Transcriptome analysis of *Sonneratia caseolaris* seedlings under chilling stress

Yong Yang [Equal first author, 1] , Chunfang Zheng [Equal first author, 2] , Cairong Zhong [3] , Tx Lu [1] , Juma Gul [1] , Xiang Jin [1] , Ying Zhang [Corresp., 1] , Qiang Liu [Corresp. 1]

1 Ministry of Education Key Laboratory for Ecology of Tropical Islands, College of Life Sciences, Hainan Normal University, Haiou, China

2 National and Local Joint Engineering Research Center of Ecological Treatment Technology for Urban Water Pollution, College of Life and Environmental Science, Wenzhou University, Wenzhou, Zhejiang, China

3 Hainan Academy of Forestry, Hainan Mangrove Research Institute, Haikou, Hainan, China

Corresponding Authors: Ying Zhang, Qiang Liu
Email address: Zhangyingred@yahoo.com, hnsylq@163.com

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

1 **Transcriptome Analysis of *Sonneratia caseolaris* Seedlings**

2 **under Chilling Stress**

3 Yong Yang[1#], Chunfang Zheng[2#], Cairong Zhong[3], Tianxin Lu[1], Juma Gul[l], Xiang Jin[1], Ying

4 Zhang[1*], Qiang Liu[1*]

5 [1] Ministry of Education Key Laboratory for Ecology of Tropical Islands, College of Life Sciences,

6 Hainan Normal University, Haikou, 571158, China

7 [2] National and Local Joint Engineering Research Center of Ecological Treatment Technology for

8 Urban Water Pollution, College of Life and Environmental Science, Wenzhou University,

9 Wenzhou, China

10 [3]Hainan Academy of Forestry, Hainan Mangrove Research Institute. Haikou, Hainan, 571100,

11 China

12 # Co-authors

13 Correspondence:

14 Prof. Ying Zhang and Qiang Liu

15 Zhangyingred@yahoo.com

16 hnsylq@163.com

18 **Abstract**

19 *Sonneratia caseolaris* is a mangrove species native to China. It is fast growing and highly

20 adaptable for mangrove afforestation, but injured by cold temperatures when grown at high

21 latitudes. To understand the response mechanisms under chilling stress, we conducted

22 physiological and transcriptomic analyses. The relative electrolyte conductivity, malondialdehyde

23 (MDA) content, soluble sugar content and soluble protein content increased significantly under

24 chilling stress. This indicated that *S. caseolaris* suffered damage and increased the levels of

25 osmoprotectants in response to the chilling stress. We performed gene expression comparison

26 analysis of *S. caseolaris* leaves after 6 h of chilling stress at the transcriptional scale using RNA-

27   Seq. A total of 168,473 unigenes and 3,706 differentially expressed genes (DEGs) were identified.

28   GO and KEGG enrichment analyses showed that the DEGs were mainly involved in carbohydrate

29   metabolism, antioxidant enzyme, plant hormone signal transduction, and transcription factors

30   (TFs). Sixteen genes associated with carbohydrate metabolism, antioxidant enzyme,

31   phytohormones and TFs were selected for qRT-PCR verification, and they indicated that the

32   transcriptome data were reliable. This study reviewed the chilling response of *S. caseolaris* at both

33   physiological and transcriptomic levels, and the results increase our understanding of stress-

34   responses in mangrove plants.

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

Introduction

38   The growth, development, and geographical distribution of plants are limited by temperatures

39   in their habitat (Jin *et al*., 2013). Low temperature stress can be divided into chilling stress (<10°C)

40   and freezing stress (<0°C) (Zhou *et al*., 2011). Plants have evolved a series of response

41   mechanisms to adapt to low-temperature environments. These include regulation of gene

42   expression and physiological and biochemical process changes that enhance cold resistance

43   (Thomshow, 1999; Sung, *et al*., 2003), These changes have been studied in model plants like

44   *Arabidopsis thaliana* and some cereal crops ((Buti *et al*., 2018; Zhang *et al*., 2004; Roman *et al*.,

45   2012). Response mechanisms enhancing cold resistance mainly include signaling cascade,

46   modification of the membrane lipid composition, accumulation of osmoprotectants, and increased

47   activity of antioxidant enzymes to scavenge reactive oxygen species (ROS) (Theocharis *et al*.

48   2012; Nievola *et al*. 2017; Nakashima *et al*., 2009). In addition, phytohormones such as auxin,

49   abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), salicylic acid(SA), gibberellin(GA),

50   cytokinin(CK), and brassinosteroid (BR) and their signal transduction related genes also involved

51   in regulating the gene expression in response to low-temperature stress (Peleg *et al*., 2011).

52   Exogenous ABA improved the cold tolerance of *Brassica napus* (He, *et al*., 2019). Some members

53   of WRKYs and MYBs transcription factors (TFs) regulated gene expression to participate in plant

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

55   Mangroves are halophytic temperature sensitive shrubs or trees that grow in the intertidal areas

56 of tropical and subtropical coasts. They include about 70 species from 20 plant families (Duke,

57 1992). Mangroves are important in carbon balancing, protecting coastlines and beaches from

58 floods and storms, reducing soil erosion, and providing habitats and shelters for animals. They also

59 provide food, wood, and medical compounds for humans (Duke, 1992; Tomlinson, 1999; Schmitt

60 and Duke, 2015). Large areas of mangroves, however, have been converted to agriculture,

61 aquaculture, and industrial use (Duke *et al*., 2007; Robertsen and Alongl, 1992; Wang and Wang,

62 2007). There are approximately 22,684 ha of mangroves in China, which is 44% of the area

63 existing in the 1950s (survey by State Forestry Administration in 2001). Since 2001, the

64 government of China has focused on mangrove protection and restoration using conservation

65 measures and large-scale afforestation in southeast China. The mangrove area in 2019 had

66 increased to 29,000 ha. *Sonneratia caseolaris* is a mangrove species that is naturally distributed in

67 the Hainan islands. Because of its rapid growth and adaptability, it was introduced to more northern

68 and colder areas in China, such as Shenzhen and Leizhou in Guangdong Province, Qinzhou and

69 Fangchenggang in Guangxin Province in 1994, where it grows well (Wang *et al.,* 2002; Zan *et al*.,

70 2003). *S. caseolaris* suffered substantial chilling injury in 2008, and there was heavy defoliation

71 in all growing areas. All of the trees in Guangxi Province died. There was also significant

72 defoliation of trees in native locations of Wenchang. Young seedlings were the most susceptible

73 growth stage and suffered the great injury (Chen *et al.,* 2017). Mangrove species with greater

74 tolerance to low temperature are needed for mangrove restoration. Understanding the molecular

75 mechanism of *S. caseolaris* tolerance to chilling stress will be of great value in restoration efforts.

76   Next-generation sequencing (NGS) is an efficient and inexpensive method for collecting

77 transcriptome data. It can be used for species that have not been sequenced. Based on RNA-Seq

78 platforms, genome-scale transcriptome analyses have been used to identify the mechanism of

79 stress response without whole-genome sequence information in different species (He *et al*., 2015;

80 Kumar *et al*., 2015). Transcriptome analysis has been used to study adaptive mechanism of

81   mangroves, such as *Acanthus ilicifolius*, *Avicennia offcinalis*, *Bruguiera gymnorhiza*, *Aegiceras*

82   *corniculatum* and *Kandelia obovata* (Fang *et al*., 2016, Krishnamurthy *et al.,* 2017, Su *et al*., 2019,

83   Yamanaka *et al*., 2009; Yang *et al*., 2015a; Yang *et al.,*2015b). The chilling stress mechanisms of

84   *S. caseolaris*, however, remains unknown.

85   To understand the mechanisms of *S. caseolaris* under chilling stress, we first measured the

86   physiological changes in *S. caseolaris* under chilling stress, Then, we used RNA-seq for

87   comparative analyses of *S. caseolaris* leaves between a control group (0 h, CK) and a chilling

88   treated group (4°C 6 h, CT). The reliability of the transcriptome results was verified by real-time

89   quantitative polymerase chain reaction (qRT-PCR). The data improved our understanding of the

90   chilling response mechanism in *S. caseolaris* and identified potential cold tolerance gene targets

91   in this, and other mangrove species.

92

93   **MATERIALS AND METHODS**

94   **Plant materials and treatments**

95   We collected ripe fruits of *S. caseolaris* in Hainan Dongzhaigang National Nature Reserve

96   (19° 57′ 23″ N, 110° 30′ 10″ E) in China. The fruit was soaked in distilled water for 7 d and then

97   individually planted in pots with a mud and sand (1:1) mixture as support. Growth chamber

98   conditions were 30/28°C (day/night) and a 14:10 h (L: D) photoperiod, 400 μmol m^{-2} s^{-1}photon

99   flux density, and 90% constant humidity. The germinated seeds were cultured in a growth chamber

100 for 5 weeks, at which time the seedlings were about 12 cm high with 12 leaves. We performed the

101 chilling stress treatment on these seedlings in a growth chamber, by setting the temperature to 4°C

102 and leaving the other conditions unchanged. The second leaf from the top of the seedlings were

103 collected as samples from 0 h (CK) 3 h, 6 h, 12 h to 24 h separately, and then frozen in liquid

104 nitrogen and stored at -80°C. Each treatment had three biological replicates.

105

106 **Physiological response assays of chilling-treated seedlings**

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

108 malondialdehyde (MDA) content was measured by the method of Yao *et al* (2011). The soluble

109 sugar content was measured by the method of Wang *et al*. (2013). The soluble protein content was

110 measured by using the Bradford method (Bradford *et al.,* 1976). Three biological replicates were

111 measured at each time point.

