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Transcriptome analysis deciphers the mechanisms of exogenous nitric oxide action on the response of melon leaves to chilling stress

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Abstract

Chilling stress is a major abiotic factor that limits the growth and productivity of melon (*Cucumis melo* L.). The application of nitric oxide (NO) can enhance plant tolerance to chilling stress; however, the underlying molecular mechanisms for this process remain poorly understood. In this study, RNA sequencing was performed on melon seedlings exposed to control conditions, chilling stress, or chilling stress in the presence of NO donor sodium nitroprusside (SNP), to identify NO-mediated transcript changes in response to chilling stress. The results identified 488, 1 012, and 1 589 differentially expressed genes (DEGs) between plants in optimum conditions (CK) and chilling stress (CS) groups, plants in the CS and chilling stress + SNP (CN) groups, and those in CK and CN groups, respectively. Through *gene ontology* (*GO*) database and *Kyoto encyclopedia of genes and genomes* (*KEGG*) enrichment analyses, the DEGs were classified as predominantly involved in saccharide metabolism, biosynthesis of other secondary metabolites, lipid metabolism, amino-acid metabolism, and signal transduction pathways. In addition, 39 genes related to sugar metabolism including those encoding UDP-glucuronate-4-epimerase, β-glucosidase, glucuronosyltransferase, α-1,4-galacturonosyl transferase, and hexokinase, were upregulated in the CK *vs*. CS comparison, and genes encoding fructose-bisphosphate aldolase and glucan-endo-1,3-β-glucosidase were upregulated in the CS *vs*. CN, and CK *vs*. CN comparisons. A gene encoding an EREBP-like factor was upregulated in the CK *vs*. CS, CS *vs*. CN, and CK *vs*. CN comparisons. The expression profiles of 10 selected genes were analyzed using real-time quantitative PCR, and the candidate gene expression patterns were consistent with the DEG classification from RNA-seq. Overall, the data provide insight into the transcriptional regulation by exogenous NO in the response of melon seedlings to chilling stress. The data from this study are relevant for further research on the molecular mechanisms that underlie chilling resistance in melon plants.

Additional key words: *Cucumis melo*, differentially expressed genes, RNA sequencing, sodium nitropruside, sugar metabolism.

Introduction

Melon is one of the most economically important and commonly consumed fruit crops in many countries, but it is sensitive to and often suffers damage from chilling stress, especially in the winter or early spring. Chilling stress is a common environmental stress that can affect crop growth and agricultural productivity, since it can induce physiological, biochemical and molecular responses (Sitnicka and Orzechowski 2014). Thus, it is extremely important to enhance the chilling tolerance of melon seedlings.

Chilling tolerance can be increased in plants by cold acclimation, which results in multiple reprogramming mechanisms, including changes in gene expression and biochemical and physiological modifications (Zhu *et al.* 2007). Evidence suggests that nitric oxide (NO) production plays a critical role in chilling tolerance (Cantrel *et al.* 2011, Zhao *et al.* 2011). Nitric oxide is a ubiquitous reactive signaling molecule that is involved in diverse developmental and physiological processes, and in various plant responses to abiotic stresses, such as drought, salt, temperature, and heavy metals (Siddiqui *et al.* 2011, Shi *et al.* 2012, 2014). NO participates in plant responses to abiotic stresses most probably by inducing antioxidant defense and by reducing the generation of reactive oxygen species (ROS) (Filippou *et al.* 2013, Sun *et al.* 2015). Recent studies have reported that NO treatment can

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Abbreviations: CK - controls; CN - chilling stress + nitric oxide; CS - chilling-stress; DEG - differentially expressed gene; GO - gene ontology; KEGG - Kyoto encyclopedia of genes and genomes; SNP - sodium nitroprusside.
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enhance plant stress resistance by influencing polyamine biosynthesis, chlorophyll content, and photosystem II activity (Chen *et al.* 2013, Fan *et al.* 2013). Furthermore, NO regulates genes that are involved in plant defense, oxidative stress responses, and plant hormone signaling (Lindermayr *et al.* 2005, Zago *et al.* 2006, Zeng *et al.* 2014, Hu *et al.* 2015, Li *et al.* 2019). Jian *et al.* (2016) and Rizwan *et al.* (2018) reported that the application of NO donor sodium nitroprusside (SNP) enhanced the expression of *CAT, APX,* and *SOD* in *Medicago truncatula* under salt stress and in rice under Ni-stress. Although numerous studies have identified NO-responsive genes, related information concerning the molecular mechanisms of NO-induced chilling tolerance in melon plants is lacking and it is crucial to improving melon breeding.

