Scientia Horticulturae 227 (2018) 169–180

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Comparative transcriptome profiling of genes and pathways related to resistance against powdery mildew in two contrasting melon genotypes

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ARTICLE INFO

Keywords:
Melon
Powdery mildew
Podosphaera xanthii
Transcriptome analysis
Defense-related genes

ABSTRACT

Powdery mildew (PM) is a catastrophic disease of melon caused by *Podosphaera xanthii* (Px). However, there is limited information available at the molecular level regarding how the melon plant develops defense mechanisms against this pathogen. We studied melon mRNA transcripts from the resistant MR-1 and susceptible Topmark cultivars through comparative transcriptome analysis. Leaves inoculated with spores of *P. xanthii* were collected from 0 to 168 hpi to identify the candidate regulators for resistance to PM at different infection stages. We identified 1888 and 2842 melon genes in MR-1 and Topmark, respectively, that were differentially expressed during the plant-pathogen interaction. Gene function analysis of these differentially expressed genes (DEGs) classified them into seven functional groups: pathogen recognition, signal transduction, transcription factors (TFs), phytoalexin biosynthesis, other primary metabolite functions, *Mildew Locus O* genes (*MLOs*) and pathogenesis-related (PR) proteins. The expression of ten of these genes in both the MR-1 and Topmark cultivars was validated via qRT-PCR. Our results revealed both similar and differential patterns of gene expression between the two cultivars. This study may provide a new understanding of the molecular mechanisms of melon resistance to PM.

1. Introduction

Melon (*Cucumis melo* L.) is one of the most important horticultural and economic crops worldwide (Garcia-Mas et al., 2012). The latest data indicated that 29.6 million tonnes of melon were produced on 1.2 million hectares around the world (http://faostat3.fao.org). Powdery mildew (PM) is a globally catastrophic disease for melon. PM can decimate the foliage of plants, thereby decreasing the yield and quality of melon fruits. PM is often caused by *Podosphaera xanthii* (Px) or *Golovinomyces cichoracearum* (Gc). Px occurs more frequently in subtropical and tropical areas and in greenhouse crops, while Gc is common in temperate and cooler areas under field conditions (Ning et al., 2013; Sathishkumar et al., 2016). Many studies have implicated Px as responsible for the occurrence of melon PM in China (Cheng et al., 2011; Ning et al., 2013; Zhang et al., 2012). Chemical prevention is currently the main method applied to control this melon disease, but this strategy is time consuming and labor intensive. The development of resistant cultivars is more effective, economic, and environmentally friendly than chemical application. To allow the marker-assisted selection (MAS) of resistant and elite cultivars, it is important to investigate PM defense mechanisms in melon. In the interactions of melon with PM, disease resistance to perturb infection is generated through rapid activation of a multitude of defense reactions, which include obvious physical changes such as cell wall thickening, callose deposition, formation of cork layers, and accumulation of tyloses in secondary xylem vessels (Cohen et al., 1990; Kuzuya et al., 2006), as well as complicated biochemical responses, including the production of reactive oxygen species (ROS) or signaling compounds such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) (Bostock, 2005). These reactions also include *de novo* synthesis of various pathogenesis-related (PR) proteins and accumulation of secondary metabolites with anti-biotic properties, such as phytoalexins and phenolics (Daayf et al., 1997; Ge et al., 2013). However, there is limited information on the genes related to these PM defense reactions in melon. Most of the disease resistance genes/quantitative trait loci (QTLs) associated with the response to PM in melon have been clustered into chromosomes/linkage groups (LGs) 2, 5, and 12, such as *Pm–1 ~ 6* (Cohen et al., 1990; Dogimont, 2011); *Pm-R/W/X/Y* (Perin et al., 2002; Pitrat, 1991; Yuste-Lisbona et al., 2011; Yuste-Lisbona et al., 2010); *Pm-x1,3,5* (Fazza et al., 2013); *Pm-Edisto47-1/2* (Ning et al., 2013); *Pm-2F* (Zhang et al., 2012); *Pm-pxA/B* (Fukino et al., 2008); *Pm-An* (Wang et al., 2011); *PmV.1*, *PmXII.1* (Perchepied et al.,

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http://dx.doi.org/10.1016/j.scienta.2017.09.033
Received 14 July 2017; Received in revised form 5 September 2017; Accepted 25 September 2017

2005); and *BPm12.1*(Li et al., 2017). Unfortunately, no resistance genes have been isolated from melon, and there is little available information regarding the genomic characteristics and expression patterns of these genes/QTLs involved in the defense response to PM in melon (Zhang et al., 2012). Gene expression profiling studies have shown that substantial changes in gene expression are associated with the resistance response to a variety of plant pathogen classes (Bolton, 2009). Comparative transcriptome analysis based on RNA-Seq has been one of the major tools for identifying genes that are differentially expressed in different states in a given tissue, and this method has been widely applied in research on plant-pathogen interactions in horticultural crops, such as tomato (Jonge et al., 2012) and cucumber (Li et al., 2016), but less so in melon.

