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Research article

Comparative transcriptome profiling of chilling stress responsiveness in grafted watermelon seedlings

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ABSTRACT

Rootstock grafting may improve the resistance of watermelon plants to low temperatures. However, information regarding the molecular responses of rootstock grafted plants to chilling stress is limited. To elucidate the molecular mechanisms of chilling tolerance in grafted plants, the transcriptomic responses of grafted watermelon under chilling stress were analyzed using RNA-seq analysis. Sequencing data were used for digital gene expression (DGE) analysis to characterize the transcriptomic responses in grafted watermelon seedlings. A total of 702 differentially-expressed genes (DEGs) were found in rootstock grafted (RG) watermelon relative to self-grafted (SG) watermelon; among these genes, 522 genes were up-regulated and 180 were down-regulated. Additionally, 164 and 953 genes were found to specifically expressed in RG and SG seedlings under chilling stress, respectively. Functional annotations revealed that up-regulated DEGs are involved in protein processing, plant-pathogen interaction and the spliceosome, whereas down-regulated DEGs are associated with photosynthesis. Moreover, 13 DEGs were randomly selected for quantitative real time PCR (qRT-PCR) analysis. The expression profiles of these 13 DEGs were consistent with those detected by the DGE analysis, supporting the reliability of the DGE data. This work provides additional insight into the molecular basis of grafted watermelon responses to chilling stress.

1. Introduction

Low temperature is one of the major environmental factors that severely limits plant growth and development, especially for the chilling sensitive cultivated watermelon (*C. lanatus*). Watermelon grows best at temperatures ranging from 21 to 29 °C, with growth ceasing at 10 °C death occurring at temperatures of 1 °C (Noh et al., 2013). Because of its low-temperature sensitivity, it is very difficult to obtain good yields and fruit quality during the cold seasons. To avoid such difficulties and improve the growth performance, watermelon seedlings are usually grafted onto rootstocks to confer resistance to low temperatures (Lee and Oda, 2003).

Grafting has been attempted in several crops to increase plant tolerance to low temperatures. Rootstocks alleviated the negative effects of low temperatures on scion performance by supplying the scion with more water, nutrients and hormones (Schwarz et al., 2010). The root system activity and SOD activity of grafted cucumber seedlings were found to be higher than those of ungrafted cucumbers under chilling stress (Li et al., 2008). Zhou et al. (2009) reported that figleaf gourd grafting significantly alleviated cucumber seedling growth inhibition by chilling at 7 °C while also increasing light utilization and reducing the accumulation of reactive oxygen species after chilling. Inhibition of the light-saturated rate of CO_2 assimilation, the maximum carboxylation activity, Rubisco content and initial Rubisco activity were all found to be weaker in grafted cucumber plants after chilling at 7 °C (Zhou et al., 2007). The relative growth rate of shoots and root mass ratios increased at 15 °C when tomato seedlings were grafted onto a cold-tolerant rootstock (Venema et al., 2008). Grafted watermelon (Ding et al., 2011) and muskmelon (Justus and Kubota, 2010) seedlings were found to have better storability under low-temperature storage than non-grafted seedlings. These studies have helped us to understand the physiological basis of increased low-temperature tolerance of rootstock-grafted plants. In addition to the physiological responses, novel proteins were identified in pumpkin rootstock grafted cucumber plants using proteomics techniques, and these

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proteins were further classified into two categories involved in stress defense and photosynthesis (Li et al., 2009). Similarly, Yang et al. (2012) found 40 differentially expressed proteins in bottle gourd rootstock-grafted watermelon seedlings compared to the self-grafted plants under salt exposure. These studies suggested the possibility that rootstocks could mediate gene expression patterns in scions under stress. However, studies on transcriptomic changes in rootstock grafted plants responding to chilling stress are lacking and are urgently required.

In the present work, the squash rootstock-grafted watermelon seedlings (RG) were found to be more tolerant of chilling stress than the self-grafted watermelon seedlings (SG). To explore the differences in the gene expression between RG and SG watermelon seedlings under chilling stress, digital gene expression (DGE) based on Illumina sequencing was applied to identify differentially-expressed genes (DEGs) between RG and SG. The transcriptome data presented here provide straight forward information regarding the molecular state of grafted plants challenged by chilling stress, which is important for understanding the transcriptomic changes of grafted watermelon plants in response to chilling.

2. Methods

2.1. Plant material and chilling stress treatment

The watermelon line MW022 was used as the scion and the squash Jingxinzhen NO.4 (JX) was used as the rootstock. An "insertion grafting" procedure described by Lee and Oda (2003) was used in this study. Watermelon plants grafted onto their own roots were used as controls. Grafted seedlings were grown in a growth chamber at 28°C/18 °C (16/8 h) day/night temperatures, a relative humidity of 70%, and a photon flux destiny of 400 μmol m^{-2} s^{-1}. After the full development of the third true leaves, the grafted plants were treated with a low temperature at 10 °C at the same relative humidity and illumination intensity. Leaves from 6 rootstock-grafted (RG) or self-grafted (SG) watermelon seedlings were pooled as one biological replicate at 0 d and 1 d after the chilling treatment, respectively. Three biological replicates were collected. All collected samples were immediately frozen in liquid nitrogen and stored at −70 °C until use.