Currently, due to the availability of the complete melon genome sequence and high-throughput RNA sequencing (RNA-seq) technologies, a lot of genomic data concerning melon fruit development and quality have been generated (Garcia-Mas *et al.* 2012, Zhang *et al.* 2016, Shin *et al.* 2017). Yano *et al.* (2017) examined the transcriptome in 30 different tissues at 7 stages of melon fruit development and identified 20 752 genes expressed in at least one tissue. In addition, RNA-seq data have revealed responsive genes or key pathways resulting from abiotic stress in melon. For example, Wang *et al.* (2016) identified the gene expression profiles in two muskmelon cultivars in response to salt stress. Genes responding to chilling stress and their corresponding pathways have been reported for other plant species, including cassava (An *et al.* 2012), rice (Zhang *et al.* 2012), watermelon (Xu *et al.* 2016), and *Populus simonii* (Song *et al.* 2013). However, to our knowledge, few transcriptome analyses have been reported on the response of melon to chilling stress. Because of the importance of melon as a crop, it is necessary to study NO-regulated and chilling stress-responsive gene networks in this species; therefore, this study investigated the transcriptional responses of melon to NO and chilling stress by RNA-seq analysis. The results represent a basis for improving its tolerance to chilling stress.

Materials and methods

Seeds of melon (*Cucumis melo* L.) cv. XL-1 were produced by our laboratory at Shanghai Academy of Agricultural Science, Shanghai, China, rinsed thoroughly with distilled water, and germinated in an incubator at a temperature of 30 °C. The germinated seedlings were then transferred to plastic plates containing *Vermiculite* and grown in a growth chamber at day/night temperatures of 30/20 °C, a 12-h photoperiod, an irradiance of 400 μmol m^{-2} s^{-1}, and a relative humidity of 80 %. Plants were watered every 2 d with a 1/8-strength Hoagland solution (Hoagland and Snyder 1933). When melon seedlings reached the second true-leaf stage, the leaves were sprayed daily with 200 cm^3 distilled water or 200 μM sodium nitroprusside (SNP) for 3 d. On the fourth day, the plants were exposed to chilling stress (6 °C for 3 h), the leaves of each group were harvested, immediately frozen in liquid nitrogen, and stored at -70 °C for further analyses. Seedlings without treatments were used as the control. All experiments consisted of three replicates.

Total RNA was extracted using a *mirVana* miRNA isolation kit (*Ambion*, Austin, TX, USA) following the manufacturer's protocol. The RNA integrity was evaluated using an *Agilent 2100* bioanalyzer (*Agilent Technologies*, Santa Clara, CA, USA). The samples with an RNA integrity number (RIN) ≥ 7 were used for subsequent analyses. The cDNA libraries were constructed using the *TruSeq Stranded mRNA LT Sample Prep* kit (*Illumina*, San Diego, CA, USA) according to the manufacturer's instructions. The libraries were sequenced on the *Illumina* sequencing platform (*HiSeqTM 2500* or *Illumina HiSeq X Ten*), and 125 bp/150 bp paired-end reads were generated. Three biological replicates were performed for RNA-seq.

Raw reads were processed by removing reads containing adapters, reads containing poly-N and low-quality reads, to obtain clean data (clean reads). The remaining high-quality clean reads were aligned to the melon reference genome sequences using *HISAT* software (Cock *et al.* 2009). The mapped reads were then reconstructed using *Cufflink* (Conesa *et al.* 2005). All RNA-seq reads were deposited at *NCBI* sequence read archive (*SRA*) database under the accession number PRJNA553119.

