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RNA-Seq analysis of ground-cover chrysanthemum provides insights into the basis of natural low-temperature stress

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Abstract

Low temperature is one of the most severe abiotic stress factors that limit chrysanthemum growth and development. Natural temperature changes are more complex, and cold stress from a laboratory incubator cannot accurately represent the natural temperature stress. Here, nine separate high-throughput mRNA sequencing technology (RNA-Seq) libraries were generated from the RNA sample of roots from different temperatures, including chilling (Ch), freezing (Fr), and control (CK). The 7 069 and 3 952 differentially transcribed genes were identified as CK *vs.* Ch and CK *vs.* Fr, respectively. The Kyoto encyclopedia of genes and genomes pathway (*KEGG*) enrichment analysis showed that significantly different flavonoid biosynthesis and linolenic acid pathways commonly appeared in CK *vs.* Ch and CK *vs.* Fr. Arginine and proline metabolism, lipid metabolism, fatty acid degradation, and fructose and mannose metabolism pathways were found in CK *vs.* Ch, and only in the CK *vs.* Fr enrichment metabolic pathway included steroid biosynthesis and monoterpenoid biosynthesis. The transcription of genes on differential metabolic pathways and MYBs were successfully validated using quantitative real-time PCR. At the same time, the antioxidant activity, malondialdehyde, and proline content were analyzed under low temperature. These datasets may aid in understanding and carrying out future studies on the molecular basis of cold stress and contribute to chrysanthemum breeding.

Keywords: ground-cover chrysanthemum, MYB, RNA-Seq, reactive oxygen species.

Introduction

Low temperature is one of the most important environmental factors that restrict plant growth, development, and geographical distribution. Low-temperature stress covers chilling (0 - 10°C) and freezing (below 0°C). In plants, chilling and freezing can cause slow growth, lack of vigor, wilting, and even death. Plants can improve their

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Abbreviations: ADC - arginine decarboxylase; AIH - agmatine to putrescine *via* agmatine iminohydrolase; BP - biological process; CAT - catalase; CBF/DREB - C-repeat binding actor/dehydration responsive element binding factor; CC - cellular component; Ch - chilling; CK - control; COR - dehydration-responsive element of downstream cold responsive; DEGs - differentially expressed genes; DTGs - quantification of transcripts and identification of differentially transcribed genes; *EF1α* - cold-dependent gene primer sequences and elongation factor 1α; FC - fold change; FDR - false discovery rate; FPKM - fragments per kilobase of transcript per million fragments mapped; FM - fresh mass; Fr - freezing; GMP - GDP-mannose pyrophosphorylase; *GO* - gene ontology; *KEGG* - Kyoto encyclopedia of genes and genomes pathway; LT_{50} - the half lethal temperature; MDA - malondialdehyde; MF - molecular function; NLP - N-carbamoylputrescine amidohydrolase; NOS - nitric oxide synthase; Nr - *NCBI* non-redundant protein; POD - peroxidase; Q30 - base ratios with quality values higher than 30 in reads; REC - relative electrical conductivity values; RNA-Seq - high-throughput mRNA sequencing technology; ROS - reactive oxygen species; SOD - superoxide dismutase.
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survival during freezing as they are exposed to chilling temperature through cold acclimation (Li *et al.* 2016). Cold acclimation is a complex process, and domesticated plants use a series of protective strategies, including synthesis of antifreeze proteins, accumulation of osmoprotectants, and scavenging of reactive oxygen species, to adapt to low temperatures (Kyu *et al*. 2019, Luo *et al.* 2020, Zulfiqar *et al.* 2020). The transcription factors C-repeat binding factor/dehydration responsive element binding factor (CBF/DREB) play an important role in plant cold stress. In *Arabidopsis*, DREB/CBF-like proteins can be classified into six subgroups according to the binding domain. Their three genes encoding DREB1B/CBF1, DREB1A/CBF3, and DREB1C/CBF2 respond to low-temperature signals. They have highly similar amino acid sequences and are functionally redundant (Maruyama *et al.* 2004). Overexpressing DREB1B/CBF1 or DREB1C/CBF2 in *Arabidopsis* showed that transgenic plants have improved survival under freezing stress (Medina *et al.* 2011). Further research indicated that CBF/DREB recognize the dehydration-responsive element of downstream cold-responsive (*COR*) genes during cold stress (Shi *et al.* 2017). Plants exposed to abiotic stress can generate reactive oxygen species (ROS), which cause oxidative damage. Meanwhile, plants also produce antioxidant enzymes that protect them from damage. Many antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), are induced for ROS detoxification (Wang *et al*. 2019). Flavonoid accumulation also can be induced for cold tolerance in plants. Flavonoids are among the most bioactive plant secondary metabolites that can suppress ROS damage and alleviate oxidative stress (Kejík *et al.* 2021). The enhancement accumulation of flavonoids leads to improved cold stress in tea (Wang *et al.* 2016). Thus, flavonoids play an important role in cold tolerance.

High-throughput mRNA sequencing technology (RNA-Seq) has been widely used to analyze gene expression under stress. Some chilling tolerance candidate genes that are mainly involved in antioxidant defense system, soluble sugar synthesis, hormone signal transduction, and photosynthesis are induced in response to low temperature as indicated by transcriptome profiling (Yang *et al*. 2019). The unsaturated fatty acid biosynthesis and jasmonic acid biosynthesis pathways participate in cold tolerance in *Camellia japonica* as indicated by RNA-Seq (Li *et al.* 2016). RNA-Seq analysis of transgenic cotton revealed that lipid biosynthesis and linoleic acid metabolism are related to chilling tolerance. Electrospray ionization mass spectrometry revealed changes in the composition of sphingolipids and glycerolipids in leaves, which might alter the fluidity of the cell membranes (Wang *et al.* 2020a). The change in outdoor temperature during winter is a complex process. In winter, the temperature decreases gradually but it fluctuates from high to low throughout the day. Stress imposed in the laboratory cannot replicate the complex natural environment. Therefore, evaluating plants' resistance was incomplete under stress from the laboratory (Des Marais *et al.* 2010). Transcriptomic and metabolomic data show that the gene expression in *Thellungiella* plants responding to stress in cabinets is different from that in their natural habitats (Guevara *et al.* 2012). In a special incubator, the molecular mechanism of cold tolerance in chrysanthemum has been reported (Lu *et al.* 2018), but the molecular mechanism of cold stress in a natural environment is still unclear.

Chrysanthemum morifolium cv. Yingjie is a perennial herbaceous flower; it has a compact size, abundant flowers, long bloom duration, diverse colors, and high cold tolerance, and is widely used in outdoor landscaping (Chen *et al.* 2013). It is one of the ground cover chrysanthemum cultivars, has red flowers, a spherical shape, and can overwinter naturally in Yanji, China. Here, the RNA-Seq platform based on *Illumina NGS* technology was used to characterize the transcriptomic response to low temperature by comparing the different transcriptomes of Yingjie plants subjected to periods of above-zero temperature or below-zero temperature in a natural environment. The findings in this work may serve as a foundation for studying the molecular mechanism of chrysanthemum cold tolerance.