112

113 **RNA extraction**

114 Based on the physiological results, we used the seedlings without chilling as the control

115 group (CK_1, CK_2, CK_3) and seedlings under chilling stress for 6 h were set as the treatment

116 group (CT_1, CT_2, CT_3) for RNA-seq. Total RNA of leaf samples was extracted with TRIzol

117 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. RNA purity was

118 checked by T6 Spectrophotometer (Puxi, Beijing, China). RNA integrity and concentration were

119 measured by the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent

120 Technologies, Santa Clara, CA, USA).

121

122 **cDNA library construction and transcriptome sequencing**

123 A 3 μg amount of total RNA of each sample was enriched using magnetic beads with Oligo

124 (dT) and then fragmented into short fragments ~200 bp by an interrupting reagent. The first strand

125 cDNA was synthesized by a random hexamer primer, and the second strand of cDNAs was

126 synthesized using the buffer, dNTPs, DNA polymerase I, and RNase H. After the double-stranded

127 cDNA was generated and processed with end-repair, A-tailed, and adapters ligation, we

128 constructed a cDNA library by PCR amplification. The cDNA library was then sequenced using

129 the BGISEQ-500 platform with paired-end reads of 100 bp, by the Beijing Genomic Institution

130 (www.genomics.org.cn, BGI, Shenzhen, China). All raw reads data from this study were submitted

131 to the National Center for Biotechnology Information (NCBI) Sequence Read Archive database

132 under the accession number PRJNA668053.

133

134 **Transcripts assembly and functional gene annotation**

135   The raw data were filtered with SOAPnuke (v1.5.2) (Li *et al.,* 2008). We discarded the low

136   quality reads such as those with adapter sequences, ambiguous bases 'N' is more than 5%, with

137   more than 20% Q<10 bases. The remaining clean data were used for *de novo* assembly with the

138   Trinity program (v2.0.6) (Grabherr *et al.,* 2011) and then the unigene sequences were aligned with

139   the following databases: NR, NT, SwissProt, KEGG, KOG, Pfam and GO by BLAST software

140   (version 2.2.26).

141

142   **Differential expression analyses**

143   The expression level of each transcript was measured by RSEM (v1.2.12), and the expression

144   level of a single gene was expressed as the value of FPKM (fragments per kilobase of transcript

145   per million mapped reads) method (Li *et al.,* 2011). For the sake of determining the differentially

146   expressed genes (DEGs), we performed differential expression analysis by the DESeq2 (Anders

147   and Huber, 2010). A P-value $\leqslant 0.05$ and an expression level $|log2(ratio)| > 2$ were regarded as

148   significant differential expression. Gene ontology (GO) enrichment analysis of DEGs was

149   conducted using the GOseq R package, GO terms with $p < 0.05$ were regarded as significantly

150   enriched in DEGs (Young *et al.,* 2010). We analyzed the KEGG pathway functional enrichment

151   of DEGs using the R package. Significant enrichment was defined as when the FDR terms were <

152   0.001.

153

154   **Quantitative real-time PCR analysis**

155   We selected 12 DEGs to verify the reliability of transcriptome sequencing through qRT-

156   PCR. The specific primers of qRT-PCR were designed by Primer3 software (http://primer3.ut.ee/)

157   and are listed in Table S1. The qRT-PCR analyses were conducted using a LightCycler 480 RT-

158   PCR system (Roche Applied Science, Mannheim, Germany) under the following conditions: 95°C

159   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

160   cDNA sample were performed for qRT-PCR analysis. We calculated the relative expression of

161   each gene by the 2^{-DDCt} method (Livak *et al.*, 2001) and used the actins gene of *S. caseolaris* as a

162   reference.

163

164   **RESULTS**

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

166   After 3 h exposure to 4°C, the leaves at the top of *S. caseolaris* seedlings began to show a

167   slight curling, and after 6 h, the leaves in the top showed evident curling. After 12 h of chilling

168   stress treatment, part of leaves had softened and exhibited loss of strength. After 24 h of 4°C

169   chilling stress, most of leaves had withered (Figure 1). Electrolyte leakage, MDA content, soluble

170   sugar content and soluble protein content increased significantly (Figure 2). The electrolyte

171   leakage increased from 6.54 ± 0.46% (0 h) to 28.56 ± 5.32% (24 h); the MDA content increased

172   from 2.15 ± 0.79 μmol/g (0 h) to 14.01 ± 1.58 μmol/g (24 h); the soluble sugar contents increased

173   from 2.79 ± 0.76 μmol/g (0 h) to 7.87 ± 1.67 μmol/g (24 h); and the soluble protein content

174   increased from 6.00 ± 0.84 μmol/g (0 h) to 33.83 ± 2.27 μmol/g (24 h). All of these parameters

175   showed a linear increase. When exposed to chilling stress for 6 h, these parameters increased

176   118.68%, 299.98%, 160.67% and 56.15% compared with the control group. On the basis of these

177   results, we selected the leaf samples exposed to 0 h and 6 h of chilling stress for RNA-seq to

178   investigate the changes in genome-wide gene expression mechanism under chilling stress. The 0

179   h time the control (CK) and 6 h was regarded as the treatment group (CT), with three biological

180   replicates.

181

182   **Sequencing and *de novo* assembly of transcriptome**

183   In total, we obtained 39.33 Gb clean bases from 6 cDNA libraries, and we obtained a total of

184   262.21 M clean reads from 280.46 M raw reads (Table S2). The clean reads were *de novo*

185   assembled by Trinity software and they yielded 168,473 unigenes. All unigenes were longer than

186   200 bp and ranged from 297 to 16,287 bp. The 200-300 bp unigenes accounted for the largest

187   proportion (13,159, 12.26%) (Figure S1A). The mean unigene length was 1,762 bp. The N50 and

188   N90 lengths were 2,849 bp and 937 bp respectively, and the GC content was 43.94%.

189

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^-

193   ^{5}. A total of 125,360 (74.41%) unigenes were predicted to be homologous with known genes from

194   the databases listed earlier. Among them, 116,619 (69.22%), 85,761 (50.90%), 90,660 (53.81%),

195   94,737 (56.23%), 100,382 (59.58%), 96,869 (57.50%) and 92,876 (55.13%) unigenes were found

196   in the seven databases. A total of 48,858 (29.00%) were found in all seven databases (Table S3).

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. These groups were classified into the following

203   three major GO categories: 18 functional groups in "biological processes", 12 functional groups

204   in "cell component", and 14 functional groups in "molecular function". The dominant

205   subcategories of the classified genes included "metabolic process (9,916)", "cellular process

206   (22,187)", and "biological regulation (10,062)" in the "biological processes" category; "membrane

207   part (29,068)", "cell (23,014)", and "organelle part (9,821)"in the "cell component" category;

208   "binding"(46,922), "catalytic activity (44,729)"and "transporter activity (5,902)"in the "molecular

209   function" category (Figure S1C). 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

216 **GO term enrichment analysis of DEGs**

217   We identified a total of 3,706 DEGs, including 2,501 that were up-regulated and 1,205 that

218 were down-regulated under the chilling stress (Figure S2). The biological function information for

219 the DEGs was analyzed by GO enrichment. A total of 2,902 DEGs were annotated in 1,411 terms

220 and a total of 125 GO terms were identified. The most enriched GO categories among these DEGs

221 were "nucleus", "DNA binding", "metal ion binding", "DNA-binding transcription factor

222 activity", and "regulation of transcription, DNA-templated", with 366, 239, 204, 128, and 125

223 DEGs respectively (Figure 3A).

224

225 **Pathway enrichment analysis of DEGs**

226   To characterize the active biological pathways of the DEGs in *S. caseolaris* under chilling

227 stress, we classified the DEGs in KEGG biological pathways and conducted enrichment analysis.

228 There were 2,557 DEGs annotated in 125 pathways. Among them, a total of nine KEGG pathways

229 were significantly enriched with a P-value≤0.05. The three most highly represented pathways

230 were "Plant hormone signal transduction (ko04075)", "Plant-pathogen interaction (ko04626)", and

231 "Glycerophospholipid metabolism (ko00564)", with 115, 88 and 41 DEGs respectively (Figure

232 3B). The largest number of DEGs included "plant hormone signal transduction", indicating that

233 plant hormones play an important role in response to chilling stress in *S. caseolaris.* The second

234 largest number of DEGs included "Plant-pathogen interaction", which suggested that low

235 temperature exposed plants are more vulnerable to be pathogen infection. The

236 "Glycerophospholipid metabolism" category had the third largest number of DEGs, suggesting

237 that fatty acid metabolism responded positively to low temperature. A considerable number of

238 biological reactions were altered in *S. caseolaris* in response to chilling stress.

239

240 **DEGs associated with chilling stress**

241 **Carbohydrate metabolism related DEGs**

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

243   enrichment analysis, 12 DEGs were enriched in "starch binding (GO:2001070)" and all were up-

244   regulated. Many DEGs that were involved in starch, fructose, sucrose, glucose, and trehalose were

245   up-regulated in chilling stress (Figure 4A). Four genes (CL1250.Contig10_All,

246   CL13708.Contig9_All, CL3523.Contig11_All, and CL5529.Contig9_All) were involved in

247   trehalose biosynthetic process, two sucrose synthase genes (CL14150.Contig25_All and

248   CL2626.Contig4_All), and one *sucrose-phosphate synthase* gene (CL2626.Contig4_All) were

249   involved in the sucrose process. Many DEGs were involved in "Pentose phosphate pathway

250   (ko00030)", "Fructose and mannose metabolism (ko00051)", "Galactose metabolism (ko00052)",

251   "Pentose and glucoronate interconversions (ko00040)", and "Starch and sucrose metabolism

252   (ko00500)" respectively, and most of these were up-regulated.

