (4,627, 3.97%) amd *Quercus suber* (1125, 0.96%) (Figure S1B).

201 We classified the functions of the *S. caseolaris* unigenes by GO analysis and categorized
202 92,876 of the unigenes into 44 functional groups (Figure S1C). These groups were classified into
203 the following three major GO categories: 18 functional groups in “biological processes”, 12
204 functional groups in “cell component”, and 14 functional groups in “molecular function”. The
205 dominant subcategories of the classified genes included “metabolic process (9,916)”, “cellular
206 process (22,187)”, and “biological regulation (10,062)” in the “biological processes” category;
207 “membrane part (29,068)”, “cell (23,014)”, and “organelle part (9,821)”in the “cell component”
208 category; “binding”(46,922), “catalytic activity (44,729)”and “transporter activity (5,902)”in the
209 “molecular function” category. To analyze the intracellular metabolic pathways and biological
210 functions in *S. caseolaris*, we conducted a KEGG analysis using the KEGG pathway tool. Genes
211 were annotated to five categories. 1 functional group in “Cellular Processes”, 2 functional groups
212 in “Environmental Information Processing”, 3 functional groups in “Genetic Information
213 Processing”, 11 functional groups in “Metabolism”, and 1 functional group in “Organismal
214 Systems” (Figure S1D).

215

GO term enrichment analysis of DEGs

We identified a total of 3,706 DEGs, including 2,501 that were up-regulated and 1,205 that were down-regulated under the chilling stress (Figure S2). The biological function information for the DEGs was analyzed by GO enrichment. A total of 2,902 DEGs were annotated in 1,411 terms and a total of 125 GO terms were identified. The most enriched GO categories among these DEGs were "nucleus", "DNA binding", "metal ion binding", "DNA-binding transcription factor activity", and "regulation of transcription, DNA-templated", with 366, 239, 204, 128, and 125 DEGs respectively (Figure 3A).

Pathway enrichment analysis of DEGs

To characterize the active biological pathways of the DEGs in *S. caseolaris* under chilling stress, we classified the DEGs in KEGG biological pathways and conducted enrichment analysis. There were 2,557 DEGs annotated in 125 pathways. Among them, a total of nine KEGG pathways were significantly enriched with a P-value$\leqslant$0.05. The three most highly represented pathways were "Plant hormone signal transduction (ko04075)", "Plant-pathogen interaction (ko04626)", and "Glycerophospholipid metabolism (ko00564)", with 115, 88 and 41 DEGs respectively (Figure 3B). The largest number of DEGs included "plant hormone signal transduction", indicating that plant hormones play an important role in response to chilling stress in *S. caseolaris*. The second largest number of DEGs included "Plant-pathogen interaction", which suggested that low temperature exposed plants are more vulnerable to be pathogen infection. The "Glycerophospholipid metabolism" category had the third largest number of DEGs, suggesting that fatty acid metabolism responded positively to low temperature. A considerable number of biological reactions were altered in *S. caseolaris* in response to chilling stress.

DEGs associated with chilling stress

Carbohydrate metabolism related DEGs

Carbohydrates played an important role in plant response to low temperatures. In GO

enrichment analysis, 12 DEGs were enriched in "starch binding (GO:2001070)" and all were up-regulated. Many DEGs that were involved in starch, fructose, sucrose, glucose, and trehalose were up-regulated in chilling stress (Figure 4A). Four genes (CL1250.Contig10_All, CL13708.Contig9_All, CL3523.Contig11_All, and CL5529.Contig9_All) were involved in trehalose biosynthetic process, two sucrose synthase genes (CL14150.Contig25_All and CL2626.Contig4_All), and one *sucrose-phosphate synthase* gene (CL2626.Contig4_All) were involved in the sucrose process. Many DEGs were involved in "Pentose phosphate pathway (ko00030)", "Fructose and mannose metabolism (ko00051)", "Galactose metabolism (ko00052)", "Pentose and glucoronate interconversions (ko00040)", and "Starch and sucrose metabolism (ko00500)" respectively, and most of these were up-regulated.

Phytohormone related DEGs

KEGG analysis of DEGs in *S. caseolaris* showed that hormone signal transduction was significantly influenced by chilling stress. There were 115 DEGs enriched in "plant hormone signal transduction" and most of them were up-regulated (Figure 5). Especially in auxin signal transduction, ABA signal transduction, EN signal transduction and JA signal transduction.

In this GO analysis of DEGs, 41 DEGs enriched in the "auxin-activated signaling pathway (GO:0009734)" term. Among them, *AUX1* (Unigene13181_All) is a key gene that encodes an auxin receptor, and down-regulated 2.50 fold. In the ABA signal pathway, PLY and PP2C are key enzymes controlled by the ABA signaling pathway. In this study, the genes encoding these two enzymes (CL1229.Contig3_All, Unigene14664_All, and CL5508.Contig1_All) were all up-regulated. Additionally, seven DEGs were enriched in the "abscisic acid-activated signaling pathway (GO:0009738)" term. In the EN signal pathway, we found that the one gene (CL2232.Contig4_All) encoding ETR down-regulated 6.81 fold. |In the Ja signal pathway, cetyl-CoA acyltransferase 1 is a key enzyme for synthesis of JA-CoA. In this study, the genes (CL10456.Contig6_All) encoding this enzyme were up-regulated by 3.93-fold.

ROS related DEGs

We found DEGs related to catalase, glutathione peroxidase, peroxidase, and superoxide dismutase were involved in chilling stress (Figure 4B). Two genes encode superoxide dismutase (CL10965.Contig3_All and CL355.Contig5_All). 3 genes encode catalase (Unigene30041_All, Unigene30063_All and Unigene30085_All), and 10 genes encode peroxidase (CL1068.Contig11_All, CL1068.Contig6_All, CL1068.Contig7_All, CL11425.Contig1_All, CL13638.Contig5_All, CL2936.Contig3_All, CL3639.Contig5_All, CL3639.Contig6_All, CL4125.Contig8_All, and Unigene3189_All). These genes were all up-regulated, indicating that they may have played a positive role in activating antioxidant enzymes in *S. caseolaris* exposed to low temperatures.