Materials and methods

Plant material and cold stress treatment: In spring, the chrysanthemum cultivar Yingjie was planted in the teaching field bases at Yanbian University, China. Common methods of cultivation and management, such as loosening soil, weeding, and fertilizing, were adopted. After flowers fell during autumn, the upper part of Yingjie was cut off. When the soil average temperature was 5°C (A) and -5°C (C) at a depth of 7 cm, the lateral root of the chrysanthemum was dug out as experimental material. Control group (D) lateral roots were harvested at a solid average temperature of 20°C. Three plants were sampled on each occasion and maintained as three biological replicates. Immediately after the samples were harvested, nonlignified lateral roots were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from the nonlignified lateral roots using a Plant RNA kit (*Tiangen*, Beijing, China), following the manufacturer's protocol. A nanophotometer spectrophotometer (*Implen*, Munich, Germany) was used to evaluate the purity of the RNA samples. The total RNA preparation was treated with RNase-free DNase I to degrade genomic DNA and mixed with oligo (dT) coated magnetic beads to concentrate the poly(A) mRNA. The library was constructed using three different temperature treatment samples (including three repetitions). The soil temperature in Yanji, China, in 2019 is shown in Fig. 1 Suppl.

RNA-Seq analysis: The library was sequenced using an *Illumina Hiseq2000* platform by *Biomarker Bio-technology Co. Ltd.* (Beijing, China). The average length of sequenced reads was 150 bp. Clean reads were obtained by removing adaptor sequences, with the poly-N of more than 10% or a lower quality. At the same time, the Q30 and GC contents of the clean reads were verified. The quality of the library has numerous main criteria,

including mRNA randomized fragmentation, sequencing saturation, and insert fragmentation size. Clean reads were mapped to the reference genome (http://www.amwayabrc.com/) using *TopHat 2* software under the criterion of not more than two mismatches in the alignment. The *NCBI non-redundant protein* (Nr) database and the *Swiss-Prot* protein database were used for *BLAST* search and annotations. Functional annotation was analyzed using *GO* terms. *KEGG* is the major public pathway-related database, in which proteins with similar functions may be classified. Quantification of the gene expression level was estimated by fragments per kilobase of transcript per million fragments mapped (FPKM). DEGs were screened in terms of $\log_2 |FC| > 2.0$ and FDR < 0.001. False discovery rate (FDR), a statistical method, was applied to correct for *P*-value in multiple tests for calculating the expression between two samples. The FC was the ratio of the average FPKM of the two groups. Here, DEGs were screened from pairwise contrasts D *vs.* A and D *vs.* C. Then, DEGs identified using this method were subjected to *GO* functional and *KEGG* pathway analyses.

RT-qPCR analysis: Total RNA was extracted from the roots of plants subjected to the various treatments described above. cDNA synthesis was performed using reverse transcriptase *FastQuant RT* kit (*TianGen*, Beijing, China). RT-qPCR was performed in an *Eppendorf* Real-Time PCR System (*Mastercycler®ep realplex*, Hamburg, Germany) using a *SYBR Premix ExTaq™ Kit* (*TaKaRa*, Beijing, China), according to the manufacturer's protocol. *EF1α* - cold-dependent gene primer sequences and elongation factor 1α gene as a reference are shown in Table 2 Suppl. Each 20 µL qPCR sample included 200 ng cDNA, 100 nM of each primer, and 10 µl *SYBR Green PCR master mix*, in which were performed PCR amplification (40 cycles of 95°C/15 s, 55°C/15 s, 72°C/15 s). Three biological replicates were performed for each sample. The data were processed using the $2^{-\Delta\Delta CT}$ method.

Assessment of freezing tolerance: After three months of planting, the third leaf of Yingjie was cut. These leaves were exposed to 4°C, 2°C, 0°C, -10°C, -16°C temperatures for 2 h, then the conductivity was measured using a conductivity meter. The relative electrical conductivity values (REC) = EC before boiling/EC after boiling × 100%, were fitted to a logistic equation $y = K/(1 + ae^{-bx})$ and calculated the semi-lethal temperature (LT50) = ln (1/a)/b (Yue *et al.* 2020).

Cold stress treatment and antioxidant enzymes activity measurement: Yingjie was planted in a pot filled with a 1:1 mixture of peat and perlite and transferred to a chamber at 25°C with a 14-h photoperiod with an irradiance of 50 µmol m^{-2} s^{-1}. Cold stress treatment was given to plants at the six-to-eight-leaf stage using a culture incubator *MIR-154* (*Sanyo*, Osaka, Japan). The low-temperature treatment consisted of 25°C for 2 h, 10°C for 2 h, 5°C for 2 h, and -5°C for 2 h. The third and fourth leaves counted from the shoot apex were collected from each treatment. The SOD, CAT, and POD activities, and proline and MDA content of the plants under cold stress were analyzed in accordance with previously described methods (Yue *et al.* 2020). Each treatment had three biological replicates. Data were normalized based on leaf fresh mass (FM).

Results

Cold tolerance of Ying Jie cultivar: Seedlings were placed in a low-temperature incubator to determine their cold tolerance. CAT, POD, and SOD activities and proline content in chrysanthemum leaves increased gradually as the temperature decreased. When the temperature dropped to 5°C, CAT, POD, and SOD activities were 6.146, 80.56, and 145.83 µg min^{-1}, respectively, and proline content was 0.0183% reaching peak value. They began to decrease gradually at -5°C. The MDA content increased rapidly from 25°C to 5°C and changed slowly at -5°C (Fig. 1). The chrysanthemum half lethal temperature (LT_{50}) was -11.97°C as measured *via* its convective conductivity (Table 1 Suppl.).

RNA-Seq libraries and read mapping: Nine mRNA libraries were constructed from plant roots at different low temperatures to generate a broad survey of genes involved in natural cold stress. The number of reads per library ranged from 4.27 million to 4.51 million. After filtering, approximately 2.13 - 2.25 million clean reads were generated. The clean reads of the pre-library comprise more than 6.40 billion clean bases, and GC percentage ranged from 42.72 - 43.65% (BioProject: PRJNA722762, https://dataview.ncbi.nlm.nih.gov/object/PRJNA722762?reviewer=e71q9o0kjcc27g5da88uc5ss40). The base ratios with quality values higher than 30 in reads (Q30) were more than 93% in all the libraries. The total clean reads were compared with the *Chrysanthemum* genome, in which all libraries mapped ranged from 50.61 to 60.40% (Table 1). Moreover, the quality of libraries was assessed by random mRNA fragmentation, length of insert fragmentation, and sequencing data saturation (Fig. 2 Suppl.). The values met the analytical criteria.