Gene expressions were calculated for each sample using *RSEM* software (McKenna *et al.* 2010). The differentially expressed genes (DEGs) among the three samples were identified using *DESeq*. The threshold of false discovery rate ≤ 0.05 and an absolute fold change value ≥ 2 were used as the threshold for DEG selection.

Gene ontology (*GO*) enrichment analysis was performed using *agriGO* (Du *et al.* 2010); the *Kyoto encyclopedia of genes and genomes* (*KEGG*) pathway enrichment analysis was implemented by the *KEGG* orthology-based annotation system (*KOBAS*) (Xie *et al.* 2011). Significantly enriched pathways with respect to DEGs were identified according to the criterion of a corrected *P*-value ≤ 0.05.

Total RNA was extracted from the same samples with three biological replicates as those subjected to *Illumina* RNA-seq using the *mirVanaTM* RNA isolation kit, according to the manufacturer's specifications. Total RNA (2 μg) from each sample was used to synthesize cDNA by *M-MLV* (*TaKaRa*, Dalian, China). Real-time PCR was performed using *LightCycler® 480* real-time PCR instrument (*Roche*, Basel, Switzerland). The expressions of mRNAs were normalized to that of the reference gene *Actin* and were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Reactions were performed in a *GeneAmp® 9700* PCR system (*Applied Biosystems*, Foster City, CA, USA). Primers used for all real-time quantitative PCR experiments are listed in Table 1 Suppl.

Results

In this study, high-throughput transcriptomic sequencing technology was used to investigate the molecular mechanisms that regulate the effect of NO on the response of *Cucumis melo* to chilling stress. Samples exposed to

chilling alone (CS), chilling in the presence of SNP (CN), or controls (CK) were sequenced. In total, approximately 412 million raw reads were generated using the *Illumina HiSeq TM 2500* platform. After removing adapters, reads containing poly-N, and low-quality reads, approximately 379 million clean reads were obtained, representing 91.99 % of the total raw reads (Table 2 Suppl.). The mean proportion of bases with mass no less than 20 or 30 after

