Plant Physiology and Biochemistry 109 (2016) 561–570

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Comparative transcriptome profiling of chilling stress responsiveness in grafted watermelon seedlings

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ARTICLE INFO

Article history:
Received 6 April 2016
Received in revised form
3 November 2016
Accepted 3 November 2016
Available online 5 November 2016

Keywords:
Watermelon
Rootstock grafting
Differentially-expressed gene (DEG)
Chilling stress

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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http://dx.doi.org/10.1016/j.plaphy.2016.11.002
0981-9428/

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