Quantification of transcripts and identification of differentially transcribed genes (DTGs): Differentially expressed genes (DEGs) were identified through pairwise comparison between various libraries where false discovery rate (FDR) = 0.001; fold change (FC) = 2. Pairwise comparisons of CK *vs.* Ch and CK *vs*. Fr yielded 7 069 and 3 952 DEGs, respectively. Among these DEGs, 3 249 and 1 860 were upregulated and 3 820 and 2 097 were downregulated in CK *vs.* Ch and CK *vs*. Fr, respectively (Fig. 2*A*). These results indicated that the number of DTGs in freezing treatment (-5°C) was less than that of the low-temperature treatment (5°C).

***GO* classification of DTGs:** DTGs for different treatments were identified by gene ontology (*GO*) to obtain their functional categorization. In CK *vs.* Ch, 3 609 (1 621 upregulated and 1 988 downregulated) of the 7 069 DTGs could be assigned to a *GO* term. Of these, 6 985 were

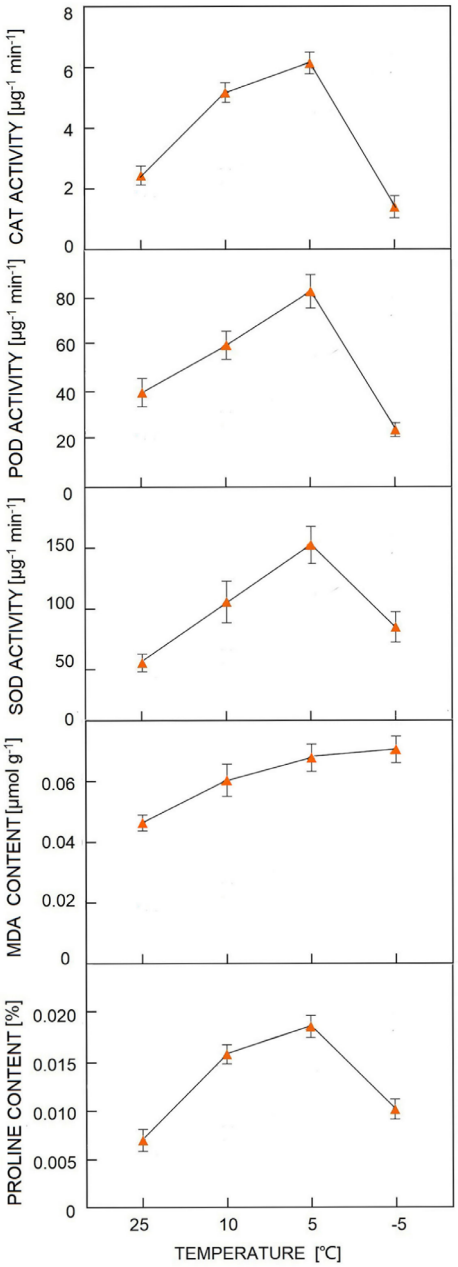

Fig. 1. Change of CAT, POD, and SOD activities and MDA and proline content in Yingjie seeding at various temperatures.

enriched in biological process (BP), 6 308 in cellular component (CC), and 4 271 in molecular function (MF). Overall, 1 929 DTGs were found in CK *vs.* Ch, with 3 810 in BP, 3 770 in CC, and 2 307 in MF (Fig. 2*B*). The number of responses to stimulus-related genes, membrane-related genes, antioxidant activity, and cell junction-related genes during freezing were 190, 746, 25, and 13, respectively, and were less than those observed during chilling (Table 3 Suppl.).

Functional annotation and *KEGG* enrichment analysis of DEGs: *KEGG* pathway database is a known base for systematic analysis of gene function through various molecular networks. In the present study, 1 390 and 876 DEGs were annotated in CK *vs.* Ch and CK *vs.* Fr, respectively, and were submitted for *KEGG* analysis. Among the different pathways of *KEGG* enrichment, we chose the pathway associated with cold tolerance. In CK *vs.* Ch, 29, 24, 20, 18, 22, and 24 DEGs were enriched in the respective pathways: flavonoid biosynthesis ko00941, linolenic acid metabolism ko00592, arginine and proline metabolism ko00330, ether lipid metabolism ko00565, fatty acid degradation ko00071, and fructose and mannose metabolism ko00051. These pathways with DEGs numbers were flavonoid biosynthesis (ko00941, 19 DEGs, 2.17%), steroid biosynthesis (ko00100, 11 DEGs, 1.26%), linoleic acid metabolism (ko00591, 9 DEGs, 1.03%), and monoterpenoid biosynthesis (ko00902, 6 DEGs 0.684%) in CK *vs.* Fr (Table 2). Flavonoid biosynthesis and linoleic acid metabolism pathways appeared in both CK *vs.* Ch and CK *vs.* Fr.

Transcription factors were affected by cold stress: The expression of several classes of transcription factors, including AP2/ERF, MYB, bHLH, and bZIP, was induced by low-temperature stress. Among them, MYB regulates flavonoid synthesis and plays an important role in cold stress. The 54 members of the MYB family were selected in CK *vs.* Ch; 34 of them had upregulated gene transcription levels, and the rest were downregulated. In CK *vs.* Ch, the MYB family consists of 25 members, 7 of which were upregulated, and 18 were downregulated (Table 4 Suppl.). The expressions of the genes (CHR00027541 and CHR00035711) between CK *vs.* Ch and CK *vs.* Fr were changed. RT-qPCR validation showed that the relative expressions of genes (CHR00027541, CHR00035711, CHR00018253, and CHR00020806) were all increased at 5°C compared with those of the control group, and the relative expression of genes (CHR00027541, CHR00035711, and CHR00046138) were increased from 20°C to -5°C (Fig. 3).

Discussion

Activities of antioxidant enzyme, content of proline, and MDA at low temperatures: The ground cover chrysanthemum cv. Yingjie has red flowers, strong cold resistance, and is a popular cultivar. Yanji City is located in the eastern part of Jilin Province in China. Temperatures are low from November to April, with the lowest temperature reaching -30°C. ROS accumulate during oxygen metabolism when plants are subjected to environmental stresses. However, antioxidants mitigate the damage caused by ROS (Liu *et al.* 2014). Antioxidants, including SOD, CAT, and POD, play different functions in plants under stress. SOD is a key antioxidant catalyzing the conversion of O_2^- to H_2O_2 and O_2, and H_2O_2 is rapidly decomposed into O_2 and H_2O by CAT and POD (Wang *et al.* 2004, Gajewska and Skłodowska 2007). SOD, CAT, and POD activities are increased under cold stress in *Avena nuda* and chickpea (Kaur *et al.* 2009, Liu *et al.*

Table 1. Overview of the RNA-Seq reads acquired from low temperature stressed Yingjie. GC percentage - the proportion of guanidine and cytosine nucleotides present, Q30 percentage - the proportion of nucleotides with quality value > 30.