Transcription factors

TFs play an important role in regulating gene expression that participates in plant stress regulatory processes, such as chilling stress-related processes. In this study, we classified a total of 232 TFs into 41 families and showed differential expression in chilling treated seedlings. A total of 161 (69.40%) were up-regulated. The five most abundant TF families were AP2-ERF, MYB, C3H, WRKY, and ARF with 35 (15.09%), 29 (12.50%) 21 (9.05%), 21 (9.05%), and 15 (6.47%) respectively (Figure 4C).

Validation of transcriptome data by RT-qPCR analyses

To validate the RNA-Seq results, we selected a total of 16 candidate genes for qRT-PCR analyses (Figure 6). Based on enrichment analyses, all of these genes were involved in the chilling response. They represented different functional categories or pathways, such as sugar metabolism, antioxidant enzyme, phytohormones and TFs. The results indicated that the transcriptome results were reliable.

DISCUSSION

297 With its rapid growth adaptability, *S. caseolaris* is a good candidate species for mangrove
298 afforestation in China. Low temperature, however, have limited its distribution in high latitude
299 areas. Understanding the physiological and molecular mechanisms of chilling resistance can aid
300 in breeding cold tolerant varieties of *S. caseolaris*. We performed physiological and transcriptomic
301 analyses of leaves under 4°C chilling stress, and then analyzed the DEGs involved in the
302 antioxidant defense system, carbohydrate metabolism, plant hormone signals, and TFs.

303 **Membrane injury of *S. caseolaris* seedlings caused by chilling stress**

304 Membrane lipids are the first place that plants perceive and respond to abiotic stress signals
305 such as low temperature. Lipids are involved in biological process, such as signal transduction,
306 energy conversion, and metabolic regulation (Xie *et al*., 2018). Damaged membrane lipids increase
307 electrolyte leakage, and a high electrolyte leakage rate reflects serious damage caused by chilling
308 stress. Membrane permeability is an important physiological index of plant resistance to stress.
309 MDA is the final degradation product of lipid peroxidation, and the MDA level reflects the damage
310 of membrane lipid peroxidation and the stress tolerance of plant cells (Mutlu *et al.*, 2013; Wise
311 and Naylor, 1987). Physiological analysis showed that the relative electrolyte conductivity and the
312 MDA level both increased significantly after chilling stress. Membrane lipid peroxidation likely
313 occurred and membrane lipids were damaged. Similar results were found in *Magnolia wufengensis*
314 (Deng *et al*., 2019). Serious member lipid injury can result in seedling death. This might explain
315 why most seedlings died when exposed to extreme low temperatures (Chen *et al*., 2017). We found
316 that DEGs were enriched in "Glycerophospholipid metabolism (ko00062)" and "Fatty acid
317 elongation (ko00564)". The genes responded to the increased level of unsaturated fatty acids and
318 membrane fluidity in the cells of *S. caseolaris* seedlings under chilling stress, which may be a
319 mechanism of cellular self-repair.

320

321 ## Carbohydrate metabolism-related DEGs

322 When exposed to low temperatures, plants usually break down starch and increase the content
323 of carbohydrates to maintain stability of cell membrane structure, this could increase the low

temperature tolerance of plant cells (Guy *et al.*, 2008). In GO enrichment analysis, 12 DEGs were enriched in "starch binding (GO:2001070)" (Figure 3B). This could result in the conversion of starch into soluble sugar under chilling stress. Physiological experiments confirmed that the soluble sugars content of *S. caseolaris* increased after chilling stress. Increased levels of sucrose, raffinose, glucose, and fructose are involved in the low-temperature resistance of other plants (Anchordoguy *et al*., 1987; Ma *et al*., 2009). The RNA-seq data showed that many DEGs that are involved in starch, fructose, sucrose, glucose, and trehalose were up-regulated in chilling stress treatment. These data were consistent with previous studies (Wang *et al*., 2018, Deng, *et al.,* 2019; Yang *et al*., 2019). Plants can accumulate trehalose for oxidative detoxification to counteract abiotic stresses (Lai *et al*., 2017). In the DEGs, four genes were involved in the trehalose biosynthetic process and all of them were up-regulated, which is similar to the report of Deng *et al*. (2019). Sucrose synthase and sucrose phosphate synthase play important roles in sucrose metabolism and biosynthesis respectively (Winter and Huber, 2000). We found that two sucrose synthase genes and one sucrose-phosphate synthase gene were up-regulated. These genes may have been involved in the response to chilling stress. A similar result was found by Yang *et al*. (2019). Carbohydrate also regulate expression of jasmonate, ABA and COR genes (Rutten and Santarius, 1992; Welling and Palva, 2006). We found that many DEGs were involved in carbohydrate metabolism, which indicated that carbohydrates play an important role in *S. caseolaris* response to chilling stress.

Antioxidant defense system

Under environment stress, ROS such as superoxide, hydroxyl radicals and hydrogen peroxide can accumulate in plant cells and cause oxidative damage (Elsheery *et al*., 2008; Suzuki and Mittler, 2006). To scavenge ROS, antioxidant systems such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) are activated in response to plant stress (Mittler, 2002). SOD is the first antioxidant enzyme that could convert O^{2-} to hydrogen peroxide (H_2O_2), and then POD and CAT could catalyze H_2O_2 and convert it to H_2O. We found

DEGs related to CAT, glutathione peroxidase, POD, and SOD were up-regulated, indicating that they may have played a key role in activating antioxidant enzymes in *S. caseolaris* exposed to low temperatures. ROS are also signal molecules that can induce gene expression and protein synthesis to increase stress tolerance in plants (Jaspers and Kangasjärvi, *et al*., 2010). ROS also is active the mitogen-activated protein kinase (MAPK) cascade pathway, which regulates low-temperature response genes involved in TFs and some hormones. (Pitzschke, *et al*., 2009; Xie, *et al*., 2009). We found 15 DEGs enriched in "MAP kinase activity" and most of them were un-regulated, which indicated that MAPK signal transduction pathway also played an important role in chilling stress responses.

These results indicated that many antioxidant genes are activated, and these would not only increase the activity of antioxidant enzymes to scavenge with ROS, but also active a sequence of signal to response chilling stress.