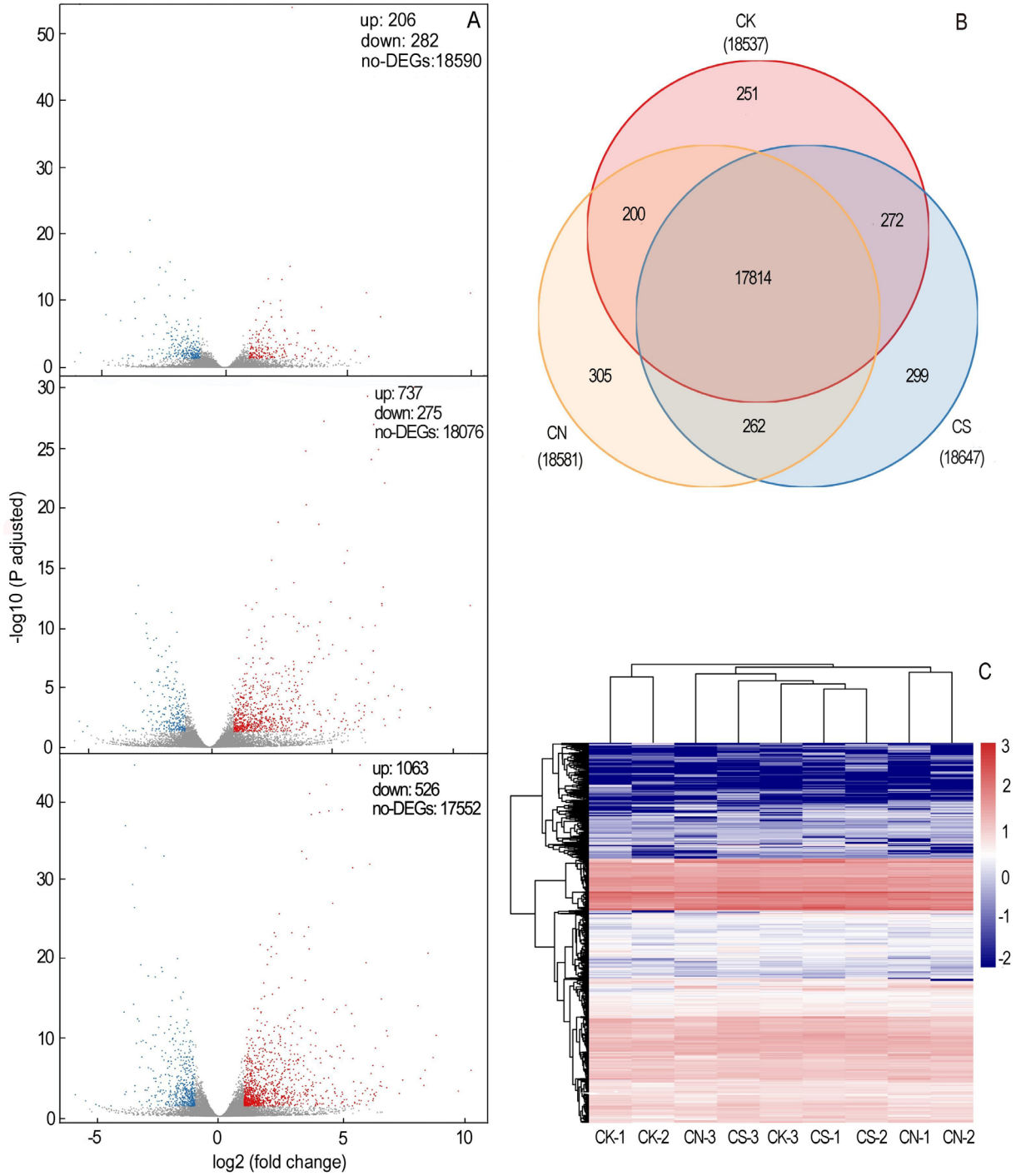

Fig. 1. Differentially expressed genes (DEGs) between samples. *A* - The Volcano plot of DEGs. The *x*-axis represents the fold-change of DEGs in different experimental groups, and the *y*-axis represents the *P* value ($\log_{10}$). *Dots* represent genes; the *red dots* represent upregulated DEGs and the *blue dots* represent downregulated DEGs. *B* - The Venn diagram of DEGs. Total DEGs for each comparison are shown in *parentheses*. *C* - The heat map of DEGs. Each *row* represents the $\log_2$ value of the expression of a gene in different treatments. Different gene expressions are displayed as a *gradient ramp*. A greater intensity of *red* indicates a higher gene expression; increasing *blue* intensity represents a decreasing gene expression. CK - control samples; CS - samples exposed to chilling stress; CN - samples exposed to chilling stress and 200 mM sodium nitroprusside. Each treatment consisted of three biological duplicates.