Samples	Total reads [kDa]	Mapped reads [kDa]	Clean reads [kDa]	Clean bases [kDa]	GC percentage	≥ Q30
A1	45 156	26 482 (58.65%)	22 578	6 721 214	42.72%	93.69%
A2	44 084	26 380 (59.84%)	22 042	6 555 120	42.74%	93.74%
A3	44 658	26 973 (60.40%)	22 329	6 674 592	42.75%	93.59%
C1	44 338	22 438 (50.61%)	22 169	6 624 345	43.13%	93.89%
C2	43 482	24 626 (56.63%)	21 741	6 500 319	43.25%	94.14%
C3	42 778	23 273 (54.41%)	21 389	6 402 008	43.35%	94.07%
D1	44 054	23 734 (53.88%)	22 027	6 579 043	43.65%	94.09%
D2	44 689	24 871 (55.65%)	22 345	6 653 202	43.11%	94.02%
D3	43 912	25 725 (58.58%)	21 956	6 529 084	42.92%	94.16%

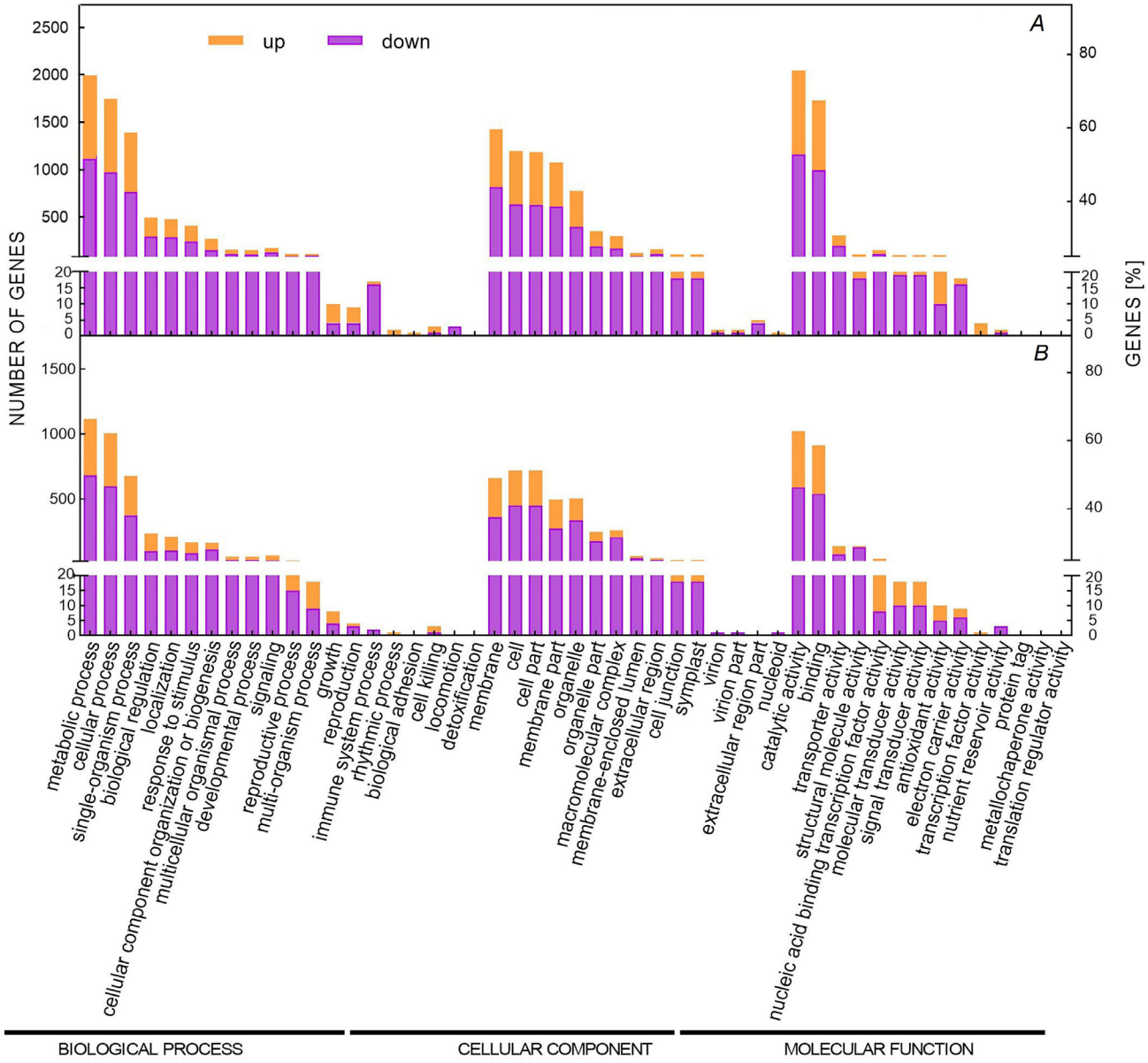

Fig. 2. *GO* classification of the DTGs identified in each comparison between pairs in libraries: *A* - control *vs*. chilling, *B* - control *vs*. freezing.

Table 2. Significant enrichment metabolic pathways and differentially expressed genes.

KO ID	*KEGG* pathway	DEGs with pathway annotation	Up DEGs	Down DEGs	Q value < 0.05
CK *vs*. Ch total DEGs with pathway annotation 1 390					
ko00941	flavonoid biosynthesis	29 (2.09%)	6	23	0.003
ko00592	α-linolenic acid metabolism	24 (1.73%)	9	15	0.041
ko00330	arginine and proline metabolism	20 (1.44%)	7	13	0.011
ko00565	ether lipid metabolism	18 (1.29%)	8	10	0.007
ko00071	fatty acid degradation	22 (1.58%)	13	9	0.020
ko00051	fructose and mannose metabolism	24 (1.73%)	10	14	0.028
CK *vs*. Fr total DEGs with pathway annotation 876					
ko00941	flavonoid biosynthesis	19 (2.17%)	7	12	0.013
ko00591	linoleic acid metabolism	9 (1.03%)	3	6	0.013
ko00100	steroid biosynthesis	11 (1.26%)	1	10	0.028
ko00902	monoterpenoid biosynthesis	6 (0.68%)	4	2	0.047

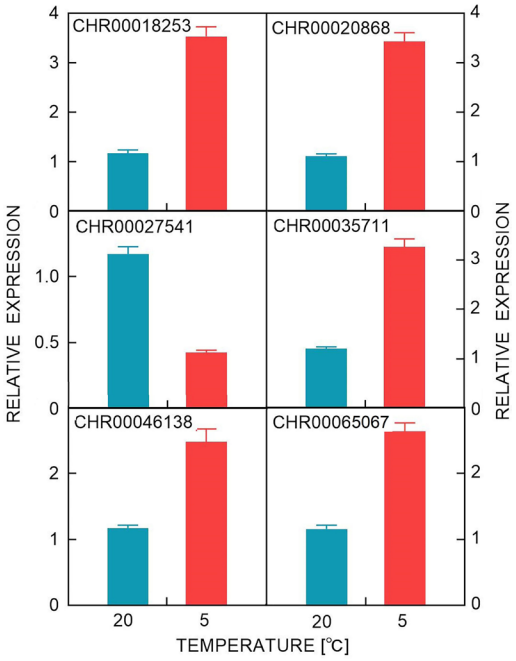

Fig. 3. The relative expression of MYB transcription factors in low temperature: *MYB41* (CHR00018253), *MYB59* (CHR00020868), *MYB4* (CHR00027541), *MYB119* (CHR00035711), *MYB2* (CHR00046138), and *MYBS3* (CHR00065067).