253

254   **Phytohormone related DEGs**

255   KEGG analysis of DEGs in *S. caseolaris* showed that hormone signal transduction was

256   significantly influenced by chilling stress (Figure 3). There were 115 DEGs enriched in "plant

257   hormone signal transduction" and most of them were up-regulated (Figure 5). Especially in auxin

258   signal transduction, ABA signal transduction, ET signal transduction and JA signal transduction.

259   In this GO analysis of DEGs, 41 DEGs enriched in the "auxin-activated signaling pathway

260   (GO:0009734)" term. Among them, *AUX1* (Unigene13181_All) is a key gene that encodes an

261   auxin receptor, and down-regulated 2.50 fold. In the ABA signal pathway, PLY and PP2C are key

262   enzymes controlled by the ABA signaling pathway. In this study, the genes encoding these two

263   enzymes (CL1229.Contig3_All, Unigene14664_All, and CL5508.Contig1_All) were all up-

264   regulated. Additionally, seven DEGs were enriched in the "abscisic acid-activated signaling

265   pathway (GO:0009738)" term. In the ET signal pathway, we found that the one gene

266   (CL2232.Contig4_All) encoding ETR down-regulated 6.81 fold. |In the Ja signal pathway, cetyl-

267   CoA acyltransferase 1 is a key enzyme for synthesis of JA-CoA. In this study, the genes

268   (CL10456.Contig6_All) encoding this enzyme were up-regulated by 3.93-fold.

269

270 **ROS related DEGs**

271   We found DEGs related to catalase, glutathione peroxidase, peroxidase, and superoxide

272 dismutase were involved in chilling stress (Figure 4B). Two genes encode superoxide dismutase

273 (CL10965.Contig3_All and CL355.Contig5_All). 3 genes encode catalase (Unigene30041_All,

274 Unigene30063_All and Unigene30085_All), and 10 genes encode peroxidase

275 (CL1068.Contig11_All, CL1068.Contig6_All, CL1068.Contig7_All, CL11425.Contig1_All,

276 CL13638.Contig5_All, CL2936.Contig3_All, CL3639.Contig5_All, CL3639.Contig6_All,

277 CL4125.Contig8_All, and Unigene3189_All). These genes were all up-regulated, indicating that

278 they may have played a positive role in activating antioxidant enzymes in *S. caseolaris* exposed to

279 low temperatures.

280

281 **Transcription factors**

282   TFs play an important role in regulating gene expression that participates in plant stress

283 regulatory processes, such as chilling stress-related processes. In this study, we classified a total

284 of 232 TFs into 41 families and showed differential expression in chilling treated seedlings. A total

285 of 161 (69.40%) were up-regulated. The five most abundant TF families were AP2-ERF, MYB,

286 C3H, WRKY, and ARF with 35 (15.09%), 29 (12.50%) 21 (9.05%), 21 (9.05%), and 15 (6.47%)

287 respectively (Figure 4C).

288

289 **Validation of transcriptome data by RT-qPCR analyses**

290   To validate the RNA-Seq results, we selected a total of 16 candidate genes for qRT-PCR

291 analyses (Figure 6). Based on enrichment analyses, all of these genes were involved in the chilling

292 response. They represented different functional categories or pathways, such as sugar metabolism,

293 antioxidant enzyme, phytohormones and TFs. The results indicated that the transcriptome results

294 were reliable.

295

296 **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

324   temperature tolerance of plant cells (Guy *et al.*, 2008). In GO enrichment analysis, 12 DEGs were

325   enriched in "starch binding (GO:2001070)" (Figure 3B). This could result in the conversion of

326   starch into soluble sugar under chilling stress. Physiological experiments confirmed that the

327   soluble sugars content of *S. caseolaris* increased after chilling stress. Increased levels of sucrose,

328   raffinose, glucose, and fructose are involved in the low-temperature resistance of other plants

329   (Anchordoguy *et al.*, 1987; Ma *et al.*, 2009). The RNA-seq data showed that many DEGs that are

330   involved in starch, fructose, sucrose, glucose, and trehalose were up-regulated in chilling stress

331   treatment. These data were consistent with previous studies (Wang *et al.*, 2018, Deng, *et al.,* 2019;

332   Yang *et al.*, 2019). Plants can accumulate trehalose for oxidative detoxification to counteract

333   abiotic stresses (Lai *et al.*, 2017). In the DEGs, four genes were involved in the trehalose

334   biosynthetic process and all of them were up-regulated, which is similar to the report of Deng *et*

335   *al*. (2019). Sucrose synthase and sucrose phosphate synthase play important roles in sucrose

336   metabolism and biosynthesis respectively (Winter and Huber, 2000). We found that two sucrose

337   synthase genes and one sucrose-phosphate synthase gene were up-regulated. These genes may

338   have been involved in the response to chilling stress. A similar result was found by Yang *et al*.

339   (2019). Carbohydrate also regulate expression of jasmonate, ABA and COR genes (Rutten and

340   Santarius, 1992; Welling and Palva, 2006). We found that many DEGs were involved in

341   carbohydrate metabolism, which indicated that carbohydrates play an important role in *S.*

342   *caseolaris* response to chilling stress.

343

Antioxidant defense system

345   Under environment stress, ROS such as superoxide, hydroxyl radicals and hydrogen peroxide

346   can accumulate in plant cells and cause oxidative damage (Elsheery *et al.*, 2008; Suzuki and

347   Mittler, 2006). To scavenge ROS, antioxidant systems such as superoxide dismutase (SOD),

348   catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) are activated in response to

349   plant stress (Mittler, 2002). SOD is the first antioxidant enzyme that could convert O^{2-} to hydrogen

350   peroxide (H_2O_2), and then POD and CAT could catalyze H_2O_2 and convert it to H_2O. We found

351   DEGs related to CAT, glutathione peroxidase, POD, and SOD were up-regulated, indicating that

352   they may have played a key role in activating antioxidant enzymes in *S. caseolaris* exposed to low

353   temperatures. ROS are also signal molecules that can induce gene expression and protein synthesis

354   to increase stress tolerance in plants (Jaspers and Kangasjärvi, *et al.*, 2010). ROS also is active the

355   mitogen-activated protein kinase (MAPK) cascade pathway, which regulates low-temperature

356   response genes involved in TFs and some hormones. (Pitzschke, *et al.*, 2009; Xie, *et al.*, 2009).

357   We found 15 DEGs enriched in "MAP kinase activity" and most of them were un-regulated, which

358   indicated that MAPK signal transduction pathway also played an important role in chilling stress

359   responses.

360   These results indicated that many antioxidant genes are activated, and these would not only

361   increase the activity of antioxidant enzymes to scavenge with ROS, but also active a sequence of

362   signal to response chilling stress.

363

364   Phytohormone signal network responses to chilling stress

365   Phytohormones are the secondary signals in plant cells that play a significant role in

366   adaptation to environmental stress (Peleg *et al.*, 2011). They can activate a sequence of signal

367   events and eventually induce the genes that directly respond to stress (Bari and Jones, 2009;

368   Hakeem *et al.*, 2014). We found four key response hormone signal transduction pathways (auxin,

369   ABA, EN, and JA) that were significantly influenced by chilling stress. Auxin is a plant growth-

370   promoting hormone (Nemhauser et al., 2006). In this study, 41 DEGs were enriched in the "auxin-

371   activated signaling pathway (GO:0009734)" term. Down-regulation of the *AUX1* gene may reduce

372   plant growth in response to chilling stress. The ABA signaling pathway is important in regulating

373   abiotic stress responses in plants (Danquah, *et al.*, 2014). It can regulate stomatal closure, reduce

374   water loss and maintain cell growth (Peleg & Blumwald, 2011). PLY and PP2C are key genes

375   controlled by the ABA signaling pathway. In present study, the genes were up-regulated in

376   response to chilling stress. This could activate high expression of PP2C and promote stomatal

377   closure and decrease transpiration to reduce water loss. Similar results have been found on

378 *Arabidopsis thaliana* (Tähtiharju and Palva, 2001) and *Oryza sativa* L (Guan *et al.*, 2019).

379 Additionally, the DEGs were also enriched in the "abscisic acid-activated signaling pathway

380 (GO:0009738)" term, which further confirmed that ABA-dependent signaling is involved in the

381 low temperature response of *S. caseolaris*. Genes related to ET biosynthesis often down-regulated

382 expressed under low temperature treatment (Chu and Lee, 1989). In this study, the key gene

383 encoding ETR was down-regulated, which was consistent with previous reports (Yang *et al.*,

384 2018). JA plays a significant role in plants responding to abiotic and biotic stress (Wasternack and

385 Song, 2017; Wasternack and Strnad, 2018). The up-regulated key genes may promote the synthesis

386 of JA-CoA and promote the synthesis of JA. Exogenous methyl salicylic acid (MeSA) or methyl

387 jasmonate (MeJA) could induce the transcription of pathogenesis-related genes, and increase

388 chilling tolerance and pathogen resistance (Ding, *et al.*, 2002). This may explain why many DEGs

389 were enriched in "Plant-pathogen interaction". Similar results have been found in *K. obovate*, *M.

390 wufengensis*, and *Camellia sinensis* under chilling stress (Deng, *et al.*, 2019; Li *et al.*, 2019; Su *et

391 al.*, 2019). However, how those plant hormones mediated signaling participates in chilling stress

392 responses in *S. caseolaris* still need to be further study.