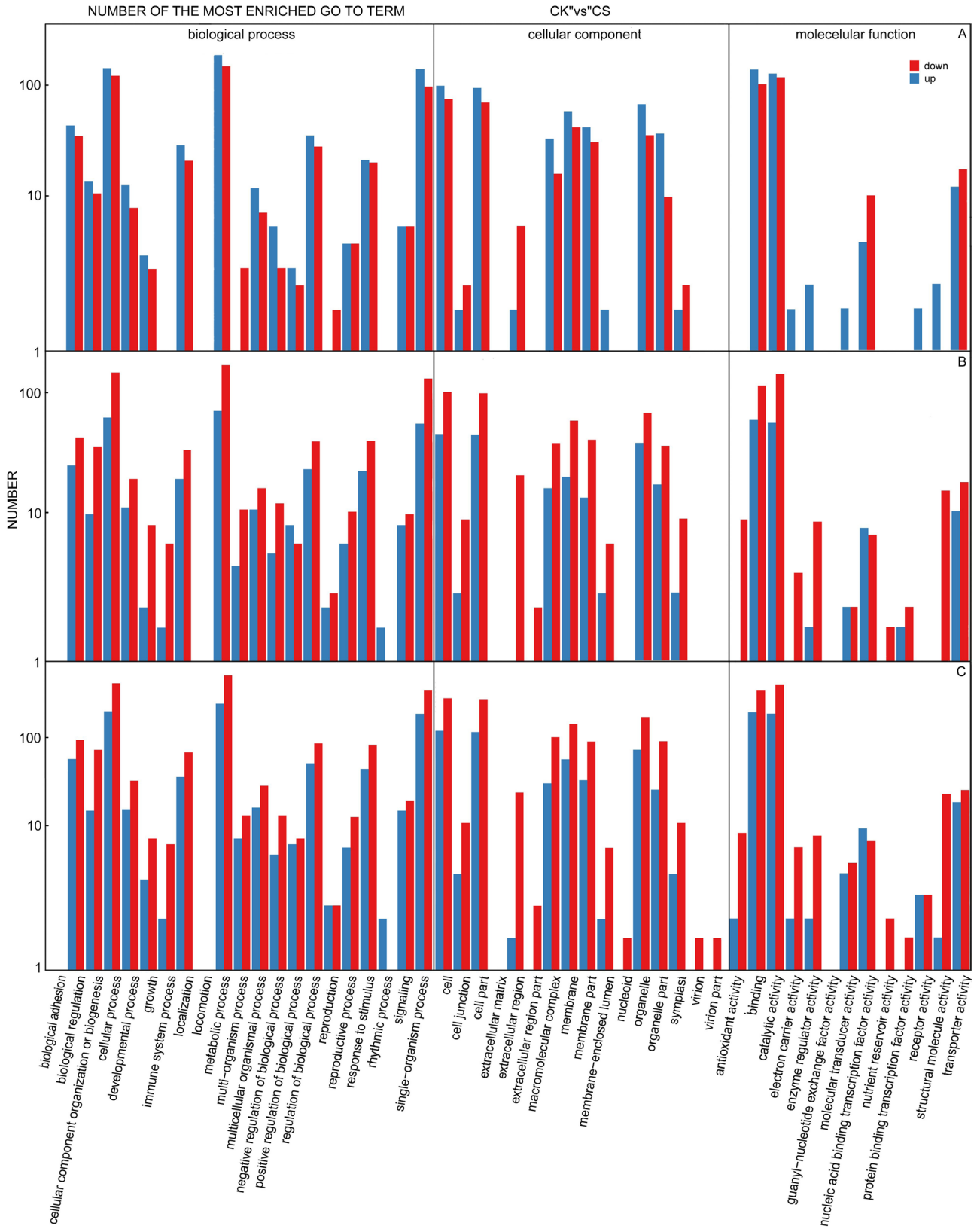

Fig. 2. *Gene ontology* (*GO*) enrichment analysis of differentially expressed genes in different treatments. *A* - Comparative *GO* terms between control (CK) and chilling stress (CS) treatment; *B* - comparative *GO* terms between CS and chilling stress + 200 mM SNP (CN) treatment; *C* - comparative *GO* terms between CK and CN. The *x*-axis displays different *GO* terms and the *y*-axis indicates the number of DEGs.