2013). In the present study, the activities of SOD, POD, and CAT increased with the decrease in temperature (Fig. 1). This phenomenon may be responsible for the high ability of the plant to eliminate ROS under cold stress and protect macromolecules from damage. However, the activities of the antioxidant enzymes decreased when the temperature was below zero. These findings indicated that enzyme activities were inhibited by freezing. MDA is one of the final products of lipid peroxidation and has been considered an indicator of cold sensitivity (Kaur *et al.* 2009). Campos *et al.* (2003) and Choudhary *et al.* (2007) reported that MDA content increases gradually under environmental stresses. The protective compound proline participates in ROS detoxification under stress conditions by maintaining redox balance in cells (Blokhina *et al.* 2003). In our experiment, an increase in both proline and MDA content as temperature decreased may be related to free radical generation. The activities of antioxidant enzymes and proline synthesis-related genes were analyzed, including peroxisome-related genes CHR00006576 and CHR00065771, hydrogen peroxide-related genes CHR00069581 and CHR00080449, MDA-related genes CHR00064596 and CHR00078080, and proline synthesis-related genes CHR00093594 and CHR00060679. The expression of these genes increased with the decrease in temperature above zero but decreased under freezing temperatures, especially CHR00006576, CHR00065771, CHR00069581, and CHR00080449 (Fig. 4).

Functional annotation and *KEGG* enrichment analysis of DEGs in low temperature: Cold stresses can have adverse effects on plants, including inhibition of seed germination, decrease in flower ornamental value, reduction of plant growth, and even plant death. To respond to low temperatures, plants alter their metabolism, including increasing osmotic pressure and membrane stability (Yue *et al.* 2020). In our study, the gene expression on the flavonoid biosynthesis pathway changed during chilling (CK *vs*. Ch) and freezing (CK *vs*. Fr, Table 2). We analyzed the relative expression of some genes involved in the major regulatory and biosynthetic steps in flavonoid biosynthesis. The relative expression of *F3H* (CHR00087441) and *FLS2* (CHR00028537) between +5 and -5°C treatments were higher than those at 20°C (Fig. 5). The increase in flavonoid content in response to cold stress might be accompanied by ROS removal and amelioration of decrease in osmotic potential in plants (Crifo *et al.* 2011, Sudheeran *et al.* 2018). Fatty acids are composed of carbon, hydrogen, and oxygen and are the main components of neutral lipids, phospholipids,

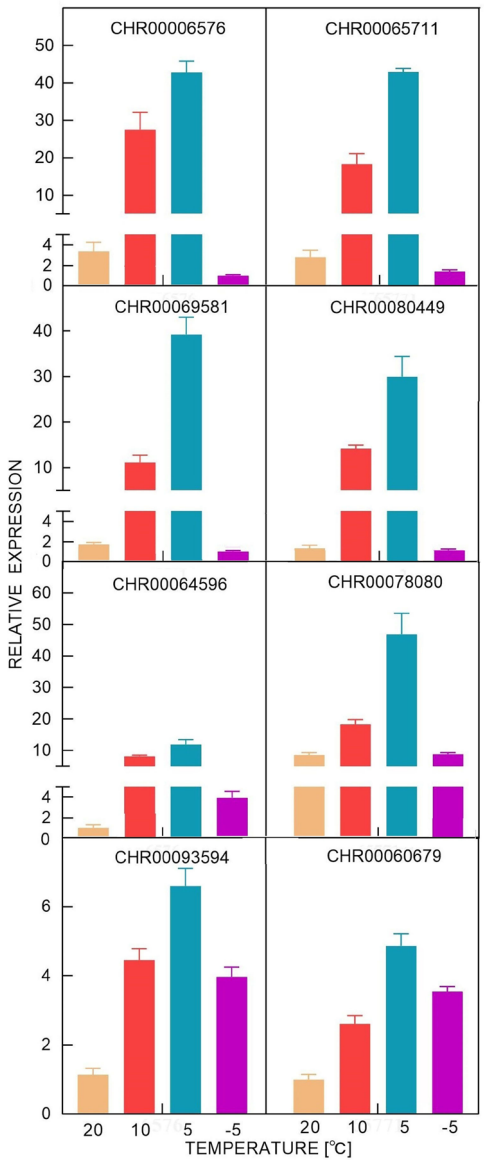

Fig. 4. Relative expression of peroxisome-related genes (CHR00006576, CHR00065771), hydrogen peroxide-related genes (CHR00069581, CHR00080449), MDA-related genes (CHR00064596, CHR00078080), and proline synthesis-related genes (CHR00093594, CHR00060679).