393

394 **TFs responding to chilling stress**

395 TFs play important roles in plant growth and stress tolerance (Lee *et al.*, 2005). When a plant

396 experiences the chilling stress, a series of signal transduction pathways could activate TFs. The

397 activated TFs will activate the expressions of a cascade of downstream resistance-related genes to

398 enhance the cold tolerance by special binding (Kasuga *et al.*, 1999). Many TF families such as

399 DREB (Ahmed *et al.*, 2017, Chen *et al.* 2012), MYB (Zhao *et al.*, 2012, Agarwal *et al.*, 2006;

400 Chen *et al.*, 2006), and WRKY (Zou *et al.*, 2010) are related to plants resistance to low temperature

401 stress. We identified 232 TFs that were classified into 41 families. AP2-ERF, MYB, C3H, WRKY,

402 and ARF were five major TF families identified as DEGs, suggest that they played a crucial role

403 in response to chilling stress. AP2-ERF genes belong to a plant-specific TF family involved in

404 regulating stress responses (Mizoi *et al.*, 2012). The AP2-ERF superfamily consists of the ERF,

405   RAV, AP2, and DREB families. Overexpression of *AP2 TF*, *JcDREB*, and *BpDREB2* increased

406   cold tolerance of *Arabidopsis* (Tang *et al*., 2011; Sun *et al*., 2014). Previous studies verified that

407   AP2-ERFs involved in a lot of plant hormone mediated abiotic stress responses, such as ABA, ET,

408   GA, CTK, and BR (Colebrook *et al*., 2014; Kazan *et al*., 2015; Tao *et al*., 2015; Nolan *et al*.,2017;

409   Sah *et al*., 2016). MYB are involved in chilling stress and regulation of ABA-responsive genes,

410   and constitute with ABA-dependent, induced and mediated types. Which plays an important role

411   in the upstream stage of low temperature transduction (Gyoungju et al., 2016). *AtMYB52*,

412   *AtMYB70*, and *AtMYB82* were ABA-dependent genes in responding abiotic stresses of *Arabidopsis*

413   (Park *et al*, 2011); *TaMYB1*, *TaMYB2*, *TaMYB3R1* were ABA-induced genes in responding abiotic

414   stresses of wheat (Cai *et al*., 2011; Tong *et al*., 2006); *AtMYB2*, *AtMYB41* were ABA-mediated

415   types that promote the accumulation of ABA in responding abiotic stresses (Abe *et al*., 2003;

416   Lippold *et al*., 2009). The WRKY genes can respond strongly and rapidly to abiotic stresses (Chen

417   *et al*., 2012). The *WRKY46* gene enhanced the low temperature tolerance of transgenic cucumber

418   by activating a series of cold-stress responsive genes in an ABA-dependent manner (Zhang *et al.,*

419   2016). *MaWRKY25* can be induced by low temperature or MeJA, which could increase the low

420   temperature tolerance of banana. It could also active the *MaLOX2*, *MaAOS3*, *MaOPR3* genes to

421   promote the synthesis of JA by special binding. Which could reduce the damage of banana fruit

422   by low temperature (Ye, *et al*., 2016). Other TFs, such as the HSF, bZIP, and GRAS families, are

423   induced by chilling stress, and members of these families can affect the cold resistance of plants

424   (Chinnusamy *et al.,* 2010). These results indicated that many TFs were involved in the seedlings

425   of *S. caseolaris* in response to chilling stress through different pathways. Besides, the both un-

426   regulated TFs and phytohormones maybe have some connection, and the regulatory network need

427   further study.

428

429   **Conclusions**

430   We analyzed at the morphological, physiological and transcriptome level of *S. caseolaris*

431   response to chilling stress. Chilling stress of *S. caseolaris* likely involves damage to the cell

432   membranes. This was supported by a significant increase in the relative electrolyte conductivity

433   and MDA contents. Up regulating genes in chilling stressed *S. caseolaris* were involved in plant

434   hormone signal transduction, TFs, carbohydrate metabolism, and antioxidant enzymes. These

435   genes might be involved in the significant increase of soluble protein content and soluble sugar

436   content in response to the chilling stress (Figure 7). These data revealed the manner in which *S.*

437   *caseolaris* responds to chilling stress, and provided insight for further research on the

438   environmental adaptations of mangrove species.

439

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

441   Figure 2 Physiological change of *S. caseolaris* in chilling stress. Error bar indicates ± SD of three

442   replicates.

443   Figure 3 The top 20 GO and KEGG enrichment scatter diagram depicting the differential

444   expression of genes. A: the top 20 GO enrichment B: KEGG enrichment.

445   Figure 4 Heat map of relative expression levels of DEGs involved in main transcription factors,

446   sugar metabolism and the antioxidant defense system. A: The main sugar metabolism-related

447   genes. B: The main antioxidant-related genes. C: The main transcription factors.

448   Figure 5 Plant hormone signal transduction and the relative heat map. A Plant hormone signal

449   transduction. B Heat map of DEGs involved with auxin, ABA, ET and JA.

450   Figure 6 Expression of *S. caseolaris* genes in response to chilling stress for 0 h (CK) and 6 h (CT)

451   as determined by qRT-PCR (blue) and RNA-seq (red).

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

453   stress.

454

455   Table S1 List of primers used in this study

456   Table S2 Summary of sequence analysis

457   Table S3 Number of annotated unigenes

458

459   Figure S1 Characteristics of unigenes. A: Distribution of unigene lengths in *Sonneratia caseolaris*.

460   B: Main species distribution of *S. caseolaris* unigenes. C: Pathway annotation of unigenes based

461   on KEGG categorization D: Functional annotation of unigenes based on Gene Ontology (GO)

462   categorization.

463   Figure S2 Volcano diagram showing the differential expression of genes. Blue represents down-

464   regulated, red indicates up-regulated

465

466   **REFERENCES**

467   Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2003. Arabidopsis *AtMYC2*

468   (bHLH) and *AtMYB2* (MYB) function as transcriptional activators in abscisic acid signaling.

469   *The Plant cell* 15(1): 63-78 DOI 10.1105/tpc.006130.

470   Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK. 2006. A R2R3 type MYB

471   transcription factor is involved in the cold regulation of *CBF* genes and in acquired freezing

472   tolerance. *The Journal of biological chemistry* 281(49): 37636-37645 DOI

473   10.1074/jbc.M605895200.

474   Ahmed MM, Shen C, Khan AQ, Wahid MA, Shaban M, Lin Z. 2017. A comparative genomics

475   approach revealed evolutionary dynamics of microsatellite imperfection and conservation in

476   genus *Gossypium*. *Hereditas* 154: 12 DOI 10.1186/s41065-017-0034-4.

477   An JP, Wang XF, Zhang XW, Xu HF, Bi SQ, You CX, & Hao YJ. 2020. An apple MYB

478   transcription factor regulates cold tolerance and anthocyanin accumulation and undergoes

479   MIEL1-mediated degradation. *Plant biotechnology journal* 18(2): 337-353. DOI

480   10.1111/pbi.13201.

481   Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. 1987. Modes of interaction of

482   cryoprotectants with membrane phospholipids during freezing. *Cryobiology* 24(4): 324-331.

483   DOI 10.1016/0011-2240(87)90036-8.

484   Anders S and Huber W. 2010. Differential expression analysis for sequence count data. *Genome

485   biology*, 11(10): R106 DOI 10.1186/gb-2010-11-10-r106.

486 Bari R and Jones J. 2009. Role of plant hormones in plant defence responses. *Plant molecular*
487   *biology* 69(4): 473-488 DOI 10.1007/s11103-008-9435-0.

488 Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of
489   protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72: 248-254
490   DOI 10.1006/abio.1976.9999.

491 Buti M, Pasquariello M, Ronga D, Milc JA, Pecchioni N, Ho VT, Pucciariello C, Perata P, Francia
492   E. 2018. Transcriptome profiling of short-term response to chilling stress in tolerant and
493   sensitive *Oryza sativa* ssp. Japonica seedlings. *Functional & integrative genomics* 18(6): 627-
494   644. DOI 10.1007/s10142-018-0615-y

495 Chen LG, Song Y, Li SJ, Zhang LP, Zou CS, Yu DQ. 2012. The role of WRKY transcription
496   factors in plant abiotic stresses. *Biochimica et biophysica acta* 1819(2): 120-128. DOI
497   10.1016/j.bbagrm.2011.09.002.

498 Chen LZ, Wang WQ, Li QQ, Zhang YH, Yang S, Osland MJ, Huang J, Peng CJ. 2017. Mangrove
499   species' responses to winter air temperature extremes in China. *Ecosphere* 8(6): e01865 DOI
500   10.1002/ecs2.1865.

501 Chen YH, Yang XY, He K, Liu MH, Li JG, Gao ZF, Lin ZQ, Zhang YF, Wang XX, Qiu XM,
502   Shen YP, Zhang L, Deng XH, Luo JC, Deng XW, Chen ZL, Gu HY, Qu LJ. 2006. The MYB
503   transcription factor superfamily of *Arabidopsis*: expression analysis and phylogenetic
504   comparison with the rice MYB family. *Plant molecular biology* 60(1): 107-124. DOI
505   10.1007/s11103-005-2910-y.

506 Chikako K and Ryozo I. 2009. Molecular basis of disease resistance acquired through cold
507   acclimation in overwintering plants. *Journal of plant biology* 52:19-26. DOI 10.1007/s12374-
508   008-9006-6

509 Chinnusamy V, Zhu JK, Sunkar R. 2010. Gene regulation during cold stress acclimation in plants.
510   *Methods in molecular biology* 639: 39-55 DOI 10.1007/978-1-60761-702-0_3.