Phytohormone signal network responses to chilling stress

Phytohormones are the secondary signals in plant cells that play a significant role in adaptation to environmental stress (Peleg *et al*., 2011). They can activate a sequence of signal events and eventually induce the genes that directly respond to stress (Bari and Jones, 2009; Hakeem *et al*., 2014). We found four key response hormone signal transduction pathways (auxin, ABA, EN, and JA) that were significantly influenced by chilling stress. Auxin is a plant growth-promoting hormone (Nemhauser et al., 2006). In this study, 41 DEGs were enriched in the "auxin-activated signaling pathway (GO:0009734)" term. Down-regulation of the *AUX1* gene may reduce plant growth in response to chilling stress. The ABA signaling pathway is important in regulating abiotic stress responses in plants (Danquah, *et al*., 2014). It can regulate stomatal closure, reduce water loss and maintain cell growth (Peleg & Blumwald, 2011). PLY and PP2C are key genes controlled by the ABA signaling pathway. In present study, the genes were up-regulated in response to chilling stress. This could activate high expression of PP2C and promote stomatal closure and decrease transpiration to reduce water loss. Similar results have been found on

Arabidopsis thaliana (Tähtiharju and Palva, 2001) and *Oryza sativa* L (Guan *et al*., 2019). Additionally, the DEGs were also enriched in the "abscisic acid-activated signaling pathway (GO:0009738)" term, which further confirmed that ABA-dependent signaling is involved in the low temperature response of *S. caseolaris*. Genes related to ET biosynthesis often down-regulated expressed under low temperature treatment (Chu and Lee, 1989). In this study, the key gene encoding ETR was down-regulated, which was consistent with previous reports (Yang *et al*., 2018). JA plays a significant role in plants responding to abiotic and biotic stress (Wasternack and Song, 2017; Wasternack and Strnad, 2018). The up-regulated key genes may promote the synthesis of JA-CoA and promote the synthesis of JA. Exogenous methyl salicylic acid (MeSA) or methyl jasmonate (MeJA) could induce the transcription of pathogenesis-related genes, and increase chilling tolerance and pathogen resistance (Ding, *et al*., 2002). This may explain why many DEGs were enriched in "Plant-pathogen interaction". Similar results have been found in *K. obovate*, *M. wufengensis*, and *Camellia sinensis* under chilling stress (Deng, *et al*., 2019; Li *et al*., 2019; Su *et al*., 2019). However, how those plant hormones mediated signaling participates in chilling stress responses in *S. caseolaris* still need to be further study.

TFs responding to chilling stress

TFs play important roles in plant growth and stress tolerance (Lee *et al.*, 2005). When a plant experiences the chilling stress, a series of signal transduction pathways could activate TFs. The activated TFs will activate the expressions of a cascade of downstream resistance-related genes to enhance the cold tolerance by special binding (Kasuga *et al.,* 1999). Many TF families such as DREB (Ahmed *et al*., 2017, Chen *et al*. 2012), MYB (Zhao *et al*., 2012, Agarwal *et al*., 2006; Chen *et al*., 2006), and WRKY (Zou *et al*., 2010) are related to plants resistance to low temperature stress. We identified 232 TFs that were classified into 41 families. AP2-ERF, MYB, C3H, WRKY, and ARF were five major TF families identified as DEGs, suggest that they played a crucial role in response to chilling stress. AP2-ERF genes belong to a plant-specific TF family involved in regulating stress responses (Mizoi *et al*., 2012). The AP2-ERF superfamily consists of the ERF,

RAV, AP2, and DREB families. Overexpression of *AP2 TF*, *JcDREB*, and *BpDREB2* increased cold tolerance of *Arabidopsis* (Tang *et al*., 2011; Sun *et al*., 2014). Previous studies verified that AP2-ERFs involved in a lot of plant hormone mediated abiotic stress responses, such as ABA, ET, GA, CTK, and BR (Colebrook *et al*., 2014; Kazan *et al*., 2015; Tao *et al*., 2015; Nolan *et al*.,2017; Sah *et al*., 2016). MYB are involved in chilling stress and regulation of ABA-responsive genes, and constitute with ABA-dependent, induced and mediated types. Which plays an important role in the upstream stage of low temperature transduction (Gyoungju et al., 2016). *AtMYB52*, *AtMYB70*, and *AtMYB82* were ABA-dependent genes in responding abiotic stresses of *Arabidopsis* (Park *et al*, 2011); *TaMYB1*, *TaMYB2*, *TaMYB3R1* were ABA-induced genes in responding abiotic stresses of wheat (Cai *et al*., 2011; Tong *et al*., 2006); *AtMYB2*, *AtMYB41* were ABA-mediated types that promote the accumulation of ABA in responding abiotic stresses (Abe *et al*., 2003; Lippold *et al*., 2009). The WRKY genes can respond strongly and rapidly to abiotic stresses (Chen *et al*., 2012). The *WRKY46* gene enhanced the low temperature tolerance of transgenic cucumber by activating a series of cold-stress responsive genes in an ABA-dependent manner (Zhang *et al.,* 2016). *MaWRKY25* can be induced by low temperature or MeJA, which could increase the low temperature tolerance of banana. It could also active the *MaLOX2*, *MaAOS3*, *MaOPR3* genes to promote the synthesis of JA by special binding. Which could reduce the damage of banana fruit by low temperature (Ye, *et al*., 2016). Other TFs, such as the HSF, bZIP, and GRAS families, are induced by chilling stress, and members of these families can affect the cold resistance of plants (Chinnusamy *et al.,* 2010). These results indicated that many TFs were involved in the seedlings of *S. caseolaris* in response to chilling stress through different pathways. Besides, the both un-regulated TFs and phytohormones maybe have some connection, and the regulatory network need further study.

Conclusions

We analyzed at the morphological, physiological and transcriptome level of *S. caseolaris* response to chilling stress. Chilling stress of *S. caseolaris* likely involves damage to the cell

membranes. This was supported by a significant increase in the relative electrolyte conductivity and MDA contents. Up regulating genes in chilling stressed *S. caseolaris* were involved in plant hormone signal transduction, TFs, carbohydrate metabolism, and antioxidant enzymes. These genes might be involved in the significant increase of soluble protein content and soluble sugar content in response to the chilling stress (Figure 7). These data revealed the manner in which *S. caseolaris* responds to chilling stress, and provided insight for further research on the environmental adaptations of mangrove species.