and glycerolipids. Fatty acids can be divided into two types, one is saturated fatty acids without carbon-carbon double bonds inside the molecule, such as stearic acid and palmitic acid. The other is unsaturated fatty acids with one or more carbon-carbon double bonds in the molecule, and the most common examples include oleic acid, linoleic acid, α-linolenic acid, and ether lipids (He *et al.* 2020). In our experiments, the expression of genes related to unsaturated fatty acid metabolism pathways had significant changes between chilling and freezing (Table 2). Under low-temperature stress, plants can improve their cold tolerance by increasing the content of unsaturated fatty acids in membrane lipids and improving membrane fluidity (Chen *et al.* 2019). Flavin adenine dinucleotide (FAD) is a key enzyme in unsaturated fatty acid biosynthesis, and *FAD* expression may improve cold tolerance (Shi *et al.* 2012). The expressions of *FAD1* (CHR00091803) and *FAD7* (CHR00079733) at 5°C were higher than those of the control (Fig. 5), which further indicated that fatty acids were involved in cold tolerance. Similar results were found in the report of Wang *et al.* (2018). The expression of genes related to the arginine, proline, fructose, and mannose metabolism pathways was significantly altered only during chilling. Arginine to agmatine by arginine decarboxylase (ADC), from agmatine to putrescine *via* agmatine iminohydrolase (AIH), *N*-carbamoylputrescine amidohydrolase (NLP), and putrescine may be further metabolized to polyamines. Another is arginine to NO *via* nitric oxide synthase (NOS) (Shi *et al.* 2015). Polyamines could modulate ROS accumulation and antioxidant enzyme activities under chilling stress. The expressions of *ADC* (CHR00093170) and *SPDS1* (CHR00025981) were also obviously increased (Fig. 5). Under low temperatures, the increase in sugar content is accompanied by the improvement of cold tolerance; sugars provide substrates for cell respiration, release osmolytes for cell homeostasis, and contribute to membrane stabilization (Reyes-Díaz *et al.* 2006, Sinha *et al.* 2015). A study reported that the soluble sugar content was correlated with acclimation and freezing tolerance in *Chrysanthemum* leaves (Wang *et al.* 2018). This finding is not consistent with our results, which may be due to the different cultivars. Sugars are mainly synthesized in plant leaves, but sugar storage in the roots is small. The L-galactose pathway is the main biosynthesis pathway of ascorbic acid biosynthesis in higher plants. GDP-mannose pyrophosphorylase (GMP) is a key enzyme in the L-galactose synthesis pathway that catalyzes the formation of GDP-D-mannose-1-phosphate. GDP-D-mannose is not only a precursor of vitamin synthesis but is also involved in cell wall polysaccharide synthesis (Tao *et al.* 2018, Li *et al.* 2019). GMP can improve response to abiotic stresses, such as cold, salt, and drought in rice and tomato (Zhang *et al.* 2011, 2015). The relative expressions of *GGPS1* (CHR00015708) and *FBA* (CHR00071374) related to fructose and mannose metabolism in 5°C-treated roots were higher than those in the control (Fig. 5). However, the expression of genes related to steroid and monoterpenoid biosynthesis was obviously changed under freezing. Steroids, also known as terpenoids, are classified as esters with a special polycyclic terpenoid complex. The high expressions of 20 genes involved in the terpenoid pathway contributed to cold stress (Zhao *et al.* 2019). Zhang *et al.* (2017) found by transcriptomic analyses that terpenoid synthase genes respond to cold stress in *Santalum album* L. Low temperature can change the expression of genes related to terpenoid biosynthesis, but increasing cold tolerance needs further studies. The results of our experiment were obviously different from the *KEGG* metabolic pathway of chrysanthemum transcriptome under low-temperature stress (special incubator) (Lu *et al.* 2018). Our differential metabolic pathways are fewer than those treated under

an indoor thermostat, but both flavonoid biosynthesis and fatty acid metabolic pathways are present. Steroid biosynthesis and monoterpenoid biosynthesis pathways were present in our results but not in the results under constant temperature treatment. This finding may be due to the natural variability in temperatures.

Role of MYB transcription factors under cold stress: The MYB proteins in plants are a large family, functionally diverse. They are characterized by a highly conserved DNA-binding MYB domain. This domain generally consists of one to four imperfect repeat sequences, each of which consists of approximately 52 amino acids. MYB proteins can be classified into three families, namely, R1/2-MYB, R3-MYB, or R2R3-MYB, and R2R3-MYB mainly exists in plants (Dubos *et al.* 2010). The R2R3-MYB family plays a significant role in the secondary metabolism of phenylpropane and in response to abiotic and biotic stresses (Lee and Seo 2015, Lai *et al.* 2019). In our study, the expressions of genes CHR00035711 and CHR00065067 were increased during chilling (Fig. 3). *NCBI BLAST* analysis showed that CHR00035711 and CHR00065067 were closely related to MYB119 and MYB14, respectively (Table 4 Suppl.). The heterologous overexpression of *MpMYBS3* in banana showed that the transgenic lines improved cold tolerance, which may be related to proline accumulation and malondialdehyde reduction (Dou *et al.* 2016). The *PtrMYB119* transcription factor from *Populus trichocarpa* accelerates flavonoid accumulation when expressed and controls the CaMV35S promoter in *Arabidopsis* (Cho *et al.* 2016). Accumulation of flavonoids increases in response to abiotic stress by enhancing the activity of enzymes that scavenge ROS (Schulz *et al.* 2015). This result was consistent with the change in the flavonoid metabolism pathway in *KEGG* analysis. CHR00018253 and CHR00020868 have high homology with MYB102 and MYB59, respectively. Tomato *SlMYB102* is considerably induced by low temperatures. *SlMYB102* overexpression in tomato plants improved cold tolerance, and the *SlMYB102* overexpressing lines show high proline accumulation and low MDA content under chilling stress (Wang *et al.* 2020b). Wang *et al.* (2016) reported that the expression of *CmMYB59* in the roots was higher than in other *Chrysanthemum* organs. After 1 h at a low temperature, the transcription of *CmMYB59* was enhanced, indicating that *CmMYB59* was responsive to low-temperature stress. CHR00046138 has a close homology with MYB2, belonging to the R2R3 type of the MYB family. The expression of *OsMYB2* was upregulated by cold, salt, and dehydration stress in *Oryza sativa*. Overexpression of *OsMYB2* in rice showed that transgenic plants had improved cold stress and accumulated high amounts of soluble sugars and proline under cold stress (Yang *et al.* 2012). CHR00027541 has a high homology with MYB308. The RT-qPCR expression analysis revealed that *JcMYB308* is expressed in different organs, abundantly

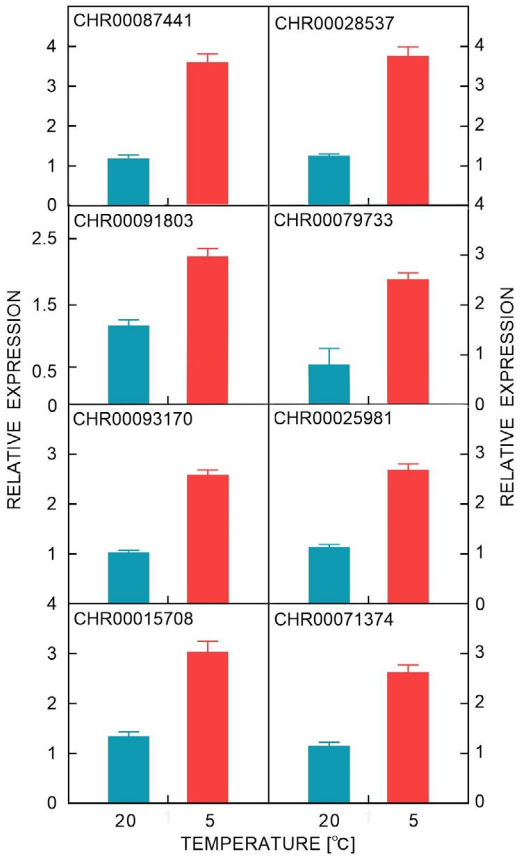

Fig. 5. Relative expression of genes associated with cold tolerance in different temperatures: *F3H* (CHR00087441), *FLS2* (CHR00028537), *FAD1* (CHR00091803), *FAD7* (CHR00079733), *ADC* (CHR00093170), *SPDS1* (CHR00025981), *GGPS1* (CHR00015708), *FBA* (CHR00071374).