511 Chu C and Lee TM. 1989. The relationship between ethylene biosynthesis and chilling tolerance
512   in seedlings of rice (*Oryza sativa*). *Bot Bull Acad Sin* 130:263-273.

513 Colebrook EH, Thomas SG, Phillips AL, Hedden P. 2014. The role of gibberellin signalling in

514   plant responses to abiotic stress. *The Journal of experimental biology*, 217(1): 67-75 DOI

515   10.1242/jeb.089938.

516 Danquah A, de Zelicourt A, Colcombet J, & Hirt H. 2014. The role of ABA and MAPK signaling

517   pathways in plant abiotic stress responses. *Biotechnology advances*, 32(1): 40-52. DOI

518   10.1016/j.biotechadv.2013.09.006.

519 Deng S, Ma J, Zhang L, Chen F, Sang Z, Jia Z, Ma L. 2019. *De novo* transcriptome sequencing

520   and gene expression profiling of *Magnolia wufengensis* in response to cold stress. *BMC plant*

521   *biology* 19(1): 321 DOI 10.1186/s12870-019-1933-5.

522 Ding CK, Wang CY, Gross KC, Smith DL. 2002. Jasmonate and salicylate induce the expression

523   of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit.

524   *Planta*, 214(6): 895-901 DOI 10.1007/s00425-001-0698-9.

525 Duke NC, Meynecke JO, Dittmann S, Ellison AM, Anger K, Berger U, Cannicci S, Diele K, Ewel

526   KC, Field CD, Koedam N, Lee SY, Marchand C, Nordhaus I, Dahdouh-Guebas F. 2007. A

527   world without mangroves? *Science* 317(5834): 41-42 DOI 10.1126/science.317.5834.41b.

528 Duke NC. 1992. Coastal and Estuarine Studies Series. American Geophysical Union, Washington,

529   DC

530 Elsheery NI, Cao KF. 2008. Gas exchange, chlorophyll fluorescence, and osmotic adjustment in

531   two mango cultivars under drought stress. *Acta Physiologiae Plantarum* 30(6): 769-777 DOI

532   10.1007/s11738-008-0179-x.

533 Fang L, Yang Y, Guo W, Li J, Zhong C, Huang Y, Zhou R, Shi S. 2016. *De novo* assembly of the

534   transcriptome of *Aegiceras corniculatum*, a mangrove species in the IndoWest Pacific region.

535   *Marine genomics* 28: 49-52 DOI 10.1016/j.margen.2016.05.004.

536 Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L,

537   Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F,

538   Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. 2011. Full-length

539   transcriptome assembly from RNA-Seq data without a reference genome. *Nature*

540   *biotechnology* 29(7): 644-652 DOI 10.1038/nbt.1883.

541   Guan SG, Xu Q, Ma DR, Zhang WZ, Xu ZJ, Zhang MG, Guo ZF. 2019. Transcriptomics profiling

542   in response to cold stress in cultivated rice and weedy rice. *Gene* 685:96-105 DOI

543   10.1016/j.gene.2018.10.066.

544   Guy C, Kaplan F, Kopka J, Selbig J, and Hincha DK. 2008. Metabolomics of temperature stress.

545   *Physiologia plantarum* 132(2): 220-235 DOI 10.1111/j.1399-3054.2007.00999.x.

546   Gyoungju N, Moonsub L, Do-Soon K, Lane RA, Thomas V and Lee DK. 2016. Transcriptome

547   analysis of *Spartina pectinatain* response to freezing stress. *PloS one* 11(3): e0152294 DOI

548   10.1371/journal.pone.0152294.

549   Hakeem KR, Rehman RU, Tahir I. 2014. Plant signaling: understanding the molecular crosstalk ||

550   signaling in response to cold stress 10:193-226 DOI 10.1007/978-81-322-1542-4_10.

551   He W, Zhuang H, Fu Y, Guo L, Guo B, Guo L, Zhang X, Wei Y. 2015. *De novo* Transcriptome

552   Assembly of a Chinese Locoweed (*Oxytropis ochrocephala*) Species Provides Insights into

553   Genes Associated with Drought, Salinity, and Cold Tolerance. *Frontiers in plant science* 2(6):

554   1086 DOI 10.3389/fpls.2015.01086.

555   Jaspers P and Kangasjärvi J 2010. Reactive oxygen species in abiotic stress signaling. *Physiologia*

556   *plantarum* 138(4): 405-413 DOI 10.1111/j.1399-3054.2009.01321.x

557   Jin J and Kim J. 2013. Cold stress signaling networks in *Arabidopsis*. *Journal of Plant Biology*

558   56(2): 69-76 DOI 10.1007/s12374-013-0903-y.

559   Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1999. Improving plant drought,

560   salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor.

561   *Nature biotechnology* 17(3): 287-291 DOI 10.1038/7036.

562   Kazan K. 2015. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. *Trends in*

563   *plant science* 20(4): 219-229. DOI 10.1016/j.tplants.2015.02.001.

564   Krishnamurthy P, Mohanty B, Wijaya E, Lee DY, Lim TM, Lin Q, Xu J, Loh CS, Kumar PP.

565   2017. Transcriptomics analysis of salt stress tolerance in the roots of the mangrove *Avicennia*

566   *officinalis*. *Scientific reports* 7(1): 10031 DOI 10.1038/s41598-017-10730-2.

567 Kumar RR, Goswami S, Sharma SK, Kala YK, Rai GK, Mishra DC, Grover M, Singh GP, Pathak

568   H, Rai A, Chinnusamy V, Rai RD. 2015. Harnessing next generation sequencing in climate

569   change: RNA-Seq analysis of heat stress-responsive genes in wheat (*Triticum aestivum* L.).

570   *OMICS* 19(10): 632-47 DOI 10.1089/omi.2015.0097.

571 Lai CP, Huang LM, Chen LF, Chan MT, Shaw JF. 2017. Genome-wide analysis of GDSL-type

572   esterases/lipases in *Arabidopsis*. Plant molecular biology 95(1-2): 181-197 DOI

573   10.1007/s11103-017-0648-y.

574 Lee BH, Henderson DA, Zhu JK. 2005. The *Arabidopsis* cold-responsive transcriptome and its

575   regulation by ICE1. *The Plant cell* 17(11): 3155-3175 DOI 10.1105/tpc.105.035568.

576 Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or

577   without a reference genome. *BMC Bioinformatics* 4(12): 323 DOI 10.1186/1471-2105-12-

578   323.

579 Li R, Li Y, Kristiansen K, Wang J. 2008. SOAP: short oligonucleotide alignment program.

580   *Bioinformatics* 24(5): 713-714 DOI 10.1093/bioinformatics/btn025.

581 Li Y, Wang X, Ban Q, Zhu X, Jiang C, Wei, C, Bennetzen JL. 2019. Comparative transcriptomic

582   analysis reveals gene expression associated with cold adaptation in the tea plant Camellia

583   sinensis. *BMC genomics* 20(1): 624. DOI 10.1186/s12864-019-5988-3.

584 Lippold F, Sanchez DH, MusialakM, Schlereth A, Scheible WR, Hincha DK, Udvardi MK. 2009

585   AtMyb41 regulates transcriptional and metabolic responses to osmotic stress in Arabidopsis.

586   *Plant physiology* 149(4): 1761-1772. DOI 10.1104/pp.108.134874

587 Livak KJ and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time

588   quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25(4): 402-408 DOI

589   10.1006/meth.2001.1262.

590 Ma YY, Zhang YL, Lu J, Shao HB. 2009. Roles of plant soluble sugars and their responses to

591   plant cold stress. *African Journal of Biotechnology* 8:2004-2010 DOI 10.1186/1471-2164-

592   10-234.

593 Marè C, Mazzucotelli E, Crosatti C, Francia E, Stanca AM, & Cattivelli L. 2004. Hv-WRKY38:

594   a new transcription factor involved in cold- and drought-response in barley. *Plant molecular*

595   *biology* 55(3): 399-416 DOI 10.1007/s11103-004-0906-7.

596   Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in plant science* 7(9):

597   405-410 DOI 10.1016/s1360-1385(02)02312-9.

598   Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. 2012. AP2/ERF family transcription factors in

599   plant   abiotic   stress   responses.   *Biochim   Biophys   Acta*   1819(2):   86-96   DOI

600   10.1016/j.bbagrm.2011.08.004.

601   Mutlu S, Karadaolu A, Nalbantolu B. 2013. Protective role of salicylic acid applied before cold

602   stress on antioxidative system and protein patterns in barley apoplast. *Biologia Plantarum*

603   57(3): 507-513 DOI 10.1007/s10535-013-0322-4.

604   Nakashima K, Ito Y, Yamaguchi-Shinozaki K. 2009. Transcriptional regulatory networks in

605   response to abiotic stresses in *Arabidopsis* and grasses. *Plant physiology* 149(1): 88-95 DOI

606   10.1104/pp.108.129791.

607   Nemhauser JL, Hong F, Chory J. 2006. Different plant hormones regulate similar processes

608   through largely nonoverlapping transcriptional responses. *Cell* 126(3): 467-75 DOI

609   10.1016/j.cell.2006.05.050.

610   Nievola CC, Carvalho CP, Carvalho V, Rodrigues E. 2017. Rapid responses of plants to

611   temperature   changes.   *Temperature*   (Austin)   4(4):371-405   DOI

612   10.1080/23328940.2017.1377812.