2.2. Assessment of chilling damage index (CI) and measurement of malonyldialdehyde (MDA) content

Eighteen rootstock-grafted (RG) or self-grafted (SG) watermelon seedlings were moved to a climate chamber at a temperature of 10 °C with a 16 h light/8 h dark cycle. After 12 d of the chilling treatment, the chilling damage index was measured according to Yang et al. (2008). The degrees of chilling tolerance were measured with 6 grades as follows: level 0: no symptom; level 1: chlorosis or crinkled at the edge of old leaves; level 2: chlorosis or crinkled at the edge of less functional leaves; level 3: chlorosis or crinkled at the edge of functional leaves with good new leaves; level 4: chlorosis or crinkled and wilting of functional leaves with damaged new leaves; level 5: severe damage of new leaves, plants wilting or death. The chilling indices (CI) of RG and SG seedlings was tabulated according to the following formula: CI = ∑(each level × number of plants with the corresponding level)/total number of measured plants.

The MDA content was measured using 2-thiobarbituric acid as described by Hodges et al. (1999).

2.3. RNA-seq library preparation and illumina sequencing

Leaves from 6 RG or SG watermelon seedlings were pooled as one single biological replicate. The experiment was repeated to obtain three biological replicates. These pooled leaf samples were used for the RNA-seq analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and digested using DNase I at 37 °C for 30 min to remove any possible genomic DNA contamination. The quality and concentration of each sample were determined using an Agilent 2100 Bioanalyzer. Six μg aliquots of total RNA from each sample were purified using oligo (dT) magnetic bead adsorption. Purified mRNAs were fragmented with endonuclease and ligated with adaptors to generate libraries with unique the 5′ and 3′ tags. After 15 cycles of linear PCR amplification, 105 bp PCR products were quantified and purified. Denatured molecules were then fixed onto an Illumina Sequencing Chip (Flow Cell) for sequencing. RNA-seq libraries were sequenced on an Illumina HiSeq™ 2000 System by BGI-Tech (Shenzhen, China).

2.4. Functional annotation of differentially-expressed genes (DEGs)

Raw sequence reads were filtered through the Illumina pipeline by BGI Shenzhen, China. Gene expression levels were calculated using the RPKM (Reads Per kb per Million reads) method (Mortazavi et al., 2008). Differentially expressed genes (DEG) among the samples were identified using the novel NOIseq method (Tarazona et al., 2011) with a probability≥0.8 and an absolute value of the |log2 Ratio| ≥ 1 as the threshold to evaluate the significance of gene expression differences. In brief, NOISeq method computes differential expression described as bellow: first, gene expression of sample in each group was used to calculate $\log_2$ (fold change) M and absolute different value D of all pair conditions (gene expression value will be substituted by 0.001 if it doesn't express in some sample). Second, average expression value of each gene standing for replicates will be used to calculate M and D. Two replicates in one of the experimental conditions is sufficient to run the algorithm: $M^i = \log_2\left(\frac{x_1^i}{x_2^i}\right)$ and $D^i = |x_1^i - x_2^i|$. Then, all these M/D values are pooled together to generate the noise distribution. If gene i differentially expresses between two groups, we set Gi = 1, otherwise set Gi = 0, and give a definition for probability of gene i differentially expressing as following formula:

$$P\left(G^i = 1 \middle| x_1^i, x_2^i\right) = P\left(G^i = 1 \middle| M^i = m^i, D^i = d^i\right) = P\left(|M^*| < \left|m^i\right|, D^* < d^i\right)$$

When P is greater than threshold value, its corresponding gene is thought to differentially express between groups.

Gene Ontology (GO) was used to analyze biological functions by mapping all DEGs to GO databases (http://www.geneontology.org/). GO terms meeting a threshold of corrected p-value≤0.05 were defined as significantly enriched. The DEGs were also mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2008) to identify significantly enriched metabolic pathways or signal transduction pathways.

2.5. Quantitative real-time PCR (qRT-PCR) analysis

Leave samples used for qRT-PCR were the same as those used for sequencing. Total RNA from watermelon leaves sampled at 0 d and 1 d after chilling treatment was extracted using the RNApure Plant Kit with DNase I (CWBiotech, Beijing, P. R. China). The BU-Superscript RT Kit was used for first-strand cDNA generation with

the oligo (dT) primer (Biouniquer, Beijing, P. R. China). Analysis of qRT-PCR was carried out using the 1 x SYBR Green PCR Master Mix (PE-Applied Biosystems, USA) and the GeneAmp® 7300 Sequence Detection System (PE-Applied Biosystems, USA) according to manufacturer's instructions. Expression profile of 13 randomly selected DEGs were analyzed. The watermelon *18S rRNA* gene (GenBank accession No. AB490410) was used as a reference gene, and relative expression levels of DEGs were normalized to the constitutive expression level of *18S rRNA* and calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001; Yin et al., 2001). Three technical replicates were performed for each biological replicate. SAS 9.2 (SAS Institute, Cary, NC) was used for all statistical analyses.

3. Results

3.1. Chilling damage index and the MDA content of SG and RG under chilling stress

To identify the effects of grafting on watermelon coffering cold tolerance, squash rootstock grafted (RG) watermelon and self-grafted (SG) watermelon seedlings were evaluated by cold stress treatment. RG seedlings exhibited less severe wilting than SG seedlings after 1d of treatment at 10 °C (Fig. 1A and B). After 12 days of treatment, the chilling damage index (CI) of RG and SG seedlings was evaluated. The CI values of SG and RG were 3.0 and 2.1, respectively (Fig. 1C). This phenotypic performance showed that RG exhibited better chilling-stress tolerance than SG. In addition, MDA content was measured to investigate physiological variation in RG and SG seedlings. The MDA contents of RG and SG seedlings significantly increased compared with their control seedlings after 1 d chilling treatment ($P < 0.05$). Compared with RG seedlings, chilled SG seedlings exhibited MDA concentrations nearly 1.2-fold higher (Fig. 1D). These results indicate that RG and SG watermelon seedlings differ in their response to chilling stress, with rootstock grafting apparently enhancing the chilling tolerance of watermelon.