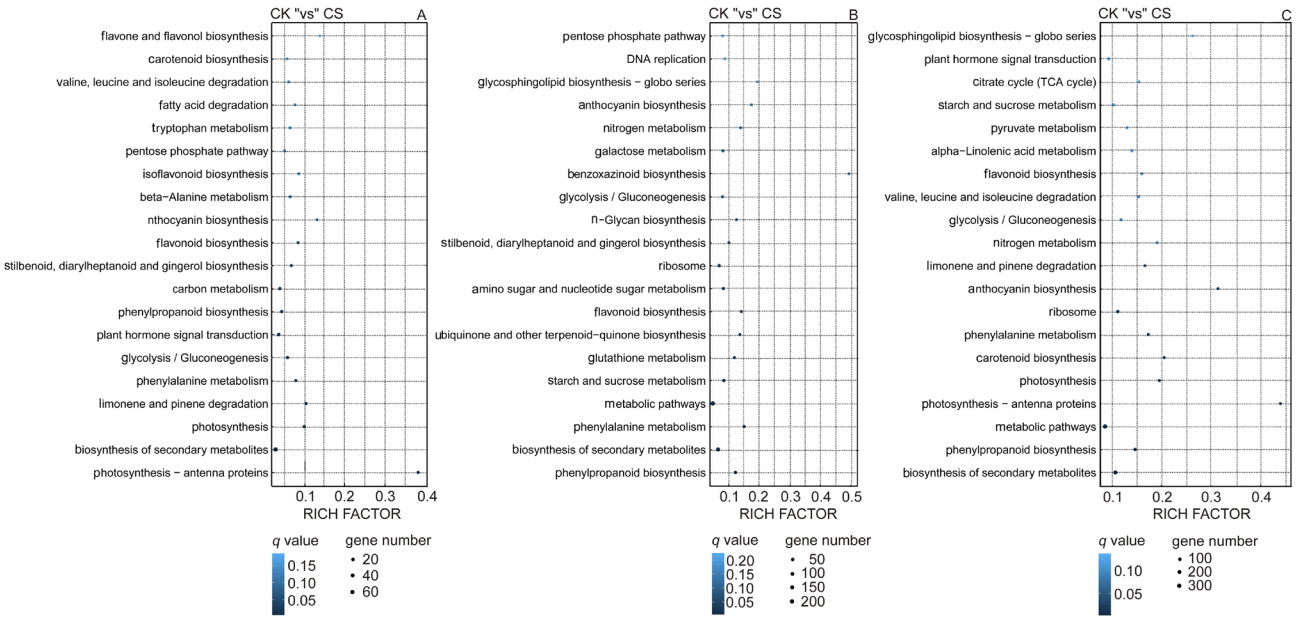

Fig. 3. *Kyoto encyclopedia of genes and genomes* enrichment analysis for different treatments. *A* - differentially expressed genes (DEGs) in control (CK) *vs*. chilling stress (CS) treatments; *B* - DEGs in CS *vs*. chilling stress + 200 mM SNP (CN) treatments; *C* - CK *vs*. N teratments. The *x*-axis represents the rich factor and the *y*-axis represents pathways. Different *colors* of the *dots* correspond to the *q* value range. The *size* of the *dot* represents the number of DEGs clustered in each pathway: larger *dots* indicate more DEGs. The rich factor indicates the degree of DEG enrichment in each pathway, the greater the rich factor, the greater the DEG enrichment.

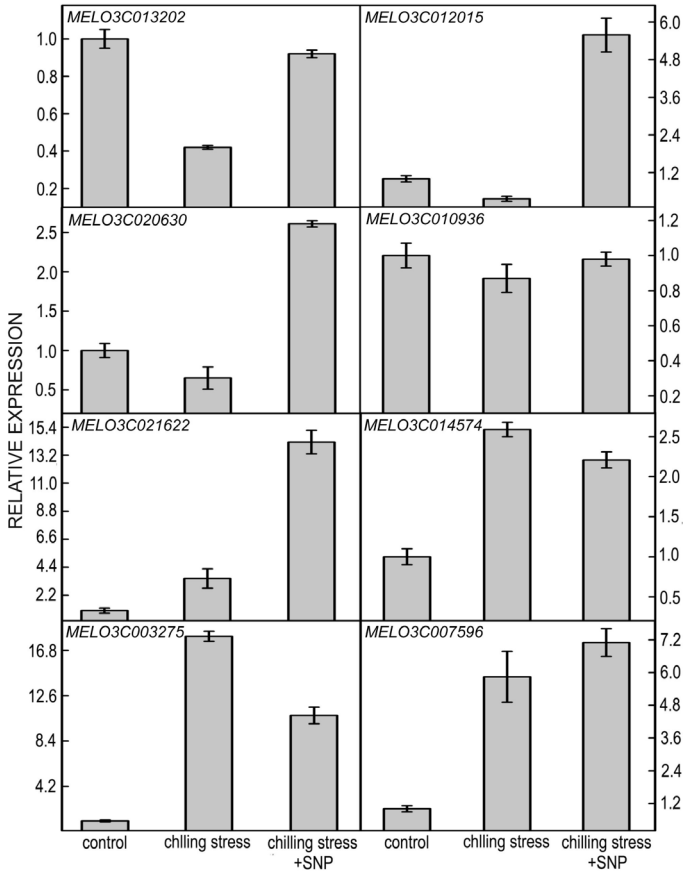

Fig. 4. Qantitative real time PCR reveals the transcripts of eight candidate genes under control, chilling stress, and chilling stress + sodium nitroprusside (SNP).

