Scientia Horticulturae 238 (2018) 1–6

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Lignification plays an important role on resistance to root-knot nematode (*Meloidogyne incognita*) based on contrastive analysis in peach

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ARTICLE INFO

Keywords:
Root-knot nematode
Resistance
Lignification
GT-1
Peach

ABSTRACT

Root-knot nematode (RKN) is a serious global pest in peach cultivation; it establishes and maintains a permanent feeding site within plant roots. To elucidate the host response to RKN infection in peach, we conducted a comparative histological and transcriptome analysis during RKN invasion in two genotypes of peach–'Honggengansutao' (resistant to *Meloidogyne incognita*) and 'Bailey' (susceptible genotype). Roots of the two genotypes infested with nematodes for 0, 12, 36 and 84 h were taken as samples. Histological analysis showed that cells concentrated with RKN were lignified. Gene ontology analysis revealed a higher percentage of differential expressed genes enriched for "catalytic" and "metabolic process". Therefore, genes with catalytic functions in lignin biosynthesis were focused on. Filtering of genes with no obvious differences compared to control revealed the resistant cultivar responded to RKN infection earlier than the susceptible one; two genes involved in upstream of lignin biosynthesis pathway were up-regulated and an additional GT-1 cis-element which can activate transcription in vivo was found in each promoter of these two genes only in the resistant cultivar. These results form a basis for understanding the mechanism of RKN resistance in peach and other plants.

1. Introduction

Root-knot nematodes (RKNs), *Meloidogyne* spp., are sedentary endoparasites of many plant species; they interact with their hosts in a subtle manner. First, the second-stage juveniles (J_2) penetrate the root, migrate down the plant cortex toward the root tip, enter the base of the vascular cylinder, and migrate up the root (Wyss et al., 1992). These obligate biotrophic pathogens establish a permanent feeding site in the differentiation zone of the root by inducing nuclear division without cytokinesis in the host cells, resulting in the formation of galls (or root-knots) as well as the development of specialized feeding cells, called "giant cells" (Caillaud et al., 2008).

Peach (*Prunus persica* L.) is one of the most important and widely cultivated stone fruit grown worldwide (Eldem et al., 2012). The global annual production of peaches and nectarines exceeded 19 million metric tons from 2010 to 2013, according to Food and Agriculture Organization (FAO) statistics (FAOSTAT, http://faostat.fao.org). For many years, RKN infection has been one of the serious problems for peach growers and nurserymen in most areas having tropical and Mediterranean climates (Lamberti, 1979). One of the most economical and environmentally sound methods for managing RKN in *Prunus* spp. is the use of RKN-resistant rootstock cultivars (Fernández et al., 1994). In recent years, many rootstocks that showed different levels of resistance to RKNs were bred, such as 'Nemared', 'Juseitou', 'Shalil', 'GF305', and Myrobalan plum (Kole, 2007). 'Honggengansutao' (*P. kansuensis*) was identified as an excellent cultivar by our laboratory; it is resistant to *Meloidogyne incognita* (Cao et al., 2011).

Previous studies mostly focused on the RKN resistance genes, e.g., the tomato *Mi*, *Mi-3*, and *Mi-9* genes (Ammiraju et al., 2003; Kaloshian et al., 1998; Yaghoobi et al., 1995), the pepper *Me3* gene (Djian-Caporalino et al., 2001), the peanut *Mae* and *Mag* genes (Garcia et al., 1996), the hot pepper *CaMi* gene (Chen et al., 2007), the cotton *rkn1* gene (Wang et al., 2006), and the peanut *Rma* gene (Nagy et al., 2010). In peach, Lu et al. developed and characterized a sequence tagged site marker, EAA/MCAT10, which is linked to RKN resistance (Lu et al., 1999). Sosinski et al. reported a simple sequence repeat marker, pchgms1, linked to the nematode resistance loci Mi and Mij of 'Nemared' (Sosinski et al., 2000). Four sequence tagged site markers, tightly linked to the resistance genes Mia/mia and Mja/mja of 'Juseitou', were successfully developed (Yamamoto and Hayashi, 2002).

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https://doi.org/10.1016/j.scienta.2018.03.036
Received 9 December 2017; Received in revised form 14 March 2018; Accepted 16 March 2018

The peach genes R_{Mia557} and R_{MiaNem}, carried by two priori unrelated resistance sources, were co-localized in a subtelomeric position on linkage group 2 (Claverie et al., 2004). An R gene (*PkMi*), including the known RKN-resistance *RMia* gene, has been characterized from *P. kansuensis* 'Honggengansutao' (Cao et al., 2011). The resistance of 'Honggengansutao' is attributed to a single dominant gene located in linkage group 2, as revealed by linkage analysis (Cao et al., 2014). However, the process of RKN resistance is complex, which contributed by many factors (e.g. R genes and metabolic pathways). R genes are widely studied, whereas the metabolic pathways involved in overcoming RKN infection are rarely manipulated.

This study aimed to identify metabolic pathways which exert an important influence on RKN resistance. A comparative histological and transcriptome analysis was performed in two peach genotypes with contrast ability of RKN resistance at three time points after infection. Histological results provided a hypothesis that lignification participates in RKN resistance. Gene ontology (GO) analysis displays a large number of differentially expressed genes (DEGs) relevant to "catalytic" and "metabolic process" are enriched. Based on the results above, expression analysis of genes involved in lignin biosynthesis in two genotypes was carried out, which presents more evidences for the hypothesis and provide new insights into RKN resistance.