Figure 1 Phenotypic changes of *S. caseolaris* under chilling stress (4 °C).

Figure 2 Physiological change of *S. caseolaris* in chilling stress.

Figure 3 The top 20 GO and KEGG enrichment scatter diagram depicting the differential expression of genes. A: the top 20 GO enrichment B: KEGG enrichment.

Figure 4 Heat map of relative expression levels of DEGs involved in main transcription factors, sugar metabolism and the antioxidant defense system. A: The main transcription factors B: The main sugar metabolism-related genes. C: The main antioxidant-related genes.

Figure 5 Expression of *S. caseolaris* genes in response to chilling stress for 0 h (CK) and 6 h (CT) as determined by qRT-PCR (blue) and RNA-seq (red).

Figure 6 Plant hormone signal transduction and the relative heat map. A Plant hormone signal transduction. B Heat map of DEGs involved with auxin, ABA, EN and JA.

Figure 7 Hypothetical model of the events occurring in the *S. caseolaris* leaves under chilling stress.

Table S1 List of primers used in this study

Table S2 Summary of sequence analysis

Table S3 Number of annotated unigenes

Table S4 Statistics of transcriptome assembly and unigenes

Figure S1 Characteristics of unigenes. A: Distribution of unigene lengths in *Sonneratia caseolaris*. B: Main species distribution of *S. caseolaris* unigenes. C: Pathway annotation of unigenes based on KEGG categorization D: Functional annotation of unigenes based on Gene Ontology (GO) categorization.

Figure S2 Volcano diagram showing the differential expression of genes. Blue represents down-regulated, red indicates up-regulated

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Figure 1

Phenotypic changes of *S. caseolaris* under chilling stress (4 ℃).

Figure 2

Physiological change of *S. caseolaris* in chilling stress.

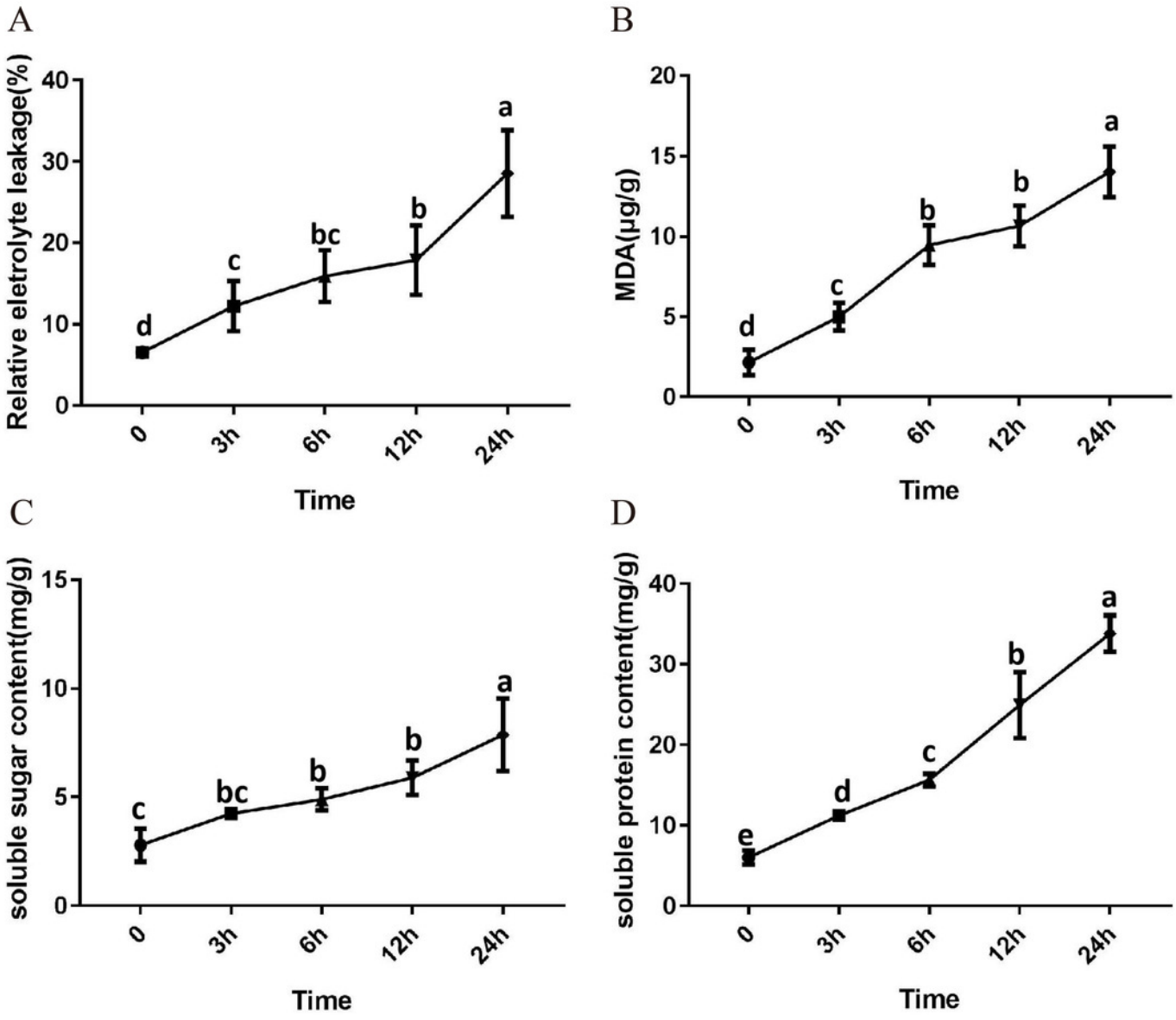


Figure 3

The top 20 GO and KEGG enrichment scatter diagram depicting the differential expression of genes. A: the top 20 GO enrichment. B: KEGG enrichment.