613   Nolan TM, Brennan B, Yang M, Chen J, Zhang M, Li Z, Wang X, Bassham DC, Walley J, Yin,

614   Y. 2017. Selective Autophagy of BES1 Mediated by DSK2 Balances Plant Growth and

615   Survival. *Developmental cell*, 41(1): 33-46.e7 DOI 10.1016/j.devcel.2017.03.013.

616   Park MY, Kang JY, Kim SY. 2011. Overexpression of *AtMYB52* confers ABA hypersensitivity

617   and drought tolerance. Molecules and cells, 31(5): 447-454 DOI 10.1007/s10059-011-0300-

618   7.

619   Peleg Z, Blumwald E. 2011. Hormone balance and abiotic stress tolerance in crop plants. *Current*

620   *opinion in plant biology* 14(3): 290-295 DOI 10.1016/j.pbi.2011.02.001

621 Peng YL, Wang YS, Fei J, Sun CC, Cheng H. 2015. Ecophysiological differences between three

622   mangrove seedlings (*Kandelia obovata*, *Aegiceras corniculatum*, and *Avicennia marina*)

623   exposed to chilling stress. *Ecotoxicology* 24(7-8): 1722-1732 DOI 10.1007/s10646-015-

624   1488-7.

625 Pitzschke A, Schikora A, & Hirt H. 2009. MAPK cascade signalling networks in plant defence.

626   *Current opinion in plant biology* 12(4): 421-426.

627 Ren H, Lu HF, Shen WJ, Huang C, Guo QF, Li ZA, Jian SG. 2002. *Sonneratia apetala* Buch.Ham

628   in the mangrove ecosystems of China: An invasive species or restoration species? *Ecological*

629   *Engineering* 35:1243-1248 DOI 10.1016/j.ecoleng.2009.05.008.

630 Robertsen AI, Alongl DM. 1992. Tropical Mangrove Ecosystem. American Geophysical Union,

631   Washington, DC, 130pp.

632 Román Á, Andreu V, Hernández ML, Lagunas B, Picorel R, Martínez-Rivas JM, & Alfonso M.

633   2012. Contribution of the different omega-3 fatty acid desaturase genes to the cold response

634   in soybean. *Journal of experimental botany,* 63(13): 4973-4982 DOI 10.1093/jxb/ers174

635 Rutten D, Santarius KA, 1992. Relationship between frost tolerance and sugar concentration of

636   various bryophytes in summer and winter. *Oecologia* 91:260-265.

637 Sah SK, Reddy KR, Li J. 2016. Abscisic Acid and Abiotic Stress Tolerance in Crop Plants.

638   *Frontiers in plant science* 7: 571. DOI 10.3389/fpls.2016.00571

639 Schmitt K, Duke NC. 2015. Mangrove Management, Assessment and Monitoring. Tropical

640   Forestry Handbook. Springer Berlin Heidelberg. pp1725-1759 DOI 10.1007/978-3-642-

641   41554-8_126-1.

642 Su W, Ye C, Zhang Y, Hao S, Li QQ. 2019. Identification of putative key genes for coastal

643   environments and cold adaptation in mangrove *Kandelia obovata* through transcriptome

644   analysis. *The Science of the total environment* 681: 191-201 DOI

645   10.1016/j.scitotenv.2019.05.127.

646 Sun J, Peng X, Fan W, Tang M, Liu J, Shen S. 2014. Functional analysis of *BpDREB2* gene

647   involved in salt and drought response from a woody plant *Broussonetia papyrifera*. *Gene*

648   535(2):140-149 DOI 10.1016/j.gene.2013.11.047.

649 Sung DY, Kaplan F, Lee KJ, Guy CL. 2003. Acquired tolerance to temperature extremes. *Trends*

650   *in plant science*, 8(4): 179-187. DOI 10.1016/S1360-1385(03)00047-5

651 Suzuki N and Mittler R. 2006. Reactive oxygen species and temperature stresses: a delicate

652   balance between signaling and destruction. *Physiologia Plantarum* 126(1): 45-51 DOI

653   10.1111/j.0031-9317.2005.00582.x.

654 Tähtiharju S, Palva T. 2001. Antisense inhibition of protein phosphatase 2C accelerates cold

655   acclimation in *Arabidopsis thaliana*. *The Plant journal* 26(4): 461-470 DOI 10.1046/j.1365-

656   313x.2001.01048.x.

657 Tang M, Liu X, Deng H, Shen S. 2011. Over-expression of JcDREB, a putative AP2/ERF domain-

658   containing transcription factor gene in woody biodiesel plant Jatropha curcas, enhances salt

659   and freezing tolerance in transgenic *Arabidopsis thaliana*. *Plant science* 181(6): 623-31 DOI

660   10.1016/j.plantsci.2011.06.014.

661 Theocharis A, Clément C, Barka EA. 2012. Physiological and molecular changes in plants grown

662   at low temperatures. *Planta* 235(6): 1091-1105 DOI 10.1007/s00425-012-1641-y.

663 Thomashow MF. 1999. Plant cold acclimation: freezing tolerance genes and regulatory

664   mechanisms. *Annual review of plant physiology and plant molecular biology* 50: 571-599.

665   DOI 10.1146/annurev.arplant.50.1.571.

666 Tomlinson PB. 1999. The Botany of Mangroves. Cambridge University Press, New York, pp126.

667 Tong GL, Jang CS, Kim JY, Dong SK, Park JH, Kim DY. 2006. A Myb transcription factor

668   (TaMyb1) from wheat roots is expressed during hypoxia: roles in response to the oxygen

669   concentration in root environment and abiotic stresses. *Physiologia Plantarum* 129: 275-385

670   DOI 10.1111/j.1399-3054.2006.00828.x.

671 Wang BS, Liao BW, Wang YJ, Zan QJ. 2002. Mangrove forest ecosystem and its sustainable

672   development in shengzhen bay. Beijing China: Science Press (in Chinese).

673 Wang H, Zou Z, Wang S, Gong M. 2013. Global analysis of transcriptome responses and gene

674   expression profiles to cold stress of *Jatropha curcas* L. *PLoS One* 8(12): e82817 DOI

675   10.1371/journal.pone.0082817.

676 Wang K, Bai ZY, Liang QY, Liu QL, Zhang L, Pan YZ, Liu GL, Jiang BB, Zhang F, Jia Y. 2018.

677   Transcriptome analysis of chrysanthemum (*Dendranthema grandiflorum*) in response to low

678   temperature stress. *BMC genomics* 19(1): 319 DOI 10.1186/s12864-018-4706-x.

679 Wang K, Zhong M, Wu YH, Bai ZY, Liang QY, Liu QL, Pan YZ, Zhang L, Jiang BB, Jia Y, Liu

680   GL. 2017. Overexpression of a chrysanthemum transcription factor gene DgNAC1 improves

681   the salinity tolerance in chrysanthemum. *Plant cell reports* 36(4):571-581 DOI

682   10.1007/s00299-017-2103-6.

683 Wang Q, Guan Y, Wu Y, Chen H, Chen F, Chu C. 2008. Overexpression of a rice *OsDREB1F*

684   gene increases salt, drought, and low temperature tolerance in both Arabidopsis and rice.

685   *Plant molecular biology* 67(6): 589-602 DOI 10.1007/s11103-008-9340-6.

686 Wang WQ and Wang M. 2007. The mangrove of China. Science Press, Beijing, China.

687 Wasternack C, Song S 2017 Jasmonates: biosynthesis, metabolism, and signaling by proteins

688   activating and repressing transcription. *Journal of experimental botany* 68(6): 1303-1321

689   DOI 10.1093/jxb/erw443.

690 Wasternack C, Strnad M. 2018. Jasmonates: News on occurrence, biosynthesis, metabolism and

691   action of an ancient group of signaling compounds. *International journal of molecular*

692   *sciences* 19(9): 2539 DOI 10.3390/ijms19092539.

693 Welling A, Palva ET. 2006. Molecular control of cold acclimation in trees. *Physiologia Plantarum*

694   127(2): 167-181 DOI: 10.1111/j.1399-3054.2006.00672.x

695 Winter H and Huber, SC. 2000. Regulation of sucrose metabolism in higher plants: localization

696   and regulation of activity of key enzymes. *Critical reviews in biochemistry and molecular*

697   *biology* 35(4): 253-289 DOI 10.1080/10409230008984165.

698 Wise RR, Naylor AW. 1987. Chilling-enhanced photooxidation: The peroxidative destruction of

699   lipids during chilling injury to photosynthesis and ultrastructure. *Plant physiology* 83(2): 272-

700   277 DOI10.1104/pp.83.2.272.

701 Xie G, Kato H, Sasaki K, & Imai R 2009. A cold-induced thioredoxin h of rice, *OsTrx23*,

702   negatively regulates kinase activities of *OsMPK3* and *OsMPK6* in vitro. *FEBS letters*
703   583(17): 2734-2738. DOI 10.1016/j.febslet.2009.07.057

704   Xie ZY, Zhou ZL, Li HM, Yu JJ, Jiang JJ, Tang ZH, Ma DF, Zhang BH, Han YH, Li ZY. 2018.
705   High throughput sequencing identifies chilling responsive genes in sweetpotato (*Ipomoea*
706   *batatas* Lam.) during storage. *Genomics* 111(5): 1006-1017
707   DOI10.1016/j.ygeno.2018.05.014.

708   Yamanaka T, Miyama M, Tada Y. 2009. Transcriptome profiling of the mangrove plant *Bruguiera*
709   *gymnorhiza* and identification of salt tolerance genes by *Agrobacterium* functional screening.
710   *Bioscience, biotechnology, and biochemistry* 73(2): 304-310 DOI 10.1271/bbb.80513.

