Host Transcriptional Profiling at Early and Later Stages of the Compatible Interaction Between *Phaseolus vulgaris* and *Meloidogyne incognita*

Luciane Santini, Carla de Freitas Munhoz, Mauro Ferreira Bonfim Jr.,
Marcelo Mendes Brandão, Mário Massayuki Inomoto, and Maria Lucia Carneiro Vieira

First, second, and sixth authors: Departamento de Genética, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, Brazil; third author: Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, Brazil; Empresa Brasileira de Pesquisa Agropecuária, Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil; fourth author: Universidade Estadual de Campinas, Centro de Biologia Molecular e Engenharia Genética, Campinas, Brazil; and fifth author: Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, Brazil.

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ABSTRACT

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The root-knot nematode (*Meloidogyne incognita*) is one of most devastating pathogens that attack the common bean crop. Although there is evidence that some cultivars have race-specific resistance against *M. incognita*, these resistance sources have not proved effective, and nematodes are able to circumvent the host's defense system. We constructed RNA-seq based libraries and used a high-throughput sequencing platform to analyze the plant responses to *M. incognita*. Assessments were performed at 4 and 10 days after inoculation corresponding to the stages of nematode penetration and giant cell development, respectively. Large-scale transcript

mapping to the common bean reference genome (G19833) resulted in the identification of 27,195 unigenes. Of these, 797 host genes were found to be differentially expressed. The functional annotation results confirm the complex interplay between abiotic and biotic stress signaling pathways. High expression levels of the wounding-responsive genes were observed over the interaction. At early response, an overexpression of the N gene, a TIR-NBS-LRR resistance gene, was understood as a host attempt to overcome the pathogen attack. However, the repression of heat shock proteins resulted in a lack of reactive oxygen species accumulation and absence of a hypersensitive response. Furthermore, the host basal response was broken by the repression of the ethylene/jasmonate pathway later in the response, resulting in a continuous compatible process with consequent plant susceptibility.

Additional keywords: plant–pathogen interaction, RNA sequencing.

The common bean (*Phaseolus vulgaris*) is a crop of economic importance and social significance worldwide for boosting the food supply and is a good alternative for reducing hunger, especially in developing countries (Broughton et al. 2003; CIAT 2013). In Brazil, which is the third largest global producer and the second largest consumer (FAOSTAT 2013), it is also an important crop, and the growing season is almost year-round in diverse environments.

Meloidogyne incognita and *M. javanica* are devastating pathogens that attack the common bean crop affecting productivity (Di Vito et al. 2004; Santos et al. 2012). Nematodes account for 90% of losses in common bean production (Abawi et al. 2005) since they capture host nutrients rendering plants more susceptible to biotic and abiotic stresses (Caromel and Gebhardt 2011).

M. incognita is a sedentary endoparasite that requires a plant host to complete its life cycle. Second-stage infective juveniles (J₂) invade plant roots and move intercellular toward the root tip and make a U-turn into the vascular cylinder, where feeding sites or giant cells are developed (Kyndt et al. 2013). Each J₂ induces differentiation of five to seven cells of the root parenchyma, which begin to enlarge and become multinucleate, forming giant cells and galls (Abad et al. 2009; Jones and Goto 2011; Perry and Moens

2011). As the nematode feeds, the xylem vessels become disrupted and the roots cannot function normally (Gosling 2005).

Despite its importance to agriculture, selection for improving plant nematode resistance is a complex procedure, particularly in tropical regions because of the large number of races and nematode species occurring in tropical soils, making it difficult to identify resistant plant varieties (Starr et al. 2002). Furthermore, plant resistance to nematodes is usually found in wild taxa and identifying resistance and its introgression into commercial varieties is a time-consuming and expensive process (Caromel and Gebhardt 2011). Some common bean genotypes have been considered resistant to *M. incognita*, and partial resistance is apparently prevalent (Ferreira et al. 2010; Saka 1990; Santos et al. 2012). However, this resistance is race-specific and strongly influenced by the environment, especially high temperature (Agrios 2005; Mullin et al. 1991; Sydenham et al. 1997). Consequently, further evaluation of resistance does not result in confirmation, especially under dissimilar environmental conditions involving different nematode populations.

Several studies have led to the identification of parasitism genes and effectors that modulate plant cell functions and promote infection (reviewed by Quentin et al. 2013), and revealed that the establishment of feeding cells is the result of extensive changes in gene expression in the infected root cells. Over the last two decades a number of studies have been done to clarify the molecular mechanisms involved in plant response to sedentary nematode infection (reviewed in Kyndt et al. 2013; Li et al. 2015). Special efforts were made to identify plant genes expressed upon nematode infection using, for instance, microarray (Barcala et al. 2010; Jammes et al. 2005) and RNA-seq assays (Beneventi et al. 2013; Ji et al. 2013; Xu et al. 2012).