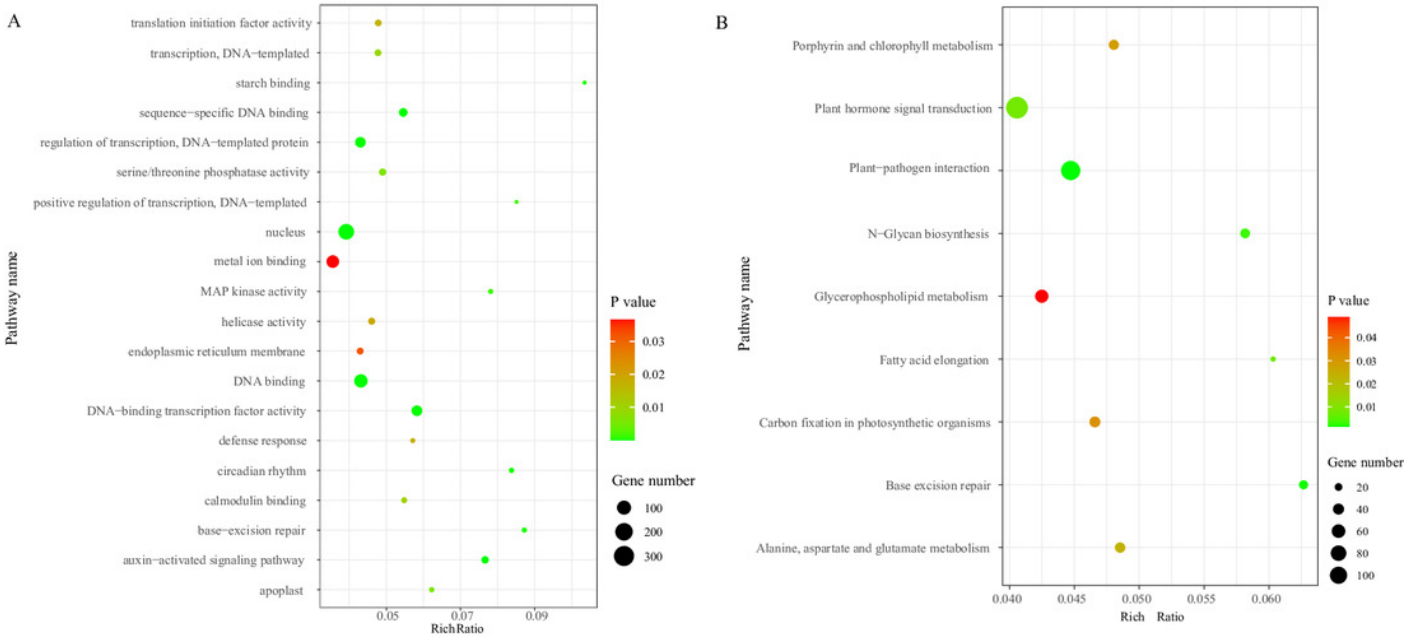
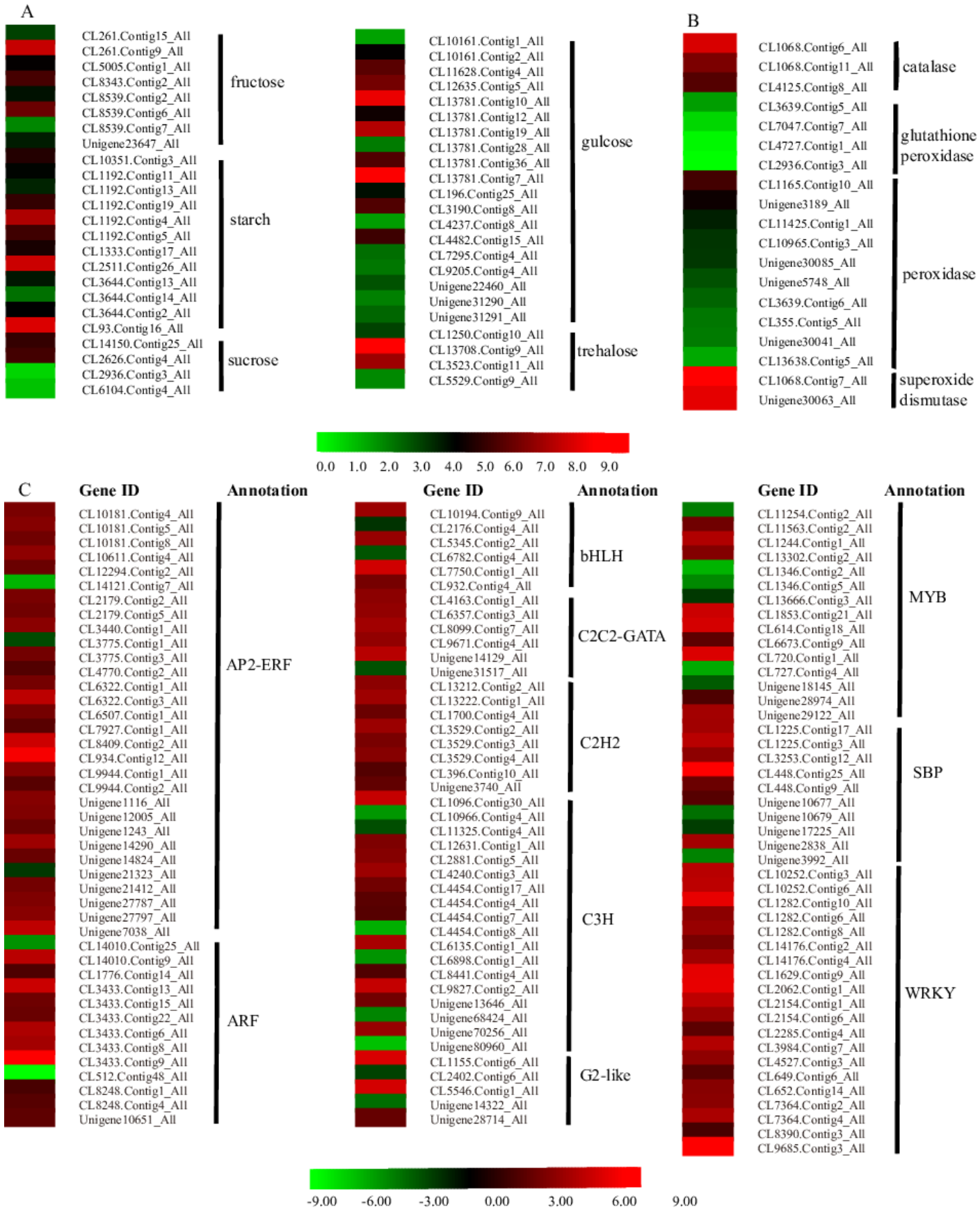