filtration (Q20, Q30, respectively), and GC content were 96.66, 92.06, and 44.25 %, respectively (Table 3 Suppl.). The high-quality reads were then mapped to the *C. melo* L. genome and the mapped clean reads of the three data sets ranged from 78.89 to 89.52 %. Among these reads, 78.05 to 88.42 % were uniquely mapped and were used for further analysis. In the control group *vs*. chilling stress treatment comparison, 488 DEGs were detected, with 206 DEGs being upregulated and 282 DEGs downregulated. The number of DEGs between the CS group and the CN group was 1 589, of which 1 063 were upregulated and 526 were downregulated. Between the CK and CN groups, 1 012 DEGs were identified, of which 737 were upregulated and 275 were downregulated (Fig. 1).

To further characterize the DEGs, they were functionally annotated using the *GO* and *KEGG* databases. The *GO* functional enrichment analysis showed that all DEGs could be classified into 45 functional groups, including 20 groups in biological process, 13 in cellular component, and 12 in molecular function (Fig. 2). Within the 'biological process' category, 'metabolic process' and 'cellular process' were predominant. In the 'cellular components category', 'cell', 'cell part', and 'organelle' were predominant and 'binding', and 'catalytic activity' were the most common subcategories in the 'molecular function' category. In the CK *vs*. CS comparison, 'cell' (148) and 'cell part' (141) were the dominant 'cellular components' subcategories. The 'binding' (189) and 'catalytic activity' (192) were the most predominant 'molecular functions' subcategories. The main 'biological processes' subcategories were 'metabolic process' (249) and 'cellular process' (209) (Fig. 2*A*). In the CS *vs*. CN comparison, DEGs were primarily involved in 'cellular process', 'metabolic process' and 'single organism process' in the 'biological process' category, and the DEGs were enriched for *GO* terms that included 'cell part', 'cell', 'binding', and 'catalytic activity' (Fig. 2*B*). As concern the CK and CN data, the *GO*-term distribution was similar to the first two groups described above (Fig. 2*C*).

To understand the biological function of genes further, we analyzed the DEGs using the *KEGG* database. In this study, *KEGG* revealed that the pathways most highly represented were 'carbohydrate metabolism', 'biosynthesis of other secondary metabolites', followed by 'amino acid metabolism', 'lipid metabolism' and 'signal transduction'. This suggests that these pathways might be important in protecting melon plants against chilling stress (Fig. 3). In the CK *vs*. CS comparison, 'biosynthesis of secondary metabolites', 'carbon metabolism', 'phenylpropanoid biosynthesis', and 'plant hormone signal transduction' were predominantly enriched (Fig. 3*A*). In the CS *vs*. CN comparison, most DEGs were enriched in three pathways: 'metabolic pathways', 'biosynthesis of secondary metabolites', and 'phenylpropanoid biosynthesis' (Fig. 3*B*). Moreover, 'metabolic pathways', 'biosynthesis of secondary metabolites', and 'phenylpropanoid biosynthesis' were the main three pathways for DEGs in the CK *vs*. CN comparison (Fig. 3*C*).

In this study, changes in the expression of transcripts encoding enzymes related to sugar metabolism pathways were identified, such as β-glucosidase, UDP-glucuronate 4-epimerase, glucose-6-phosphate isomerase, triose phosphate isomerase, and hexokinase (Fig. 4). Comparing transcriptome data for control and chilling-stressed plants, most of these genes were downregulated. The expression of genes encoding fructose-bisphosphate aldolase, glucan endo-1,3-β-glucosidase, and raffinose synthase were upregulated in the CS vs. CN comparison. In the CK vs. CN comparison, DEGs encoding fructose-bisphosphate aldolase, glucan endo-1,3-β-glucosidase and UDP-glucuronate 4-epimerase were also upregulated.

In addition to an activation of genes involved in sugar metabolism, an enrichment in transcripts associated with hormone signalling was observed. These DEGs included genes that encoded EREBP-like factors, WRKY transcription factors, an auxin-responsive GH3 protein, an auxin-responsive IAA protein, gibberellin 2-oxidase, an abscisic acid receptor PYR/PYL family protein, a myb proto-oncogene protein, and abscisic acid 8′-hydroxylase, which were all upregulated in the CK vs. CS group (Table 3 Suppl.).