Corresponding author: M. L. C. Vieira; E-mail address: mlcvieir@usp.br

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Although some aspects of the plant–nematode interaction have been elucidated in well-studied species, many questions remain to be answered about host gene expression changes that result in a compatible reaction, mainly with regard to tropical crops. In particular, the transcriptome of *P. vulgaris* expressed in response to *M. incognita* attack remains unidentified, especially when the RNA-seq technique is used. This study presents valuable information about common bean genes involved in responding to this pathogen infection, comparing the global transcriptome from infected roots with the transcriptome from uninfected plant roots.

MATERIALS AND METHODS

Plant material and nematode inoculation. The common bean cultivar IPR Saracura, released by the Instituto Agrônômico do Paraná, Brazil (IAPAR 2004), was used in this study. Seeds were sown in moistened Germitest (CIAL, Paulínia, Brazil) paper rolls and kept for 3 days in the dark at 28°C in a Dual Program Illuminated Incubator 818 (Precision Scientific, Chicago, IL). After germination, plantlets were transferred to plastic grow bags (1 liter) containing an autoclaved (120°C, 2 h) soil mixture (73% sand, 20% clay, and 7% silt). Four days later, the plantlets were inoculated based on the following protocol.

First, a nematode population of *M. incognita* (race 3) collected from a cotton field in western-central Brazil (15°33'60"S 55°10'08"W) was used to isolate nematodes from plant roots using the Coolen and D'Herde (1972) method. Then successive multiplication cycles were performed in order to obtain an inoculum bank, alternately kept in susceptible cotton (*Gossypium hirsutum* 'Fibermax 966', Bayer CropSciences, Lubbock, TX), carrot (*Daucus carota* 'Brasília RL', Sakata, Bragança Paulista, Brazil), and tomato (*Solanum lycopersicum* 'Santa Clara VF5600', Sakata) plants. These plants were kept in the greenhouse, in pots (2 liter) containing the same autoclaved soil mixture, to which an aqueous suspension containing eggs and J₂ nematodes was added.

Subsequently, roots from six tomato plants were processed for 1 min in a blender containing 1% (vol/vol) commercial NaOCl solution. The solution was filtered and the processed roots collected from the 60/500 mesh sieves, and rinsed in tap water. Next, a modified Baermann technique was used for nematode (J₂) extraction (Hooper 1986). The inoculum was quantified under light microscopy, using a counting slide. The number of nematodes was counted twice to calculate an average expressed per a given volume (J₂ ml⁻¹). Each plant was inoculated with an aqueous suspension containing 1,000 J₂, distributed in two holes (20 and 40 mm deep) opened in soil (7 mm in diameter). After inoculation, they were covered with vermiculite to prevent the holes drying up.

Nematode development in common bean roots. A histological analysis was conducted with the aim of identifying appropriate time points for studying plant gene expression in response to nematode infection. *M. incognita* development in 'IPR Saracura' roots was evaluated at eight time points: 1, 2, 4, 10, 16, 22, 30, and 36 days after inoculation (DAI). The experimental design was entirely randomized with four replicates per time point, giving a total of 32 plants (or plots) kept in the greenhouse. Plant roots were collected, stained with acid fuchsin (Byrd et al. 1983), examined under optical microscopy (Olympus BX50, Olympus, Hamburg, Germany), and photographed with an OPTIKAM B3 digital camera (Optika SRL, Bergamo, Italy).

Transcriptome assay and estimation of nematode reproduction factor. Based on the histological analysis results, two time points were chosen to perform the transcriptome assay, 4 and 10 DAI. This experiment was conducted in anti-aphid cage in an entirely randomized greenhouse arrangement and with three biological replicates of each treatment (inoculated and control), giving a total of 12 plants. The individual plants were harvested and the roots were washed in tap water and immersed in a 20% (vol/vol) commercial NaOCl solution (4 min). The NaOCl residue was

removed by surface washing and immersing the roots in water (15 min), leaving the plants intact to prevent tissue oxidation and RNA degradation. Finally, the roots were longitudinally divided into two portions. One half was frozen in liquid nitrogen for total RNA extraction and the other half was stained with acid fuchsin to verify the presence of nematodes in all biological replicates.