2. Materials and methods

2.1. Nematode culture

M. incognita was cultured on tomato in a greenhouse under conditions described by Cao et al. (2011). Nematode eggs were collected by root bleaching and concentrated with 36% (w/v) sucrose flotation. Next, the eggs were hatched in sterile water at 25 °C, with 24 h dark for 3 days. The J_2 nematodes were separated from the concentrated fluid by discarding the supernatant with gentle centrifugation (1208 × *g*) for the following assays.

2.2. Nematode infection and sampling

Nematode infection experiments on the two peach genotypes, 'Honggengansutao' (resistant to *M. incognita*) and 'Bailey' (susceptible one), were performed according to Wang et al. (2009). Six-well tissue culture plates containing 5 mL of 23% (w/v) Pluronic F-127 (Sigma-Aldrich, St. Louis, MO, USA) and 500 J_2 nematodes or H_2O (control) were prepared. One 10-day-old peach seedling was added to each well. The incubation condition of the plates was 25 °C, 70% humidity, and a moderate photoperiod (12 h light:12 h dark).

Samples of root tips were collected from three seedlings at each time point (0, 12, 36 and 84 h post-inoculation), which were used for stereoscopic observation and transcriptome analysis.

2.3. Paraffin sectioning and stereoscopic observation

Samples of root tips were collected from three seedlings at 0, 12, 36 and 84 h post-inoculation (hpi). Next, 1.5 cm of the root tips were cut, fixed in formaline acetic acid, and then dehydrated successively in 30%, 50%, 70%, 80%, 90%, 95%, and 100% (v/v) ethanol. After clearing in xylene, the root fragments were embedded in paraffin. A microtome (YD-1508B, Zhejiang, China) was used to obtain 12 μm paraffin sections that were adhered on glass slides. The paraffin sections were dried at 42 °C, stained with safranine and fast green, sealed with neutral gum, and then observed and photographed using a microscope (OLYMPUS DP71, Tokyo, Japan).

2.4. RNA isolation and mRNA sequencing

Total RNA was extracted from the root tip samples by using an extraction kit (Aidlab, Beijing, China) and treated with RNase-free DNase I (Takara, Dalian, China). The total RNA content was quantified using an ND-1000 spectrophotometer (Thermo, Waltham, MA, USA). Oligo (dT)25 magnetic beads were used to isolate poly-(A) tail-containing mRNAs from the total RNA (20 μg), and then fragmentation buffer was added to obtain short mRNA fragments for 5 min at 70 °C. These short fragments were used as templates to synthesize first-strand cDNA by using reverse transcriptase and random hexamer primers. Second-strand cDNA fragments were obtained using a buffer containing DNA polymerase I, dNTPs, and RNase H. The cDNA library was obtained by ligating the cDNA fragments to sequencing adapters (Genomic DNA Sample Preparation Kit, Illumina, San Diego, CA, USA). Thereafter, cDNA fragments with a size of about 350 bp were gel-purified and used as templates in polymerase chain reaction (PCR) amplification (Illumina Genomic Sample Preparation Kit). Finally, the mRNA sequencing was performed using an Illumina HiSeq 2000 platform (Macrogen Bioinformatics Technology Co., Ltd., Shenzhen, China).

2.5. Transcript assembly and DEG analysis

Raw reads were filtered to remove those containing adapter and reads with more than 5% unknown nucleotides. Low quality reads were also remove, in which the percentage of low Q-value (≤10) base was more than 20%. Clean reads were mapped to the *P. persica* genome v.1.0 (http://www.rosaceae.org/node/355) by using programs Tophat, Bowtie, and BWA (Langmead et al., 2009; Li and Durbin, 2009; Trapnell et al., 2009).

HTSeq (Anders et al., 2015) was used to count the number of reads mapped to reference transcripts. Cufflinks (http://cufflinks.cbcb.umd.edu/) and RSEM (Li and Dewey, 2011) were used to calculate the reads per kilobase per million mapped reads and normalize values. DEGseq package was used to identify the DEGs between treated samples (12, 36 and 84 hpi) and control (0 h) with *P* values of < 0.05 and fold change of > 2 or ≤ 2 (Anders and Huber, 2010).

AgriGO (http://bioinfo.cau.edu.cn/agriGO/) was used to identify the GO annotation of DEGs. WEGO software (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) was used to perform GO functional classification.

2.6. Validation by qRT-PCR

The results from the RNA-seq experiment were validated by analyzing 5 DEGs from lignin biosynthesis pathway using qRT-PCR with 'Honggengansutao' cDNA as template. The gene-specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Supplementary Table 1. PCR was performed using the Roche Light Cycler 480 (Roche, Basel, Switzerland) with the following cycling conditions: 7 s at 95 °C, 10 s at 57 °C, and 15 s at 72 °C. RNA polymerase II was used as a housekeeping gene (Tong et al., 2009). Relative transcript levels for each sample were obtained using the comparative Ct method (Livak and Schmittgen, 2001).

2.7. Sequencing of the promoters of two candidate genes

Three gene-specific primers for each genes' promoter were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Supplementary Table 1. PCR productions were used for Sanger sequencing (Sangon Biotech Co., Ltd., shanghai, China).

3. Results

3.1. Structural changes of root tips of the resistant and susceptible cultivars

The results of paraffin section (Fig. 1) showed that the root tips were infected by RKN at 12 hpi when the structure of the root tips was still