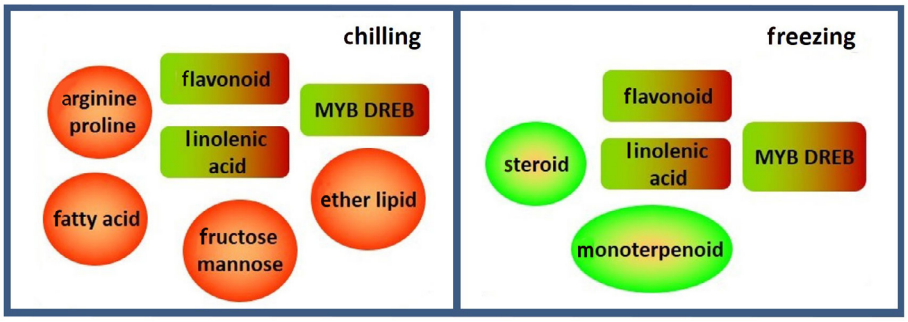

Fig. 6. Model of plants strategy in response to natural chilling and freezing stresses.

in the root, but scarcely in leaves, and it is responsive to low-temperature stress (Hu *et al.* 2008). In addition, we focused on the cold-resistant AP2/DREB transcription factor. Fifty-eight members of the AP2/ERF family were selected in CK *vs*. Ch, and 11 of them belong to the DREB subfamily. In CK *vs.* Fr, the AP2/ERF family has 28 members, 13 of which belong to the DREB subfamily. RT-qPCR validation showed that the relative expression of genes CHR00044059, CHR00069918, CHR00090843, CHR00090844, and CHR00092416 (DREB subfamily members) was increased at 5°C compared with that of the control group. The relative expression of genes CHR00020948, CHR00020949, CHR00020956, CHR00020961, CHR00020962, CHR00020963, CHR00020971, CHR00020972, CHR00052675, CHR00069918, CHR00090843, and CHR00090844 were increased from 20°C to -5°C (Fig. 4 Suppl.).

Chrysanthemum has some strategies to cope with natural chilling stress, including flavonoid biosynthesis, changes in linolenic acid metabolism, arginine and proline metabolism, ether lipid metabolism, fatty acid degradation, and fructose and mannose metabolism pathway, MYB, and DREB transcription factor. Flavonoid biosynthesis, linolenic acid metabolism, steroid biosynthesis, monoterpenoid biosynthesis metabolism pathway, and MYB and DREB transcription factors play an important role in natural freezing stress (Fig. 6).

References

Blokhina O., Virolainen E., Fagerstedt K.V.: Antioxidants, oxidative damage and oxygen deprivation stress: a review. - Ann. Bot.-London **91**: 179-194, 2003.

Campos P.S., Quartin V., Ramalho J.C., Nunes M.A.: Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. - J. Plant Physiol. **160**: 283-292, 2003.

Chen L., Yu H., Yang S. *et al.*: Study on the mechanism of cold tolerance of the strain *Shewanella putrefaciens* WS13 through fatty acid metabolism nanoscience and nanotechnology. - Nanosci. Nanotech. Lett. **11**: 1718-1723, 2019.

Chen X.J., Sun M., Liang J.G. *et al.*: Genetic diversity of species of *Chrysanthemum* and related genera and groundcover cultivars assessed by amplified fragment length polymorphic markers. - HortScience **48**: 539-546, 2013.

Cho J.-S., Nguyen V.P., Jeon H.-W. *et al.*: Overexpression of *PtrMYB119*, a R2R3-MYB transcription factor from *Populus trichocarpa*, promotes anthocyanin production in hybrid poplar. - Tree Physiol. **36**: 1162-1176, 2016.

Choudhary M., Jetley U.K., Khan A.M. *et al.*: Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. - Ecotox. Environ. Safe. **66**: 204-209, 2007.

Crifo T., Puglisi I., Petrone G. *et al.*: Expression analysis in response to low temperature stress in blood oranges: implication of the flavonoid biosynthetic pathway. - Gene **476**: 1-9, 2011.

Des Marais D.L., Juenger T.E.: Pleiotropy, plasticity, and the evolution of plant abiotic stress tolerance. - Ann. N. Y. Acad. Sci. **1206**: 56-79, 2010.

Dou T.-X., Hu C.-H., Sun X.-X. *et al.*: *MpMYBS3* as a crucial transcription factor of cold signaling confers the cold tolerance of banana. - Plant Cell Tiss. Org. Cult. **125**: 93-106, 2016.

Dubos C., Stracke R., Grotewold E. *et al.*: MYB transcription factors in *Arabidopsis*. - Trends Plant Sci. **15**: 573-581, 2010.

Gajewska E., Skłodowska M.: Effect of nickel on ROS content and antioxidative enzyme activities in wheat leaves. - Biometals **20**: 27-36, 2007.

Guevara D.R., Champigny M.J., Tattersall A. *et al.*: Transcriptomic and metabolomic analysis of Yukon *Thellungiella* plants grown in cabinets and their natural habitat show phenotypic plasticity. - BMC Plant Biol. **12**: 175, 2012.

He M., Qin C.-X., Wang X., Ding N.-Z.: Plant unsaturated fatty acids: biosynthesis and regulation. - Front. Plant Sci. **11**: 390, 2020.

Hu X., Yan Y.H., Ding X.M. *et al.*: [Genome-wide dentification of MYB gene family and cloning & expression analysis of *JcMYB308* gene from *Jatropha curcas*.] - Biotechnol. Bull. **34**: 110-118, 2008. [In Chinese]

Kaur S., Gupta A.K., Kaur N. *et al.*: Antioxidative enzymes and sucrose synthase contribute to cold stress tolerance in chickpea. - J. Agron. Crop Sci. **195**: 393-397, 2009.

Kejík Z., Kaplánek R., Masařík M. *et al.*: Iron complexes of flavonoids – antioxidant capacity and beyond. - Int. J. Mol. Sci. **22**: 646, 2021.

Kyu S.Y., Naing A.H., Pe P.W. *et al.*: Tomato seeds pretreated with Antifreeze protein type I (AFP I) promotes the germination under cold stress by regulating the genes involved in germination process. - Plant Signal. Behav. **14**: 1682796, 2019.

Lai B., Du L.N., Hu B. *et al.*: Characterization of a novel litchi R2R3-MYB transcription factor that involves in anthocyanin biosynthesis and tissue acidification. - BMC Plant Biol. **19**: 62, 2019.