3.2. DGE sequencing in grafted watermelon leaves under chilling stress

RNAs from RG and SG watermelon seedlings with no chilling treatment (C) or with chilling stress treatment (LT) were extracted to prepare four cDNA libraries for RNA-seq analysis. 11,695,581 (99.22%), 11,806,210 (99.38%), 12,074,690 (99.22%) and 12,275,446 (99.35%) clean reads remained in the SG-C, SG-LT, RG-C and RG-LT libraries, respectively, when reads containing adaptors, poly-N, and low quality reads were filtered. This data indicates that the sequencing depth was sufficient for the transcriptome coverage in watermelon (Table 1). A total of 10,256,476 (87.7%), 10,410,835 (88.18%), 10,614,574 (87.91%) and 10,839,819 (88.3%) clean reads in SG-C, SG-LT, RG-C and RG-LT libraries were mapped to the reference watermelon genome by allowing a 3-bp mismatch. This suggests that the DGE data are reliable and sufficient for subsequent bioinformatics analysis.

3.3. DEGs responding to low temperature in SG and RG

The SG and RG transcriptome profiles were analyzed to find differentially expressed genes. Gene expression alterations in SG

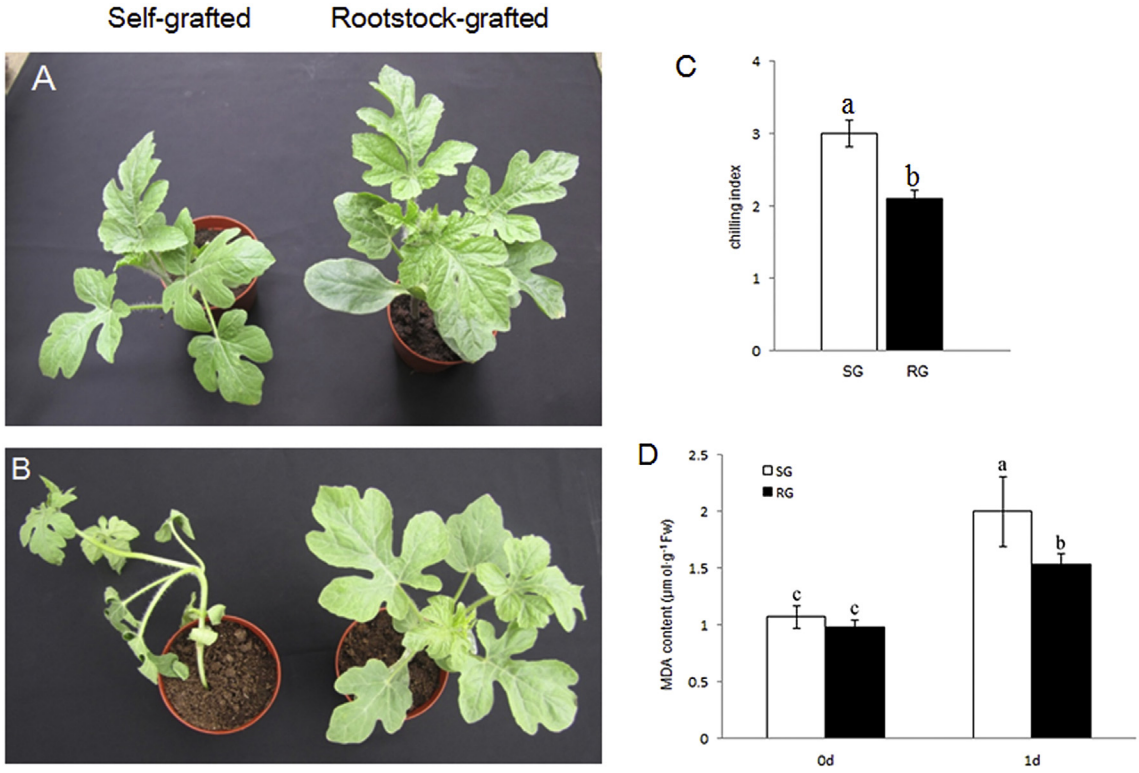


Fig. 1. Phenotypes of grafted watermelon seedlings under chilling stress.
A. Comparison of self-grafted and rootstock-grafted watermelon seedlings in control. B. Comparison of self-grafted and rootstock-grafted watermelon seedlings treated at 10 °C for 1d. C. Chilling damage index of SG and RG watermelon seedlings treated at 10 °C for 12d. D. MDA content of SG and RG watermelon seedlings treated at 10 °C for 1d. Values are means of 3 replicates. Vertical bars indicate standard error. Three biological replicates were used, with six plants per replicate. Values are presented as mean ± SE of three biological replicates. Lowercase letters (a, b and c) indicate the significant differences between RG and SG genotypes.

Table 1
Summary of sequencing data.

sample	Raw tag	Clean tag TN	TP (%)	Tags mapped to genome TN	TP (%)
SG-T	11,879,434	11,806,210	99.38	10,410,835	88.18
RG-T	12,356,129	12,275,446	99.35	10,839,819	88.3
SG-C	11,787,043	11,695,581	99.22	10,256,476	87.7
RG-C	12,169,968	12,074,690	99.22	10,614,574	87.91

and RG after 1 day of chilling stress were compared to their respective controls. The significance of gene expression difference between SG and RG was assessed using the NOISeq method (Tarazona et al., 2011). A probability ≥0.8 and an absolute value of the |log2 Ratio| ≥ 1 were used as thresholds to define the significant differences in transcript abundance between two libraries. At control temperature, 36 genes were differentially expressed in RG plants as compared to SG plants (Table S1, Fig. 2A). After 1d of chilling stress, a total of 1491 (973 up- and 518 down-regulated) (Table S2) and 702 (522 up- and 180 down-regulated) (Table S3) chilling-responsive genes were identified in SG and RG, respectively. 953 chilling-responsive genes (574 up- and 379 down-regulated) were exclusively identified in SG (Table S4), whereas 164 chilling-responsive genes (123 up- and 41 down- regulated) were uniquely observed in RG (Table S5). 538 genes (399 up- and 139 down-regulated) were commonly regulated by chilling stress in both SG and RG (Table S6, Fig. 2B).