MR-1 (resistant) and Topmark (susceptible) cultivars were selected for the present study. These cultivars have often been used as important resistant and susceptible hosts for the identification and analysis of physiological races of PM in previous studies (Kuzuya et al., 2006; Ning et al., 2013; Perchepied et al., 2005). We conducted comparative transcriptome profiling on the leaves of susceptible and resistant Px genotypes of melon at different time stages of Px infection to evaluate gene regulation at the transcriptome level and to identify differential changes in gene expression. The aims of this study were to explore two major questions: 1) what are the main discrepancies between susceptible and resistant genotypes in transcription-level responses to Px infection at different stages of Px infection; and 2) how does the genetic defense mechanism of resistance to Px infection differ between the two contrasting genotypes? Based on our bioinformatics analysis, we expect that the experimental results of this study may offer some new insights into the molecular defense mechanisms of the high resistance to Px infection in melon, which may help melon breeders explore new approaches for achieving more efficient MAS in melon and other closely related crops.

2. Materials and methods

2.1. Plant growth, Px strains and pathogen inoculation

MR-1 (PM-resistant genotype) and Topmark (PM-susceptible genotype) were grown in a plastic greenhouse at the Xiangfang farm (Northeast Agriculture University, Harbin, Heilongjiang, China) under a photoperiod of 16 h light/8 h dark and air temperatures of 28/18 °C (day/night). Approximately 100 Px strains were collected from the main melon-growing areas of this farm using the single sporangiophore transfer method. These strains were inoculated onto seedlings with two or three unfolded leaves in the 13 melon powdery mildew race international differential lines, and their virulence were evaluated according to standard protocols (Mccreight, 2006). The second to fourth unfolded leaves behind the stem tips were inoculated with the sporangial suspension of race '2F' at a concentration of 1×10^6/mL as previously described (Cohen et al., 1990). The leaves of MR-1 and Topmark plants inoculated with Px were harvested for observation at 0, 24, 72, and 168 h post-inoculation (hpi). Six infected leaves were collected from six independent melons and pooled as a single biological replicate at every inoculation stage. Two individual biological replicates for each treatment were sequenced.

2.2. RNA-Seq library construction and Illumina sequencing

Total RNA was extracted from leaves using the RNA plant Plus Reagent kit (TIANGEN, Beijing, China) reagent according to the manufacturer's instructions. The quality, quantity, and integrity of the total RNA were evaluated using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), the Qubit® RNA Assay Kit with a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA), and the RNA Nano 6000 Assay Kit with the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Briefly, 6 μg of RNA per sample was used as input material for the RNA sample preparations. Total RNA samples were treated with RNase-free DNaseI before poly-A RNA enrichment using poly-T oligo-attached magnetic beads. First-strand cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Then, we carried out cDNA library construction and cluster generation according to the manufacturer's instructions. The library preparations were sequenced on the Illumina HiSeq™ 2500 system at the Novogene Bioinformatics Institute in Tianjin, China. High-quality reads (clean reads) with lengths of 125 bp were obtained by removing low-quality reads with ambiguous nucleotides, and adaptor sequences were filtered from the raw reads. The raw sequence reads are available for download from the NCBI sequence read archive database (Accession number: SRX2442184 and SRX2442273)

2.3. Reads mapping and data analysis

The quality of the raw sequenced reads from all samples was checked using FastQC v0.11.2. Then, all clean reads from each sample were mapped to the melon reference genome (DHL92, https://melonomics.net/files/Genome/Melon_genome_v3.5.1/) using the default setting of TopHat v2.0.11 (Trapnell et al., 2012). Uniquely mapped reads for each specific transcript were counted in HTSeq v0.6.1 software (Anders et al., 2015) and normalized with the edgeR package v2.6.0 (Robinson et al., 2010). Pearson's correlation coefficients between the independent biological replicates for each sample were calculated and demonstrated using R package (pheatmap). Differentially expressed genes (DEGs) were identified using edgeR. Absolute values of log2 (fold change) > 1 and false discovery rate (FDR) < 0.01 were the criteria for defining DEGs. To investigate the function of the DEGs, the Gene Ontology (GO) enrichment of these DEGs was analyzed with Blast2GO v4.0 (Fisher, P-value < 0.05) (Conesa et al., 2005). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs was performed with the web bioinformatic tool KOBAS v2.0 (hypergeometric test, P-value < 0.05) (Wu et al., 2006). Transcription factors (TFs) under PM stress were identified and classified into families based on the sequence similarity in the plant TF database (PlantTFcat) (Dai et al., 2013). Clustering analysis of the DEGs was computed with Short Time-Series Expression Miner (STEM) using default parameters (STEM Clustering Method) (Ernst and Bar-Joseph, 2006).

2.4. Validation of quantitative reverse-transcription PCR

Quantitative reverse-transcription PCR (qRT-PCR) analysis was performed for ten putative disease-resistant candidate genes chosen from the DEGs according to functional annotation to validate the RNA-seq. The specific primers for these genes, listed in Table S1, were designed with Primer Premier v6.0 software. For primer design, small amplified fragments (80–200 bp) within the first third of the cDNA sequences were chosen. Whenever possible, the forward and reverse primers bound to different exons, and the reverse primer was designed to hybridize with two consecutive exons to avoid amplification of genomic DNA.

A total of 1 μg of RNA was reverse transcribed for first-strand cDNA synthesis using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Then, 20-μL reactions were prepared containing 10 μL of SYBR Green Master mix (TOYOBO, OSAKA, JAPAN), 1 μL of each primer pair, and 1 μL of the cDNA template. PCR amplification of target genes was carried out in 96-well optical reaction plates in an iQ5 Gradient Real Time PCR system (Bio-Rad, CA, USA). Two biological replicates and three technical replicates were performed for each cultivar and ripening stage assayed. The thermal cycling program started with a step of 10 s at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, with a final melting curve analysis consisting of ramping from 55 °C to 95 °C with a 0.5 °C increase