In order to estimate the nematode reproduction factor (RF), an additional experiment was carried out concomitantly with the one described above and under the same conditions. RF was estimated according to Oostenbrink (1966) at 68 DAI using a set of 10 common bean plants. Nematodes were extracted from all roots as described in Coolen and D'Herde (1972), observed under an optical microscope, and quantified using a counting slide. The number of nematodes was counted twice to calculate an average expressed per a given volume (J₂ ml⁻¹). Finally, the RF was calculated as follows: RF = final population/initial population. Another set of 10 plants was used to verify absence of contamination.

RNA extraction, library construction, and sequence analyses. Approximately 200 mg of frozen root tissue was macerated in liquid nitrogen and used for total RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The quality of the total RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the Agilent RNA 6000 nano assay protocol, considering the RNA integrity number (RIN). Only RNA samples that presented suitable quality scores (RIN > 8) were used for the RNA library preparation.

Subsequently, libraries were constructed following the Illumina manufacturer's protocol in the "TruSeq RNA sample preparation v2 low throughput (LT)" kit; a unique barcode was assigned to each biological sample (12). Paired-end sequencing was performed on the Illumina platform (MiSeq), generating 101-bp long reads. Six samples per lane of the flow cell were used. Sequencing data were examined based on the transcriptome analysis algorithms implemented in CLC Genomics Workbench V7.0.1 (CLC Bio, Aarhus, Denmark). Reads were analyzed by QC reports and low quality bases were removed using Quality Trimmer with the following settings: (i) quality score = 0.01; (ii) maximum number of ambiguous bases = 2; and (iii) minimum read length = 50 bp. To ensure that only high-quality sequence regions were selected, five bases from the 5' ends and one base from the 3' ends were eliminated from all reads.

Next, the transcripts from the 4 DAI and 10 DAI plant materials were recovered from the total number of reads by mapping to the complete genome of both *P. vulgaris* (Schmutz et al. 2014) and *M. incognita* (Abad et al. 2008) using the RNA-Seq analysis tool implemented in CLC Genomic Workbench. In particular, our reference was the Pvulgaris_218 genome sequence of the Andean common bean genotype G19833, available on the Phytozome comparative platform (<ftp://ftp.jgi-psf.org/pub/compugen/phytozome/v9.0/Pvulgaris>). For *M. incognita*, our reference was m_incognita.PRJEA28837.WS241, available in WormBase (http://www.wormbase.org/species/m_incognita#10-10). The settings were as follows: alignment penalties of mismatch: 2, insertion: 3, deletion: 3, minimal fraction of length: 0.90, minimum similarity fraction: 0.95, maximum number of hits per read: 10, and automatic paired distance estimation. Next, the number of host transcripts mapped per kilobase of exon model per million mapped reads (RPKM) was computed.

Using the Set Up Experiment tool, we defined the groups to be compared, i.e., inoculated transcripts and respective controls captured at both time points (4 and 10 DAI). Then, we performed the empirical analysis of differential gene expression based on the exact testing procedure (Robinson and Smyth 2008), which takes a negative binomial distribution in order to search for and generate a list of differentially expressed genes.

Absolute fold-change (FC) cutoffs of ≥2 and *P* values adjusted for multiple testing using a false discovery rate (FDR) (Benjamini and Hochberg 1995) correction for a given significance level of $\alpha \leq 0.05$ were used to check the significance of differential gene expression.

Finally, the significant expression values were converted at $\log_2$ FC scale using the R software (R Core Team 2012).

Quantitative gene expression analysis by real-time polymerase chain reaction (PCR). A subset of 15 differentially expressed genes was selected to perform a relative quantification by real-time PCR, according to Munhoz et al. (2015). Briefly, primers were manually designed and primer quality verified based on Gene Runner parameters (<http://www.generunner.net/>) as follows: 100 to 200 bp as the ideal amplicon size range; primer length from 18 to 22 bases with a 40 to 60% GC content, absence of secondary structures (such as dimers and hairpins), and melting temperature from 58 to 62°C (Table 1).

Initially, the total RNA was treated with DNase (Promega, Madison, WI) and ss-cDNA was synthesized from 1 µg of RNA. The reaction mixture contained 1× of M-MLV reverse transcription (RT) buffer, 0.6 mM of each dNTP, 25 U of RNasin (Promega), 200 U of M-MLV RT (Promega), 0.25 µM of primer CDS (5' AAG CAG TGG TAT CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT TTN V3'). DEPC-treated water (0.01%) was added to a final volume of 25 µl, and the reaction was incubated at 42°C for 1 h.