To verify the RNA-seq based gene expression levels, 13 DEGs with differential expression patterns were randomly selected from SG or RG DEGs for qRT-PCR analysis. Before the qRT-PCR assay, we testified the stability of the reference gene. Results showed that expression of *18S rRNA* was stable (Fig. S1) and thus was used as reference gene for data normalization. As expected, expression of these genes verified by qRT-PCR showed the same expression patterns as in DGE analysis except for expansion protein and aquaporin (Fig. 3, Table S7). Differences between the two methods were on the scale of the fold changes, which may be due to the different sensitivity and algorithms between the two assay methods.

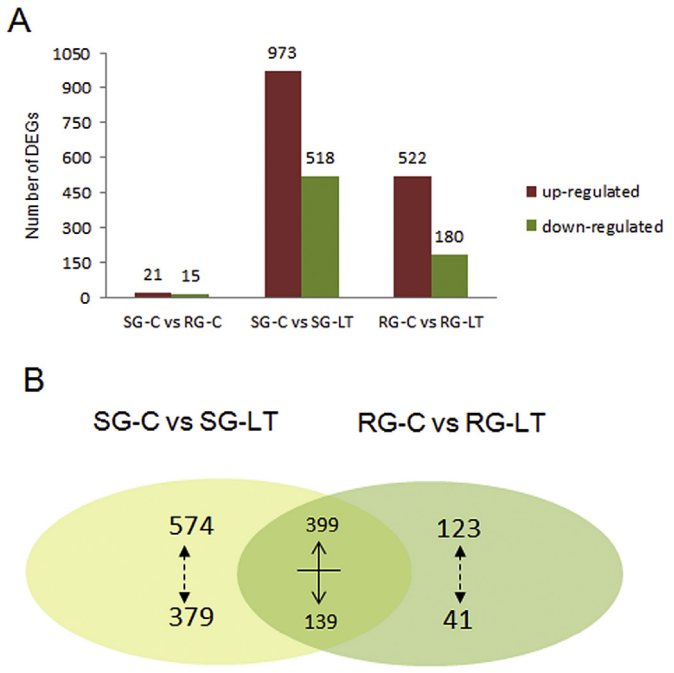


Fig. 2. Differentially expressed genes (DEGs) among different libraries.
A. DEG numbers among no treatment (C) and low-temperature treatment (LT) libraries of RG and SG watermelon seedlings. B. Venn diagram of DEGs under the control and low-temperature conditions. The upwards or downwards arrows indicate the up- or down-regulated DEGs, respectively.

3.4. Functional classifications of DEGs

To determine the similarities and differences in chilling-induced transcriptomes between RG and SG, DEGs were analyzed for GO classification and enrichment. A total of 2193 DEGs (1491 in SG and 702 in RG) between control and chilling stress treated SG and RG watermelon seedlings were categorized into 43 functional groups using GO classifications (Fig. 4). In the biological process category, metabolic process, single-organism process, cellular process, and response to stimulus were the main groups. For the cellular components category, cell, cell part and organelle were the main groups. Among the molecular function category, binding and catalytic activity were the main groups. Additionally, we found that very few differences could be observed between rootstock grated watermelon plants and self-grafted plants in either up-regulated DEGs or down-regulated DEGs. These results indicate that the majority of DEGs responding to chilling stress were involved in the metabolic process, cell and catalytic activity, which suggest that chilling stress treatment mainly affects physiological metabolism and cell differentiation in grafted watermelon.

To further understand the biological functions of DEGs, pathway enrichment analysis was performed by identifying the metabolic pathways or signal transduction pathways that were significantly enriched in DEGs. Analysis of DEGs in SG plants assigned 575 DEGs to 104 KEGG pathways. In RG plants, 274 DEGs were assigned to 77 KEGG pathways. Based on KEGG analysis, up-regulated DEGs in SG were highly enriched in protein processing in the endoplasmic reticulum (ko04141), plant-pathogen interaction (ko04626) and the spliceosome (ko03040). Although the number of significantly enriched terms for up-regulated DEGs in RG was less than that in SG, the main significantly enriched terms of up-regulated DEGs in RG were similar to those in SG. The photosynthesis-antenna proteins (ko00196) pathway was the most common significantly enriched term for down-regulated DEGs in SG and RG (Table S8).

3.5. DEGs encoding transcription factors

Among the DEGs of SG, 141transcription factors were identified (Table S9), including WRKY (15), C3H (13), C2H2 (12), NAC (12), bHLH (9), ERF (8), MYB (2) and MYB-related (9) families, etc. 62 transcription factors were identified in the DEGs of RG, including WRKY (6), C3H (4), C2H2 (5), NAC (4), bHLH (6), ERF (5), and HSF (5) families, etc. Among the transcription factors that responded to low temperature in SG and RG, 48 transcription factors were identified in both SG and RG. 14 transcription factors were found to be uniquely regulated in RG, including the ERF (3) and HSF (4) families.