711   Yang T, Huang XS. 2018. Deep sequencing-based characterization of transcriptome of *Pyrus*
712   *ussuriensis* in response to cold stress. *Gene* 661:109-118 DOI10.1016/j.gene.2018.03.067.

713   Yang X, Zhao T, Rao P, Gao K, An X. 2019. Transcriptome profiling of *Populus tomentosa* under
714   cold stress. *Industrial Crops and Products* 135:283-293 DOI10.1016/j.indcrop.2019.04.056.

715   Yang Y, Yang S, Li J, Deng Y, Zhang Z, Xu S, Guo W, Zhong C, Zhou R, Shi S. 2015a
716   Transcriptome analysis of the Holly mangrove *Acanthus ilicifolius* and its terrestrial relative,
717   *Acanthus leucostachyus*, provides insights into adaptation to intertidal zones. *BMC Genomics*
718   16(1): 605 DOI10.1186/s12864-015-1813-9.

719   Yang Y, Yang S, Li J, Li X, Zhong C, Huang Y, Zhou R, Shi S. 2015b. De novo assembly of the
720   transcriptomes of two yellow mangroves, *Ceriops tagal* and *C. zippeliana*, and one of their
721   terrestrial relatives, *Pellacalyx yunnanensis*. *Mar Genomics* 23:33-6
722   DOI10.1016/j.margen.2015.04.003.

723   Yao YX, Dong QL, Zhai H, You CX, Hao YJ. 2011. The functions of an apple cytosolic malate
724   dehydrogenase gene in growth and tolerance to cold and salt stresses. *Plant physiology and*
725   *biochemistry* 49(3): 257-64 DOI10.1016/j.plaphy.2010.12.009.

726   Ye YJ, Xiao YY, Han YC, Shan W, Fan ZQ, Xu QG, Kuang JF, Lu WJ, Lakshmanan P, & Chen
727   JY. 2016. Banana fruit VQ motif-containing protein5 represses cold-responsive transcription
728   factor *MaWRKY26* involved in the regulation of JA biosynthetic genes. *Scientific reports* 6:

729   23632. DOI 10.1038/srep23632

730 Young MD, Wakefield MJ, Smyth GK, Oshlack A. 2010. Gene ontology analysis for RNA-seq:

731   accounting for selection bias. *Genome biology* 11(2): R14 DOI10.1186/gb-2010-11-2-r14.

732 Zan QJ, Wang BS, Wang YJ, Li MG. 2003. Ecological assessment on the introduced *Sonneratia*

733   *caseolaris* and *Sonneratia apetala* at the mangrove forest of Shenzhen Bay, China. *Acta*

734   *Botanica Sinica* 45:544-555.

735 Zhang X, Fowler SG, Cheng H, Lou Y, Rhee, SY, Stockinger EJ, & Thomashow MF. 2004.

736   Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon

737   that differs from that of freezing-tolerant Arabidopsis. *Plant journal*, 39(6): 905-919 DOI

738   10.1111/j.1365-313X.2004.02176.x.

739 Zhang Y, Yu H, Yang X, Li Q, Ling J, Wang H, Gu X, Huang S, Jiang W. 2016. *CsWRKY46*, a

740   WRKY transcription factor from cucumber, confers cold resistance in transgenic-plant by

741   regulating a set of cold-stress responsive genes in an ABA-dependent manner. *Plant*

742   *physiology and biochemistry* 108: 478-487 DOI10.1016/j.plaphy.2016.08.013.

743 Zhao L, Nicholson JK, Lu A, Wang Z, Tang H, Holmes E, Shen J, Zhang X, Li JV, Lindon JC.

744   2012. Targeting the human genome-microbiome axis for drug discovery: inspirations from

745   global systems biology and traditional Chinese medicine. *Journal of proteome research*

746   11(7): 3509-3519 DOI10.1021/pr3001628.

747 Zhou MQ, Shen C, Wu LH, Tang KX, Lin J. 2011. CBF-dependent signaling pathway: a key

748   responder to low temperature stress in plants. *Critical reviews in biotechnology*. 31(2): 186-

749   192 DOI10.3109/07388551.2010.505910.

750 Zou C, Jiang W, Yu D. 2010. Male gametophyte-specific *WRKY34* transcription factor mediates

751   cold sensitivity of mature pollen in Arabidopsis. *Journal of experimental botany* 61(14):

752   3901-3914 DOI10.1093/jxb/erq204.

753

Figure 1

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

Figure 2

Physiological change of *S. caseolaris* in chilling stress. Error bar indicates ± SD of three replicates.
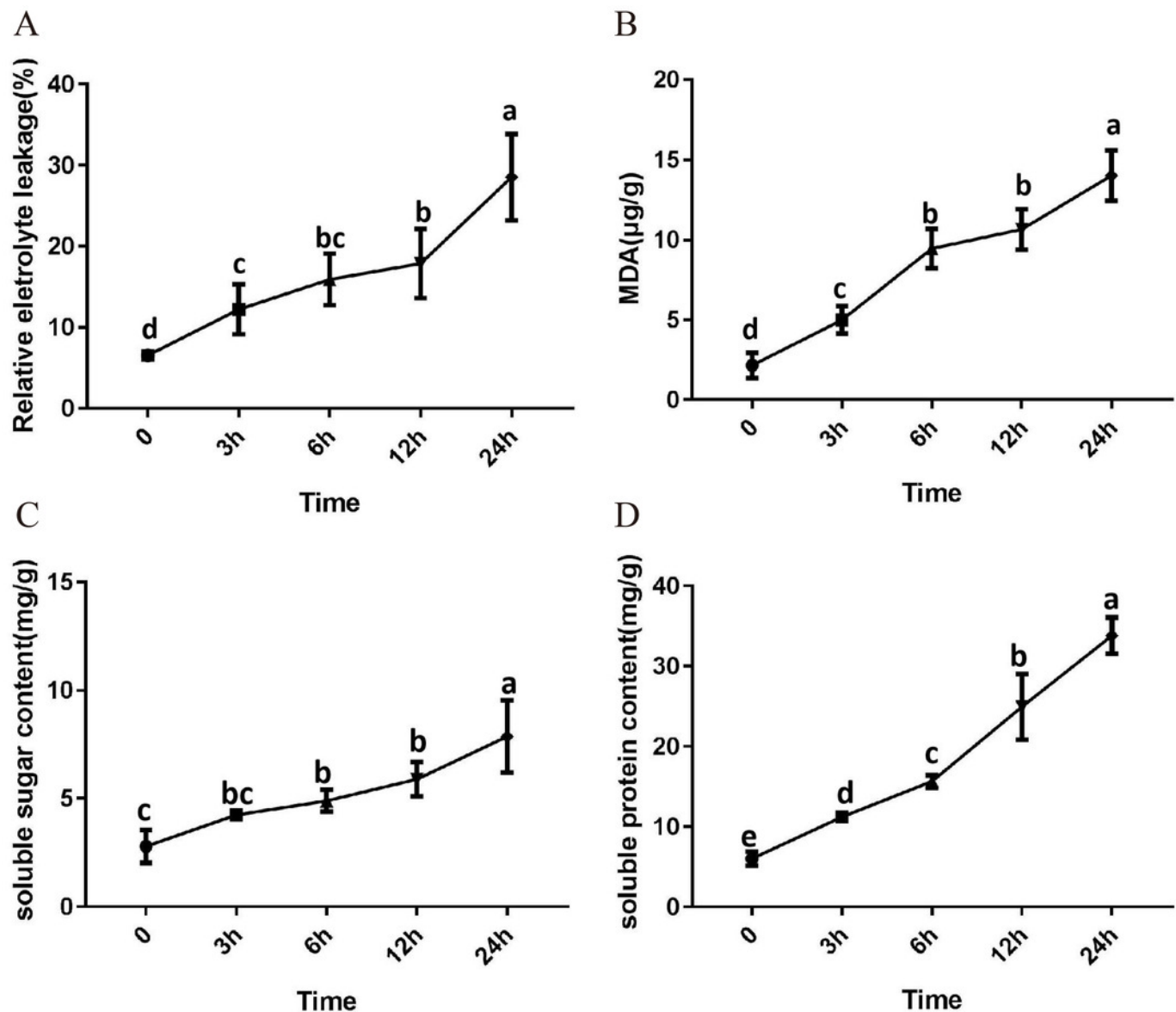
Figure 3