Subsequently, the 12 biological samples (three inoculated and three control plants for each time point) were amplified by quantitative PCR (qPCR), using technical triplicates. Amplifications were performed on a StepOne Real-Time PCR System (ABI, CA). The reaction mix contained 2.5 μ l of ss-cDNA 20 $\times$ diluted, 5.1 μ l of GoTaq qPCR Master mix (Promega), 0.3 μ M of each primer, and water was added to a final volume of 10 μ l. Cycling conditions were as follows: 95°C for 5 min; 40 cycles of 95°C for 5 s and 60°C for 1 min. Reaction specificity was confirmed by obtaining the dissociation curve for every reaction. Four common

TABLE 1. Sequence of the forward (F) and reverse (R) primers designed from the common bean transcripts detected by RNA-seq approach and used in assays for quantifying gene expression by quantitative polymerase chain reaction (qPCR)

Gene	Primer	Primer sequence F (5'-3')	Primer sequence R (5'-3')	Amplicon length (bp)	Sequence ID	BLASTx description
HSP ^a	Ch1HSP	CGATGGCTGCTACACCTG	CACCCCTTCTTTTCTTCTTC	164	Phvul.001G039700	Heat shock protein
ERF ^a	Ch1ERF	GCAGAAATGGTGGTAAAAGG	CAAACATCTCCCGAAGCATC	170	Phvul.001G160200	Ethylene-responsive transcription factor 1b-like
EXPA ^a	Ch2EXPA	CGTATGTTTCTCCAGCCTTC	GTGTGGACTGCTACCTCTC	136	Phvul.002G004100	Expansin-like b1-like
PR ^a	Ch3PR	GAAAGGAAAGACAGTGTACG	GGAGGATCAGTTGTAAAGTTG	193	Phvul.003G109400	Pathogenesis-related protein
CLV3 ^a	Ch5CLV3	TAACCTCTCCAATCTTCCTC	CCAAACTCTTCACCCATCAT	132	Phvul.005G067900	Clavata3 esr -related protein 5-like
WRP ^a	Ch5WRPa	TCACTTCCCACTCTAAAACC	CATCAGATCATACACTCAGG	154	Phvul.005G087200	Wound-induced protein
WRP ^a	Ch5WRPb	TCTGCCTTGCTTCTCTTCTC	CTGTTGGAGTAGTGGAGACG	202	Phvul.005G108400	Wound-responsive family protein
UGT ^a	Ch6UGT	CGAAGGATCTTAGCAAGTTG	TTACCCCTACACACATCAC	190	Phvul.006G017400	UDP-Glycosyltransferase 85a5-like
Chi ^a	Ch6Chi	GATACCCACTGTTTCCACG	GTGTCCCTTTCCTGATGAG	147	Phvul.006G109000	Acidic endochitinase
Stig1 ^a	Ch6Stig1	GTCTCTCCGAGGAACAAGC	GTGGAAAAC TTCACACACTTC	132	Phvul.006G147200	Stigma-specific Stig1 family
MYB ^a	Ch7MYB	TCTCTGCCACCAACTCTAAG	TTCGCCGAGGAGGTTGACG	192	Phvul.007G211900	MYB transcription factor 76
N ^a	Ch8N	GTGGTAATATGCTTCCCTGG	TGTAGATCCCTCAGATATACG	150	Phvul.008G195000	TMV resistance protein n-like
WRKY ^a	Ch9WRKY	AGCAAATGGGGAGGGAAAAAC	GAACAACACAAATCAGCAGC	195	Phvul.009G137500	Probable WRKY transcription factor 53-like
BRN ^a	Ch11BRN	GATGAGAGTGTAAGAGAGAG	AGGGATAAACGAATGGGGTG	195	Phvul.011G003900	Protein bearskin1-like
POX ^a	Ch11POX	GCTTGGAAACCGAAGGAGAG	ATTGGCGTAACATAAGAGACC	133	Phvul.011G105900	Peroxidase 3-like
WOX4 ^b	Ch1WOX4	AAGTAGTAGGTGCGTGGAC	CTTTCTTCTTTGGTTGTGTCTG	204	Phvul.001G023600	Wuschel-related homeobox
TEF ^b	Ch5TEF	GGTTAGTTAAACGGAAGGAG	GGAGAAGTGAGCATGTTATG	151	Phvul.005G146900.2	Transcription elongation factor b
Fd ^b	Ch6Fd	AGAGGAAGAAGGCATTGACC	CAGAAGTGGGATAAGCAACG	167	Phvul.006G216600	Ferredoxin
P26S ^b	Ch10P26S	CTCAGTCAGGGGAAAATAAAG	CAGAAGTTCAGTGCTACTCC	107	Phvul.010G122200	26S protease regulatory subunit

^a Differentially expressed genes detected by RNA-seq approach.