3.6. Differentially expressed genes in RG watermelon leaves relative to SG leaves under chilling stress

There were 52 DEGs induced by rootstock when RG seedlings

were compared with SG seedlings under chilling stress (Table S10). Among these genes, 42 were up-regulated, including 3 genes (WMU41743, WMU41479, WMU50993) encoding phloem protein, 1 gene (WMU49190) encoding LRR serine/threonine-protein kinase, 1 gene (WMU78566) encoding methyltransferase, 1 gene (WMU36427) encoding early nodulin-like protein 3-like and 1 gene (WMU31369) encoding expansin-A1-like. Two genes (WMU75582, WMU62304) showing the greatest increase in expression in RG have no annotation in the NCBI database. Ten genes were down-regulated, including 2 genes (WMU44051, WMU41261) encoding transcription factor, 2 genes (WMU45207, WMU16709) encoding HARBI1-like gene, and 1 gene (WMU62127) encoding HSP25.5.

Among the DEGs induced by rootstock under control condition, 6 genes in SG and 6 genes in RG were also responsive to low temperature too. Two genes (WMU22881, WMU28938) encoding aquaporin PIP2-1-like were up-regulated by rootstock in RG and up-regulated by low temperature in SG. Two genes (WMU63563, WMU11846) encoding HSP were down-regulated by rootstock and up-regulated by low temperature in RG.

4. Discussion

4.1. Rootstock grafted seedlings shown less sensitivity to chilling stress than self-grafted seedlings

Grafting is regarded as a promising tool to increase the resistance of watermelon seedlings to chilling stress. To better understand the molecular mechanisms of how rootstock regulate the scion's low temperature tolerance, we used the Illumina HiSeq 2000 system to investigate changes in transcriptomes in response to low-temperature stress between rootstock grafted and self-grafted watermelon seedlings. The sequence data of the four analyzed libraries (SG-LT, SG-C, RG-LT, RG-C) consisted of approximately 12 million tags per library (Table 1), and the distribution of total clean tags was uniform across all sequenced libraries in the whole dataset.

To compare the transcriptome variation in RG and SG seedlings under low-temperatures, DEGs among the four libraries were checked with a $|\log2\ \text{Ratio}| \geq 1$. Relatively fewer DEGs (702 DEGs) related to chilling response were obtained in RG than that (1491 DEGs) in SG seedlings. Additionally, 52 DEGs were found when comparing RG-LT and SG-LT libraries, which is fewer than the number of DEGs found in RG and SG when comparing chilling and control treatments. The number did not support our hypothesis that rootstock grafting may intensively alter the regulation of the scion's transcriptome to respond to chilling stress. A similar result was also reported in previous comparative transcriptome work in freezing-sensitivity and cold-hardness for grapes under cold stress (Xin et al., 2013). In this study, the authors explained that the lower number of DEGs was because of poor alignment and annotation of wild grape genes to the reference genome (43.61% in cold treated and 39.4% in control libraries). While this explanation does not apply to our work, as approximately 88% of the unique tags were found to be aligned to reference genes in both RG and SG libraries (Table 1). Thus, we suggest that rootstock grafting reduced the sensitivity of watermelon seedlings to chilling stress and modified gene expression patterns at the transcriptome level in a different manner during chilling stress. Moreover, it was found that more of the DEGs in RG libraries (75%) were up-regulated under chilling treatment compared to SG (60%), which indicates that these genes are involved in the chilling stress response and that rootstock grafting could induce the expression of some essential genes related to chilling tolerance to enhance plant response to low-temperature stress (Gu et al., 2015).

4.2. Specific responses to low-temperature stress in RG plants

Differences in DEG numbers and functional annotation in RG and SG libraries under chilling stress indicate that rootstock grafting enhanced the plant response to chilling stress in a different way. Further analysis found a subset of DEGs in response to chilling stress that were specifically expressed only in rootstock-grafted seedlings (Table S5). Most of these genes were up-regulated under chilling stress, suggesting they may play roles in enhancing the chilling tolerance of rootstock-grafted seedlings.