Discussion

Sugars are important regulators of plant growth, not only as carbon and energy source but also as signaling molecules (Rosa *et al.* 2009, O'Hara *et al.* 2013, Ruan 2014). Furthermore, increasing numbers of studies have demonstrated that sugars can regulate photosynthesis, lipid metabolism, osmotic homeostasis, and gene expression in response to various abiotic stresses (Keunen *et al.* 2013, Sami *et al.* 2016). Genes associated with sugar metabolism are regulated differently by cold acclimation in tea plants, whereby *CsBAM*, *CsINV5*, *CsRS2* are upregulated and *CsSWEET2, 3, 16, CsERD6.7* and *CsINT2* are downregulated (Yue *et al.* 2015). Therefore, varying content of sugars and activities of related metabolic pathways are crucially important to confer tolerance against diverse abiotic stresses. In this study, sugar metabolism was predominantly enriched upon chilling stress in melon leaves (CK *vs*. CS), including 16 upregulated genes (Fig. 3). Among these, four genes (encoding UDP-glucuronate 4-epimerase, β-glucosidase, glucuronosyltransferase, and α-1,4-galacturonosyl transferase) were upregulated in response to chilling stress. Glucose and fructose are monosaccharides and can be phosphorylated by hexokinase (HXK) and/or fructokinase (FRK) during glycolysis, to provide substrates for numerous physiological pathways. In this study, *CmHXK* was upregulated and 6-phosphate fructokinase was downregulated by chilling stress. Genes encoding UDP-glucuronate 4-epimerase, fructose-bisphosphate aldolase, glucan endo-1,3-β-glucosidase were upregulated by combined chilling stress and SNP treatment (CS *vs*. CN). Uridine diphosphate glucose dehydrogenase (UGD) catalyzes oxidation of UDP-glucose to UDP-glucuronate, which is then converted to UDP-galacturonic acid, UDP-xylose, UDP-arabinose, and UDP-apiose (Seifert 2004). These sugars are precursors for the synthesis of polymers such as hemicellulose and

pectin in plant cell walls. Therefore, we propose that the induced metabolism of sugars in response to SNP potentially provides an osmoprotectant function against membrane injury, which thereby contributes to chilling tolerance in melon seedlings.

Phytohormones, including ABA, SA, and auxin, are involved in plant adaptation to stress (Gan 2010, Davies 2010). The data here indicate that many genes related to hormone pathways, including those encoding abscisic acid 8′-hydroxylase, a PYR/PYL family member, an auxin responsive GH3 family member, and gibberellin 2-oxidase were upregulated during chilling stress (Table 3 Suppl.). Consistent with previous studies, GH3 and auxin-related gene families have been reported to be involved in responses to biotic and abiotic stresses in rice (Hagen and Guilfoyle 2002). Many studies have demonstrated that transcription factor networks regulate abiotic stress responses (Chen *et al.* 2012). In this study, genes encoding an EREBP-like factor, WRKY transcription factor 2 and a myb proto-oncogene protein were significantly upregulated after exposure to chilling stress. Taken together, our results suggest that hormones might play crucial roles in plant chilling stress responses.

Conclusions

In this study, we performed RNA-seq to comparatively analyze the transcriptome of melon seedlings exposed to chilling stress or chilling stress combined with NO treatment. A large number of DEGs were identified in response to NO treatment under chilling stress. The results indicate that exogenous NO upregulates many genes related to sugar metabolism, including UDP-glucuronate 4-epimerase, β-glucosidase, glucuronosyltransferase, α-1,4-galacturonosyl transferase, as well as *ERF*/*EREBP*, *WRKY*, *GH3* and *PYR*/*PYL* genes, which are involved in diverse signaling pathways. A significant positive correlation between qPCR results and the RNA-seq data for 10 genes confirmed the reliability of the transcriptome data. Collectively, these results provide an important contribution to the current understanding NO mediation of the chilling tolerance of melon seedlings but the specific regulatory mechanisms require further study.

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