Figure 4

Figure 4 Heat map of relative expression levels of DEGs involved in main transcription factors, sugar metabolism and the antioxidant defense system. A: The main sugar metabolism-related genes. B: The main antioxidant-related genes. C: The main transcripti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CL8343.Contig2_All
CL8539.Contig2_All
CL8539.Contig6_All
CL8539.Contig7_All
Unigene23647_All
fructose
CL10351.Contig3_All
CL1192.Contig11_All
CL1192.Contig13_All
CL1192.Contig19_All
CL1192.Contig4_All
CL1192.Contig5_All
CL1333.Contig17_All
CL2511.Contig26_All
CL3644.Contig13_All
CL3644.Contig14_All
CL3644.Contig2_All
CL93.Contig16_All
starch
CL14150.Contig25_All
CL2626.Contig4_All
CL2936.Contig3_All
CL6104.Contig4_All
sucrose
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CL10161.Contig2_All
CL11628.Contig4_All
CL12635.Contig5_All
CL13781.Contig10_All
CL13781.Contig12_All
CL13781.Contig19_All
CL13781.Contig28_All
CL13781.Contig36_All
CL13781.Contig7_All
CL196.Contig25_All
CL3190.Contig8_All
CL4237.Contig8_All
CL4482.Contig15_All
CL7295.Contig4_All
CL9205.Contig4_All
Unigene22460_All
Unigene31290_All
Unigene31291_All
gulcose
CL1250.Contig10_All
CL13708.Contig9_All
CL3523.Contig11_All
CL5529.Contig9_All
trehalose
B
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CL1068.Contig11_All
CL4125.Contig8_All
catalase
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CL7047.Contig7_All
CL4727.Contig1_All
CL2936.Contig3_All
glutathione peroxidase
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Unigene3189_All
CL11425.Contig1_All
CL10965.Contig3_All
Unigene30085_All
Unigene5748_All
CL3639.Contig6_All
CL355.Contig5_All
Unigene30041_All
CL13638.Contig5_All
peroxidase
CL1068.Contig7_All
Unigene30063_All
superoxide dismutase
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0
C
Gene ID
Annotation
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CL10181.Contig8_All
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CL934.Contig12_All
CL9944.Contig1_All
CL9944.Contig2_All
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Unigene12005_All
Unigene1243_All
Unigene14290_All
Unigene14824_All
Unigene21323_All
Unigene21412_All
Unigene27787_All
Unigene27797_All
Unigene7038_All
AP2-ERF
CL14010.Contig25_All
CL14010.Contig9_All
CL1776.Contig14_All
CL3433.Contig13_All
CL3433.Contig15_All
CL3433.Contig22_All
CL3433.Contig6_All
CL3433.Contig8_All
CL3433.Contig9_All
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CL8248.Contig1_All
CL8248.Contig4_All
Unigene10651_All
ARF
Gene ID
Annotation
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CL5345.Contig2_All
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CL932.Contig4_All
bHLH
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CL8099.Contig7_All
CL9671.Contig4_All
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Unigene31517_All
C2C2-GATA
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CL13222.Contig1_All
CL1700.Contig4_All
CL3529.Contig2_All
CL3529.Contig3_All
CL3529.Contig4_All
CL396.Contig10_All
Unigene3740_All
C2H2
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CL11325.Contig4_All
CL12631.Contig1_All
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CL4240.Contig3_All
CL4454.Contig17_All
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C3H
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G2-like
Gene ID
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Unigene28974_All
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MYB
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CL448.Contig9_All
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Unigene10679_All
Unigene17225_All
Unigene2838_All
Unigene3992_All
SBP
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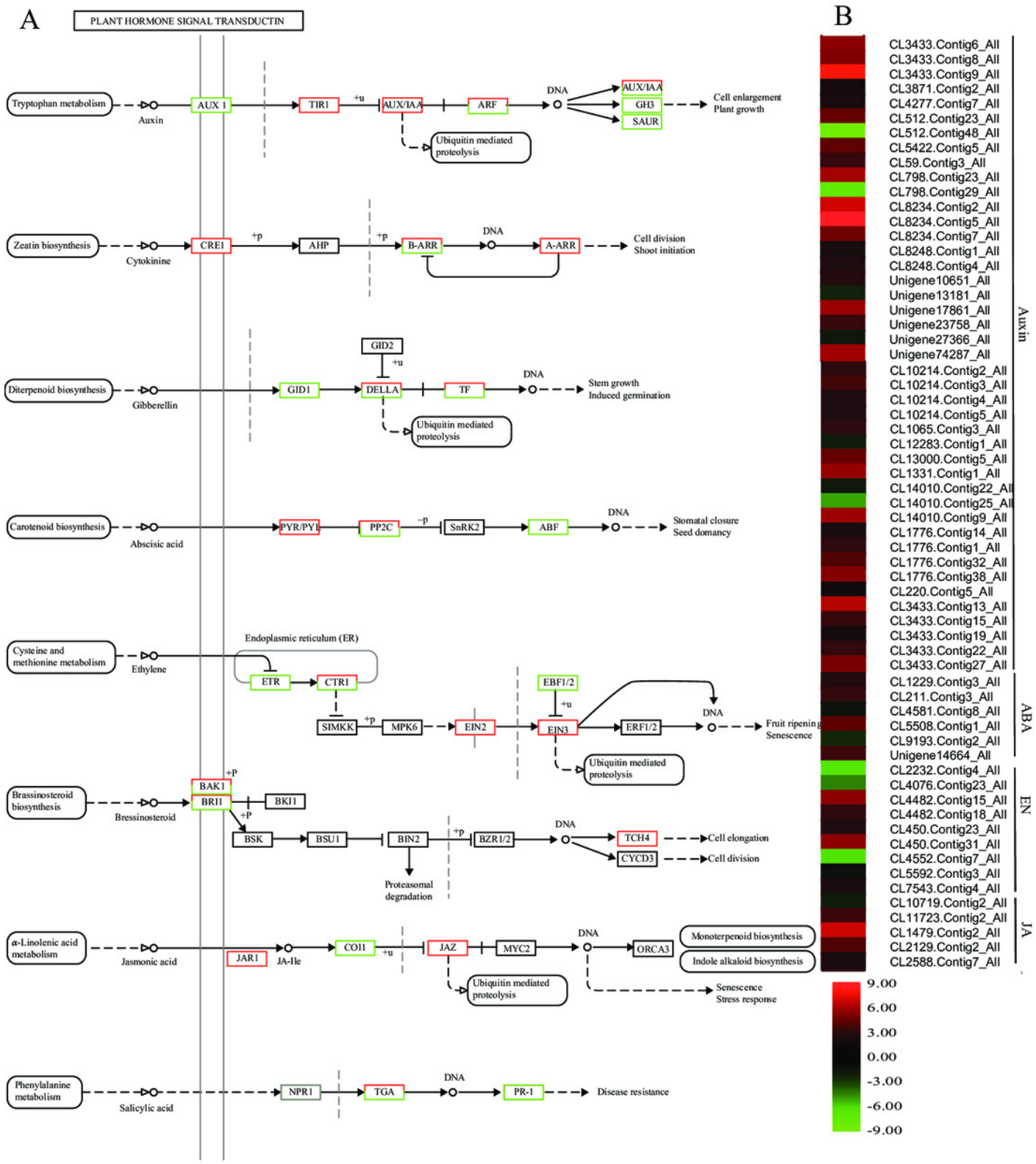
WRKY
-9.00 -6.00 -3.00 0.00 3.00 6.00 9.00