Lee H.G., Seo P.J.: The MYB96-HHP module integrates cold and abscisic acid signaling to activate the CBF-COR pathway in *Arabidopsis*. - Plant J. **82**: 962-977, 2015.

Li Q.Y., Lei S., Du K.B. *et al.*: RNA-seq based transcriptomic analysis uncovers α-linolenic acid and jasmonic acid biosynthesis pathways respond to cold acclimation in *Camellia japonica*. - Sci. Rep.-UK **6**: 36463, 2016.

Li X.J., Ye J., Munir S. *et al.*: Biosynthetic gene pyramiding leads to ascorbate accumulation with enhanced oxidative stress tolerance in tomato. - Int. J. Mol. Sci. **20**: 1558, 2019.

Liu N., Lin Z.F., Guan L.L. *et al.*: Antioxidant enzymes regulate reactive oxygen species during pod elongation in *Pisum sativum* and *Brassica chinensis*. - PLoS ONE **9**: e87588, 2014.

Liu W.Y., Yu K.M., He T.F. *et al.*: The low temperature induced physiological responses of *Avena nuda* L., a cold-tolerant plant species. - Sci. World J. **2013**: 658793, 2013.

Lu J., Bi H., Zhang A. *et al.*: Comparative transcriptome analysis by RNA-Seq of the regulation of low temperature responses in *Dendranthema morifolium*. - Hortic. Environ. Biotech. **59**: 383-395, 2018.

Luo H., Liang X.F., Li J. *et al.*: Effect of long-chain saturated and unsaturated fatty acids on hypothalamic fatty acid sensing in Chinese perch (*Siniperca chuatsi*). - Comp. Biochem. Physiol. B **241**: 110395, 2020.

Maruyama K., Sakuma Y., Kasuga M. *et al.*: Identification of cold-inducible downstream genes of the *Arabidopsis DREB1A/CBF3* transcriptional factor using two microarray systems. - Plant J. **38**: 982-993, 2004.

Medina J., Catalá R., Salinas J.: The CBFs: Three *Arabidopsis* transcription factors to cold acclimate. - Plant Sci. **180**: 3-11, 2011.

Reyes-Díaz M., Ulloa N., Zúñiga-Feest A. *et al.*: *Arabidopsis thaliana* avoids freezing by supercooling. - J. Exp. Bot. **57**: 3687-3696, 2006.

Schulz E., Tohge T., Zuther E. *et al.*: Natural variation in flavonol and anthocyanin metabolism during cold acclimation in *Arabidopsis thaliana* accessions. - Plant Cell Environ. **38**: 1658-1672, 2015.
Shi H.-T., Chan Z.-L.: *In vivo* role of *Arabidopsis* arginase in arginine metabolism and abiotic stress response. - Plant Signal. Behav. **8**: e24138, 2015.
Shi J., Cao Y., Fan X. *et al.*: A rice microsomal delta-12 fatty acid desaturase can enhance resistance to cold stress in yeast and *Oryza sativa*. - Mol. Breeding **29**: 743-757, 2012.
Shi Y., Huang J., Sun T. *et al.*: The precise regulation of different *COR* genes by individual CBF transcription factors in *Arabidopsis thaliana*. - J. Integr. Plant Biol. **59**: 118-133, 2017.
Sinha S., Kukreja K., Arora P. *et al.*: The omics of cold stress responses in plants. - In: Pandey G. (ed.): Elucidation of Abiotic Stress Signaling in Plants. Pp. 143-194. Springer, New York 2015.
Sudheeran P.K., Feygenberg O., Maurer D., Alkan N.: Improved cold tolerance of mango fruit with enhanced anthocyanin and flavonoid contents. - Molecules **23**: 1832, 2018.
Tao J., Wu H., Li Z. *et al.*: Molecular evolution of GDP-D-mannose epimerase (*GME*), a key gene in plant ascorbic acid biosynthesis. - Front. Plant Sci. **9**: 1293, 2018.
Wang C., Zhang Z., Zhang X. *et al.*: [Molecular cloning and expression of *CmMYB59* from *Chrysanthemum morifolium*.] - J. Nanjing Agric. Univ. **39**: 63-69, 2016. [In Chinese]
Wang K., Bai Z.Y., Liang Q.Y. *et al.*: Transcriptome analysis of chrysanthemum (*Dendranthema grandiflorum*) in response to low temperature stress. - BMC Genomics **19**: 319, 2018.
Wang L., Yao L.N., Hao X.Y. *et al.*: Transcriptional and physiological analyses reveal the association of ROS metabolism with cold tolerance in tea plant. - Environ. Exp. Bot. **160**: 45-58, 2019.
Wang M., Hao J., Chen X., Zhang X.: *SlMYB102* expression enhances low-temperature stress resistance in tomato. - Peer J. **8**: e10059, 2020b.
Wang Q., Xue D., Zhou Y. *et al.*: The β-ketoacyl-CoA synthase KCS13 regulates the cold response in cotton by modulating lipid and oxylipin biosynthesis. - J. Exp. Bot. **71**: 5615-5630, 2020a.
Wang Y., Ying Y., Chen J., Wang X.: Transgenic *Arabidopsis* overexpressing Mn-SOD enhanced salt-tolerance. - Plant Sci. **167**: 671-677, 2004.
Yang A., Dai X., Zhang W.-H.: A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. - J. Exp. Bot. **63**: 2541-2556, 2012.
Yang X., Zhao T., Rao P. *et al.*: Transcriptome profiling of *Populus tomentosa* under cold stress. - Ind. Crop. Prod. **135**: 283-293, 2019.
Yue Y.Y., Ren M.R., Quan Y.J. *et al.*: Autopolyploidy in *Chrysanthemum* cv. 'Gongju' improved cold tolerance. - Plant Mol. Biol. Rep. **38**: 655-665, 2020.
Zhang C., Liu J., Zhang Y. *et al.*: Overexpression of *SlGMEs* leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. - Plant Cell Rep. **30**: 389-398, 2011.
Zhang G.-Y., Liu R.-R., Zhang C.-Q. *et al.*: Manipulation of the rice L-galactose pathway: evaluation of the effects of transgene overexpression on ascorbate accumulation and abiotic stress tolerance. - PLoS ONE **10**: e0125870, 2015.
Zhang X., Teixeira da Silva J.A., Niu M. *et al.*: Physiological and transcriptomic analyses reveal a response mechanism to cold stress in *Santalum album* L. leaves. - Sci. Rep.-UK **7**: 42165, 2017.
Zhao H., Feng S., Zhou W., Kai G.: Transcriptomic analysis of postharvest toon buds and key enzymes involved in terpenoid biosynthesis during cold storage. - Sci. Hortic.-Amsterdam **257**: 108747, 2019.
Zulfiqar F., Akram N.A., Ashraf M.: Osmoprotection in plants under abiotic stresses: new insights into a classical phenomenon. - Planta **251**: 3, 2020.