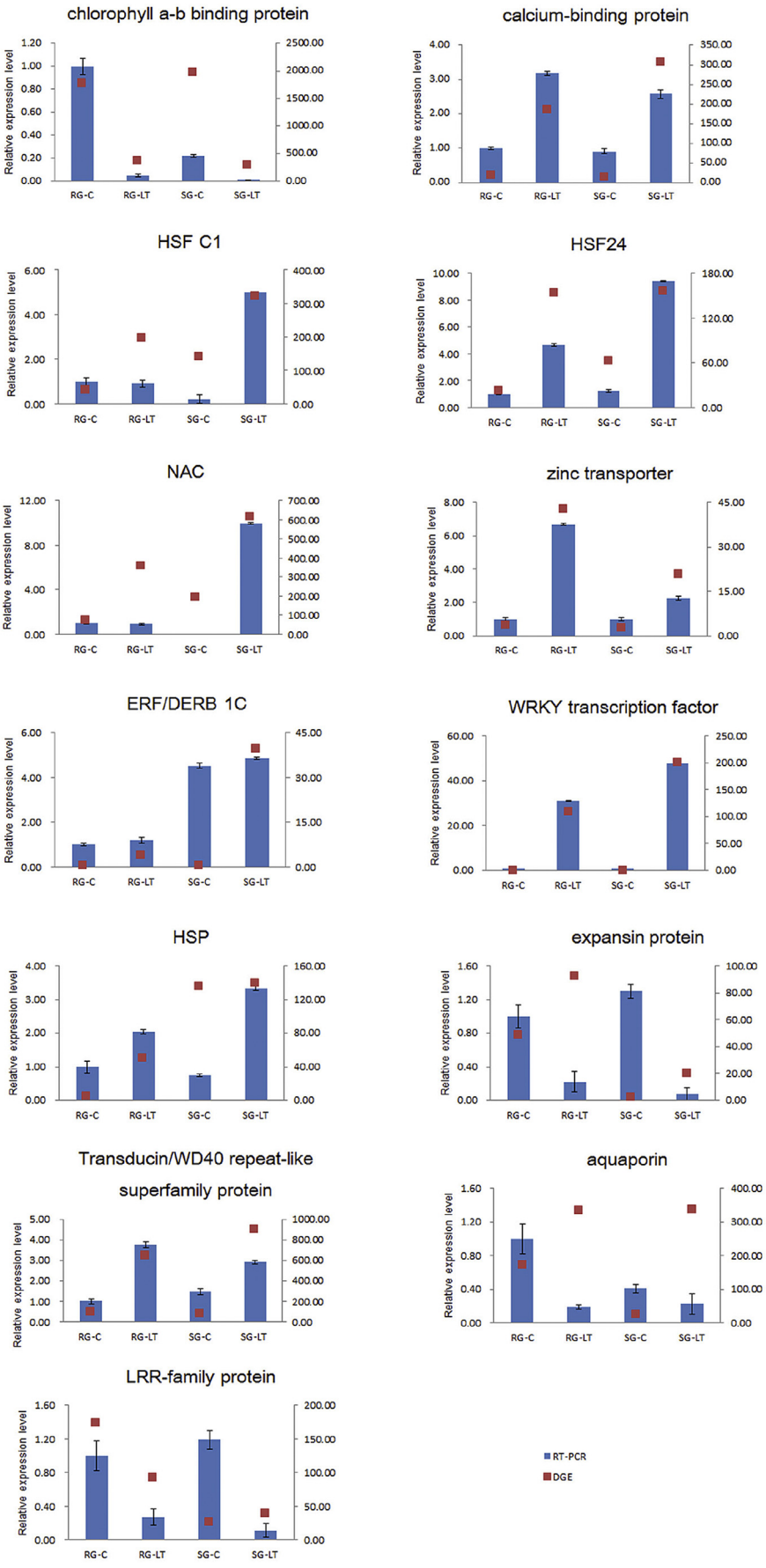
4.2.1. Metabolism

In the present work, 27 transcripts related to metabolism were detected in RG seedlings under chilling stress. Of these, 19 genes were up-regulated and 8 genes were down-regulated. Glutathione S-transferases (GSTs), rate-limiting enzymes of the MAP pathway, conjugate glutathione to several electrophilic substrates, and therefore, GSTs function in the plant detoxification system. The detoxifying activity of GSTs was found to be associated with pathogen attack, oxidative and heavy-metal stress, and environmental stresses. Overexpression of *GST* improved the growth rate of transgenic tobacco seedlings exposed to chilling stress (Roxas et al., 1997). Expression of *Scgst1* and GST enzyme activity accumulated in cold tolerant potato and in its hybrid with a sensitive line but declined in a freezing sensitive potato (Seppänen et al., 2000). Here, we observed two up-regulated *GSTs* after chilling stress in RG, suggesting that rootstock grafting activates GST accumulation in response to chilling stress, perhaps through its detoxifying property.

1-aminocyclopropane-1-carboxylate (ACC) oxidase catalyzes the conversion of ACC to ethylene, which is the second step of the ethylene biosynthetic pathway (Yang and Hoffman, 1984). It has been reported that ACC oxidase activity could be stimulated when apple fruit is exposed to low temperatures (Lelièvre et al., 1995). Down-regulation of ethylene biosynthesis related genes resulted in uneven ripening in cold-stored tomatoes (Rugkong et al., 2011). In this report, two genes encoding ACC oxidase protein were up-regulated in RG. This may indicate that the chilling tolerance of rootstock grafted seedlings may be a consequence of chilling-induced ethylene accumulation caused by activation of ACC oxidase gene, or perhaps due to the protection elicited by the interaction of ethylene and methyl jasmonate (MeJA) (Yu et al., 2011).

Protein disulfide isomerase (PDI) is a member of the thioredoxin super family and is considered a major catalysts for protein folding in the lumen of the endoplasmic reticulum (ER). It is well established that PDI possesses multiple function, including acting as a molecular chaperone (Huang et al., 2005). Transgenic rice seedlings with high Hg tolerance and more effective photosynthesis showed a correlation with overexpression of the *PDI* gene *MTH1745* (Chen et al., 2012). In this experiment, two *PDIs* were up-regulated in RG seedlings under chilling stress. This may suggest that the accumulation of PDI may increase the stability of proteins and therefore enhance chilling tolerance in RG seedling.