Figure 5

Figure 5 Plant hormone signal transduction and the relative heat map. A Plant hormone signal transduction. B Heat map of DEGs involved with auxin, ABA, EN and JA.


A
PLANT HORMONE SIGNAL TRANSDUCTIN
Tryptophan metabolism
Auxin
AUX 1
TIR1
+u
AUX/IAA
ARF
DNA
AUX/IAA
GH3
SAUR
Cell enlargement
Plant growth
Ubiquitin mediated proteolysis
Zeatin biosynthesis
Cytokinine
CRE1
+P
AHP
+P
B-ARR
DNA
A-ARR
Cell division
Shoot initiation
Diterpenoid biosynthesis
Gibberellin
GID2
+u
GID1
DELLA
TF
DNA
Stem growth
Induced germination
Ubiquitin mediated proteolysis
Carotenoid biosynthesis
Abscisic acid
PYR/PYL
PP2C
−P
SnRK2
ABF
DNA
Stomatal closure
Seed domancy
Cysteine and methionine metabolism
Ethylene
Endoplasmic reticulum (ER)
ETR
CTR1
SIMKK
+P
MPK6
EIN2
EBF1/2
+u
EIN3
DNA
ERF1/2
Fruit ripening
Senescence
Ubiquitin mediated proteolysis
Brassinosteroid biosynthesis
Bressinosteroid
+P
BAK1
BRI1
BKI1
+P
BSK
BSU1
BIN2
+P
BZR1/2
DNA
TCH4
Cell elongation
CYCD3
Cell division
Proteasomal degradation
α-Linolenic acid metabolism
Jasmonic acid
JAR1
JA-Ile
COI1
+u
JAZ
MYC2
DNA
ORCA3
Monoterpenoid biosynthesis
Indole alkaloid biosynthesis
Ubiquitin mediated proteolysis
Senescence
Stress response
Phenylalanine metabolism
Salicylic acid
NPR1
TGA
DNA
PR-1
Disease resistance
B
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CL3433.Contig8_All
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CL1776.Contig32_All
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CL3433.Contig19_All
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CL10719.Contig2_All
CL11723.Contig2_All
CL1479.Contig2_All
CL2129.Contig2_All
CL2588.Contig7_All
Auxin
ABA
EN
JA
9.00
6.00
3.00
0.00
-3.00
-6.00
-9.00

Figure 6

Expression of *S. caseolaris* genes in response to chilling stress for 0 h (CK) and 6 h (CT) as determined by qRT-PCR (blue) and RNA-seq (red).