The top 20 GO and KEGG enrichment scatter diagram depicting the differential expression of genes. A: the top 20 GO enrichment. B: KEGG enrichment.
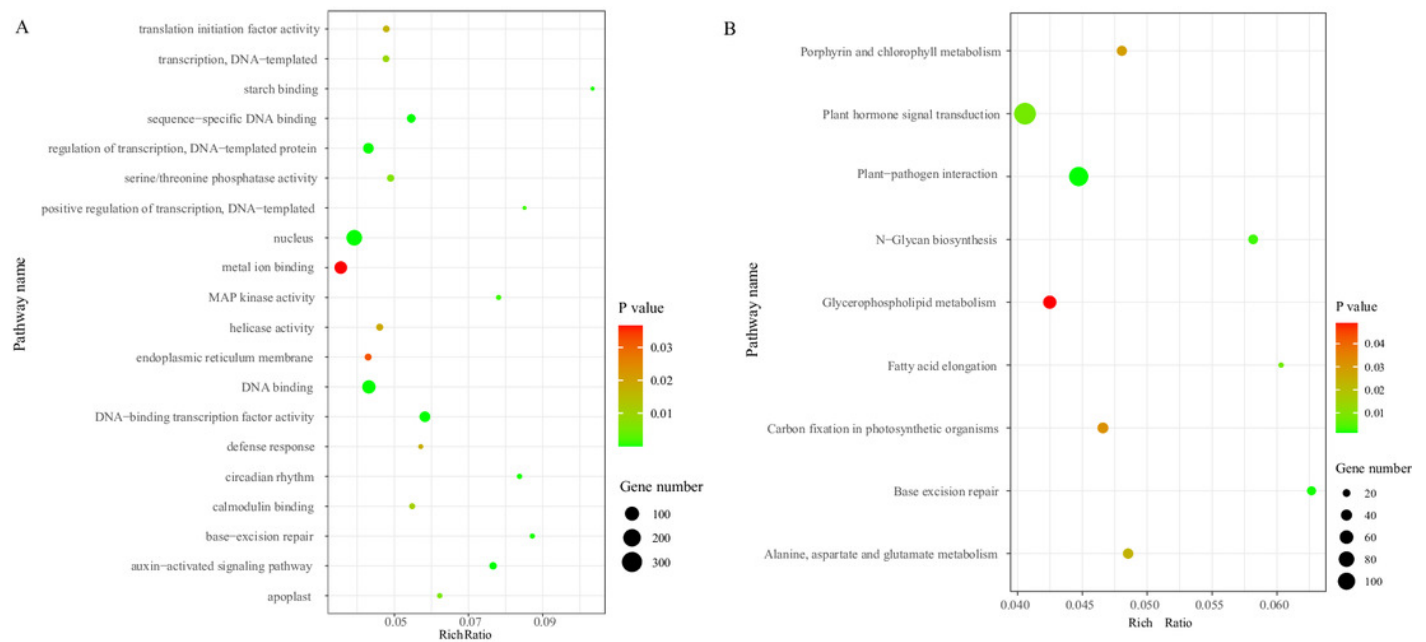
Figure 4

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
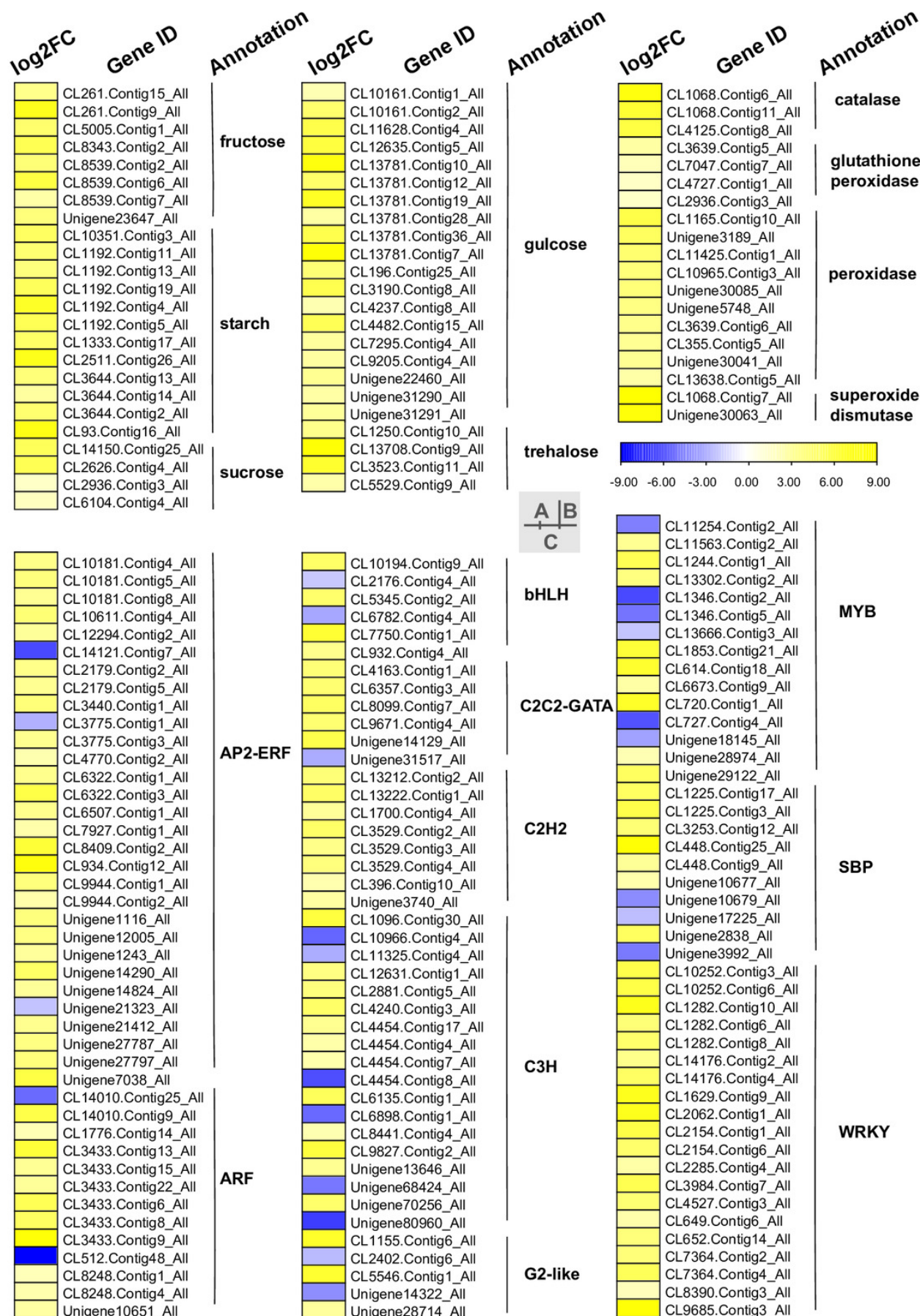
Figure 5

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.
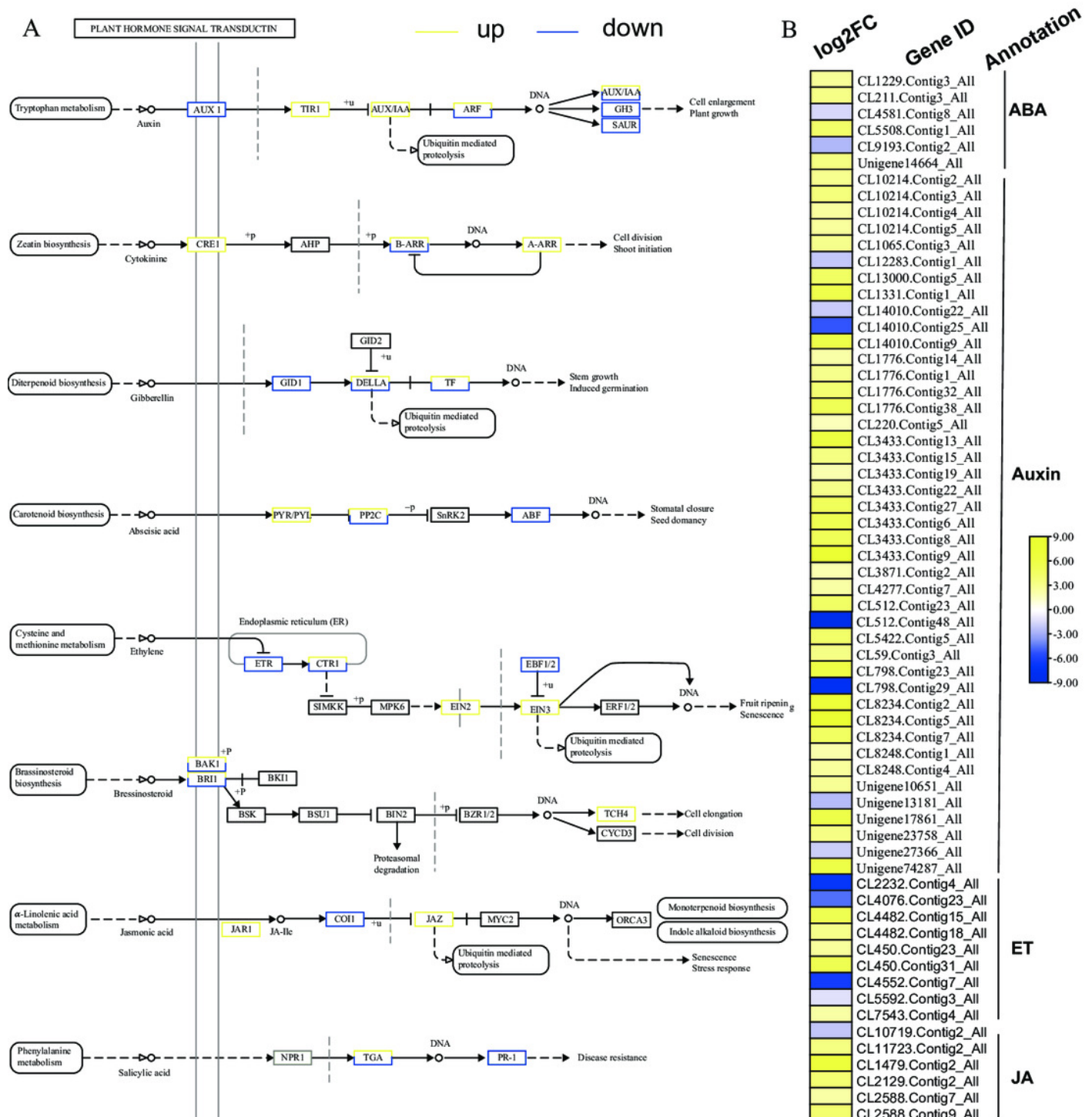
Figure 6

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.