Two 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) genes were found to be up-regulated in the chilling-tolerant rootstock grafted watermelon seedlings. Previous studies have shown that expression of NCED genes can be induced by environmental stresses such as drought, cold, salt and osmotic stresses (Gómez-Cadenas et al., 2003; Zhang et al., 2014). Overexpression of *NCED* has been found to enhance plant tolerance to multiple abiotic stresses (Xian et al., 2014). NCED indirectly catalyzes the conversion of C_{40}-carotenoids to ABA, which is considered the key regulatory step in the ABA biosynthesis. Therefore, it is presumed that enhanced plant tolerance to chilling stress from rootstock grafting may be associated with the increase in ABA accumulation which is caused by expression of *NCED* genes.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HSF24
NAC
zinc transporter
ERF/DERB 1C
WRKY transcription factor
HSP
expansin protein
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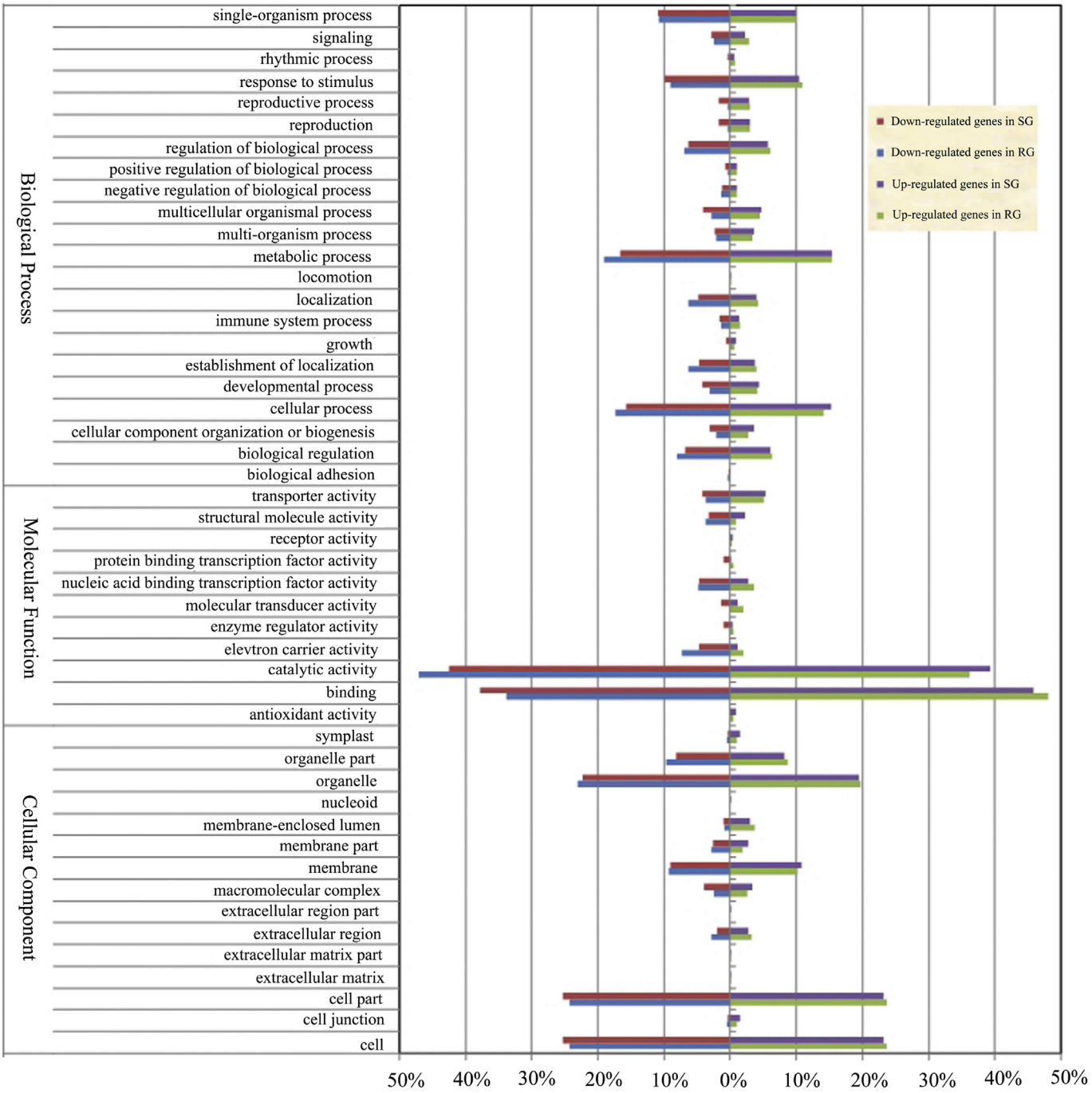

Fig. 4. Gene ontology functional analysis of DEGs in RG-LT and SG-LT libraries. The x-axis indicates the percentage of up- or down-regulated genes in RG or SG libraries under chilling stress in each GO term. The y-axis shows the names of functional categories.

4.2.2. Transport

In this work, four genes related to transport were up-regulated in RG seedlings under chilling stress as follows: metal ion binding protein, heavy metal-associated isoprenylated plant protein (HIPP), DnaJ protein and zinc transporter. Metal ion binding proteins not only play important roles in many biological processes but also help to maintain structural integrity by using metal ions as scaffolds (Mohan et al., 2010). The strong expression of this gene might be one of the chilling tolerance mechanisms exhibited by rootstock grafted plants.

HIPP proteins are a new class of plant proteins with the following two conserved domains: a heavy metal associated

Fig. 3. Confirmation of expression of 13 randomly-selected DEGs by qRT-PCR analysis. The x-axis indicates the four samples. RG: rootstock grafted watermelon; SG: self-grafted watermelon; C: no treatment; LT: chilling stress treatment. The y-axis shows the expression level. Squares represent expression from DEG analysis. Columns indicate relative expression as measured by qRT-PCR. Relative levels of gene expression were calculated using 18sRNA. PCR reactions were performed three times. Error bars indicate standard error of the mean.

domain (HAM) and an isoprenylation motif at the C-terminal end. HAM proteins have been reported to play important roles in metal transport and metal homeostasis processes. Isoprenylation acts in post-translational modification of regulatory proteins (Barth et al., 2009). Barth et al. (2004) observed the induction of barley *HvFP1*, a group III HIPP gene, during cold stress. In the present work, a *HIPP* gene was up-regulated after 1-d of low temperature treatment in RG seedlings, suggesting that *HIPP* also plays a role in the chilling stress response.

DnaJ proteins are a family of conserved co-chaperones for HSP70s and are involved in responses to various stresses (Rajan and D'Silva, 2009), chloroplast movement (Suetsugu et al., 2005), and developmental processes (Shen et al., 2011). Plants with overexpressed DnaJ in transgenic tobacco exhibited higher tolerance to drought stress and resistance to *P. solanacearum* (Wang et al., 2014). In the present work, we observed the up-regulation of a DnaJ protein in RG seedlings under chilling stress. This may indicate that low sensitivity of RG seedlings to chilling stress might be a consequence of reduced accumulation of H_2O_2 and O_2^- caused by *DNAJ* gene expression.