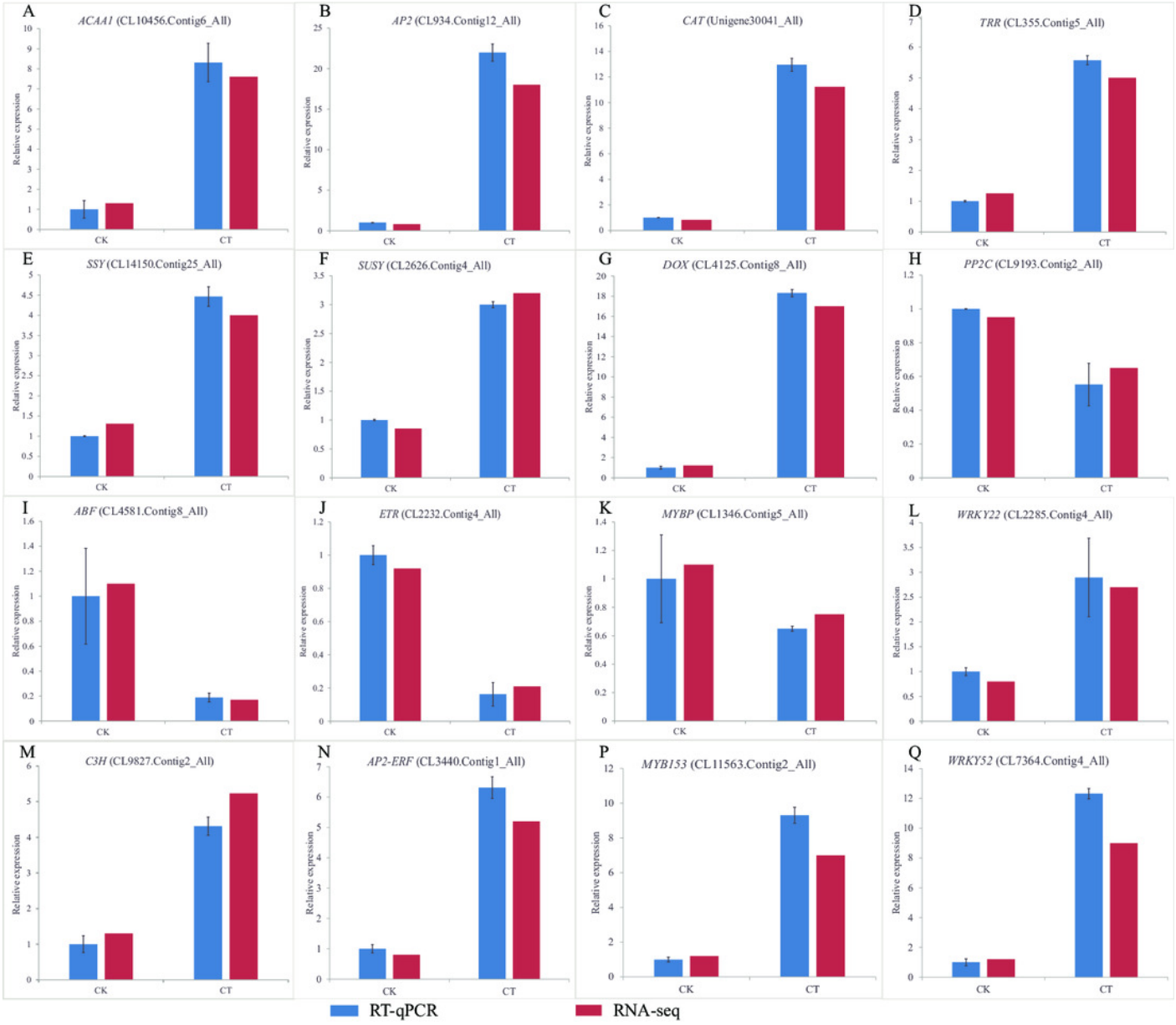
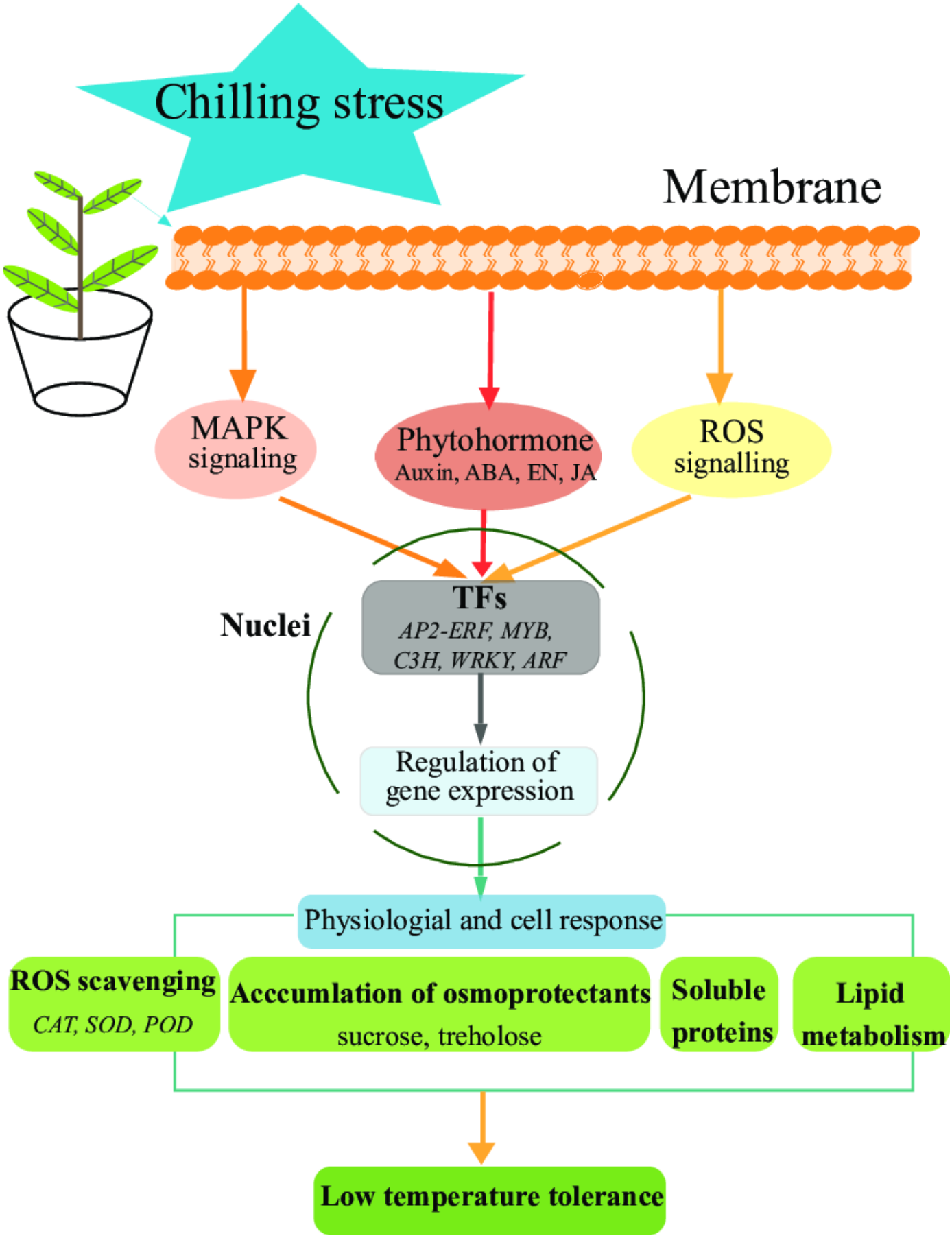


Figure 7

Hypothetical model of the events occurring in the *S. caseolaris* leaves under chilling stress.


Chilling stress
Membrane
MAPK signaling
Phytohormone
Auxin, ABA, EN, JA
ROS signalling
Nuclei
TFs
AP2-ERF, MYB, C3H, WRKY, ARF
Regulation of gene expression
Physiologial and cell response
ROS scavenging
CAT, SOD, POD
Acccumlation of osmoprotectants
sucrose, treholose
Soluble proteins
Lipid metabolism
Low temperature tolerance