4.2.3. Signal transduction

Four genes function as signal transduction, and two of these were found to be up-regulated while one was down-regulated. Two (WMU07974, WMU31278) of them were identified as two-component response regulator-like proteins, which are involved in the His-Asp phosphorelay signal transduction and are reported play important roles in various developmental and environmental conditions by regulating different stress-responsive genes (Mason et al., 2010). WMU37022 was predicted to be a receptor-like protein kinase (RLK). It is well established that RLKs contribute greatly in plant responses to both biotic and abiotic stresses. Overexpression of *GsLRPK* enhanced the resistance of yeast and *Arabidopsis* to cold stress and increased the expression of a number of cold responsive gene markers (Yang et al., 2014). The up-regulation of these genes suggests that several signal transduction pathways are involved in the chilling tolerance of RG seedlings.

4.2.4. HSP

Heat shock proteins (HSPs) constitute a stress-responsive family of proteins that are essential for maintaining cellular homeostasis under stressful conditions in plants. The accumulation of HSPs protects plants against stress and against any subsequent stressful situation (Aghdam et al., 2013). There are five families of HSPs as follows: HSP60s, HSP70s, HSP90s, HSP100s and small HSPs (sHSPs). In this work, 9 genes were found to encode HSPs, and all of them were up-regulated. In addition to heat shock, other environmental stresses including low temperatures that cause protein misfolding and aggregation, may trigger the accumulation of HSPs. Expression of *CsHSP17.5* was quickly activated when chestnut seedlings were exposed to cold conditions. Moreover, purified CsHSP17.5 significantly protected the cold-labile enzyme lactate dehydrogenase from freeze-induced inactivation (Lopez-Matas et al., 2004). Recently, it was reported that sHSPs are essential for maintaining membrane quality attributes under chilling stress (Aghdam et al., 2013). These results suggest that HSPs can play relevant roles in the acquisition of cold tolerance.

4.2.5. Stress related genes

Molecular and cellular responses of plants to low temperature stress are complex. A variety of stress-inducible genes were induced, which function not only in protecting plant cells by producing functional proteins but also in regulating genes that are involved in signal transduction pathways. Cross-talk has been shown to exist between the following two different stress signaling pathways: low temperature and dehydration (Shinozaki and Yamaguchi-Shinozaki, 2000). In this work, one elicitor-responsive protein, one salt tolerance protein and one CASP-like protein were identified specifically up-regulated in RG seedlings under chilling stress, which suggests that rootstock grafting take a multiple stress-related networks that can identify and react to elicitors in a more efficient manner, thus eliciting the defense response of grafted plants.

4.2.6. Transcription factors

Transcription factors (TFs) mediate the gene transcription processes by initiating transcription of defense genes in plant innate immune systems. Many TFs have been shown to function in plant resistance against various stresses, especially low temperature (Luo et al., 2012). In this work, 11 stress related TFs were found to be uniquely present in RG libraries under chilling stress, including 5 HSFs, 4 bHLHs and 2 other TFs. The heat shock transcription factor (HSF) family has been implicated in various abiotic stresses (Mittal et al., 2009). Rice *Hsf* genes showed inducible expression in response to low temperature stress.

Basic helix–loop–helix (bHLH) proteins are the second largest transcription factor familiy in plant genomes (Castilhos et al., 2014; Jin et al., 2014). The bHLH family has been shown to be one of the major plant regulators involved in various plant physiological and developmental processes (Sajeevan and Nataraja, 2016) and abiotic stresses, such as drought, low temperature, and osmotic stress (Dong et al., 2014; Chinnusamy et al., 2003; Feng et al., 2012; Liu et al., 2013). Many studies have shown that the stress tolerance of plants is a complex stress signaling and response networks of plants. It has been reported that *HsfA* genes exclusively induced the expression of *DREB2A* (Liu et al., 2011). HaDREB2 and HaHSFA9 interact *in vitro* and mediate synergistic transactivation (Mizoi et al., 2012). The apple bHLH gene *MdClbHLH1* activated *MdCBF2* gene expression through the CBF pathway and promoted the cold tolerance of transgenic apple plants (Feng et al., 2012). Similar results were also obtained by Feng et al. (2013), where SlICE1a interacted with CBF/DREB and enhanced the freezing tolerance of transgenic tobacco. These works suggest that various transcription factors cross talk with one another for their maximal response to environmental stresses and play crucial roles in the signal transduction network (Yamaguchi-Shinozaki and Shinozaki, 2006).

5. Conclusion

A number of differentially expressed genes related to rootstock grafting regulated chilling tolerance were identified using the Illumina sequencing based DGE, and the expression patterns of 13 randomly selected DEGs were further validated by qRT-PCR. These DEGs were involved in multiple biological functions, including the induction of protein processing, plant-pathogen interaction, the spliceosome and suppression of photosynthesis. We also investigated the transcription factors respond to low-temperature stress in rootstock grafted plants. This work provides comprehensive insight into the molecular basis of the gene expression profiles of rootstock grafted watermelon seedlings in response to chilling stress. Further research will focus on the functional characterization of these candidate genes of grafted watermelon seedlings under chilling stress via transgenic approaches.

Contributions

J.X., X.Y. and X.H. conceived the idea and led the study design. J.X. carried out the experiment, performed analyses and wrote the pater. M.Z. assisted with data analysis and writing. G.L. assisted with material preparation. All authors contributed to the editing of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.11.002.

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