^b Endogenous reference genes used to normalize relative gene expression quantification.

addition, BLASTx was used to align the unigene sequences against all plant databases embedded in the Mercator web tool (Lohse et al. 2014). Each transcript was assigned to a single BIN (the multiple BIN assignment was not selected) and a BLAST cutoff of 80 was chosen. Mapping files generated by Mercator were used to assign the unigene sequences to functional pathways in MapMan v.3.5.1R2 (Thimm et al. 2004; Usadel et al. 2005).

Finally, the differential expressed unigenes common to both time points were graphically represented by a hierarchically clustered heatmap. The $\log_2$ FC values were clustered using the McQuitty hierarchical clustering method (McQuitty 1966) implemented on the hclust package and the heatmap was produced by running the heatmap.2 function in the gplots package (Warnes et al. 2014), both in the R software suite (R Core Team 2012).

RESULTS

Nematode development in common bean roots. In the trial used to determine suitable time points for RNA isolation, an absence of nematodes was observed on the first and second day after inoculation. Mobile J_2 were found to have invaded root tissues at 4 DAI (Fig. 1A), and the establishment of feeding sites at 10 DAI

(Fig. 1B). From 10 to 30 DAI, the sedentary stage development (Fig. 1B to E) and the subsequent onset of a new infection cycle was seen at 36 DAI (Fig. 1F). We therefore chose to analyze the host transcriptional responses to *M. incognita* infection at two time points: 4 DAI and 10 DAI.

Transcriptome assay and estimation of nematode reproduction factor. In the trial used for library construction, the presence of nematodes was confirmed in all inoculated plant roots, and absence of nematodes in control plant roots. The average nematode reproduction factor was 22.6, confirming the compatibility between 'IPR Saracura' and *M. incognita*.

Library features. The Illumina platform produced a total of 595.08 million reads of 101 bp in length, including 288.81 million (48.5%) from 4 DAI samples and 306.27 million (51.5%) from 10 DAI samples (Table 2). Raw sequence data are available at NCBI BioProject database (<http://www.ncbi.nlm.nih.gov/bioproject>) under accession number PRJNA288189.

After trimming, the percentage of high-quality sequences in each treatment was 73% (inoculated roots) and 80% (controls). Approximately 327 million (327/595, 55.0%) RNA-seq reads were mapped to the genome of *P. vulgaris* as reference, and 27,195 unigenes were identified. In regard to *M. incognita*, $\leq 0.07\%$ reads

Fig. 1. Photomicrographs of the developmental stages of *Meloidogyne incognita* within the 'Saracura' common bean roots. **A**, Infecting juveniles (J_2) migrating to vascular cylinder (4 days after inoculation [DAI]); **B**, sausage-shaped juveniles initiating feeding sites (FS) (10 DAI); **C**, sausage-shaped juvenile feeding itself and galling (G) occurrence as a response to parasitism (16 DAI); **D**, young female (22 DAI); **E**, adult female (30 DAI); and **F**, adult female producing egg masses (EM) and the onset of a new infection cycle (arrow indicates a J_2) (36 DAI). Bars = 100 μ m. N, nematode.

(Table 2) were mapped to its reference genome (2,929 reads from 4 DAI and 32,691 from 10 DAI samples). For this reason, only the common bean transcriptome was subsequently taken into account.

We were able to identify 797 differentially expressed genes, 508 at 4 DAI, 210 at 10 DAI, and 79 at both time points (based on $\log_2 FC \geq 1$) (Supplementary Table S1). As shown in Figure 1, the first time point correlates with nematode penetration and the second corresponds to giant cell development. Compared with the control, in the early period of interaction, 293 genes were found to be up-regulated and 294 down-regulated. The most up-regulated was a *bearskin1-like* (BRN) gene (Phvul.011G003900), identified in *Arabidopsis* as a gene related to root cap maturation (Bennett et al. 2010). On the other hand, the most down-regulated gene was the stigma-specific *Stig1* (Phvul.006G147200), whose function is unknown. As far as we know, this is the first time that *bearskin1-like* and *Stig1* have been identified among the genes involved in plant response to nematode infection. In the late period of the interaction, 117 genes were found up-regulated and 172 down-regulated. At this time point (10 DAI), genes related to the defense response were the ones with the highest FC: a wound-responsive gene (Phvul.005G108400) was found highly up-regulated, and a peroxidase gene highly down-regulated (Phvul.011G105900).

The relative gene expression data using real-time qPCR was consistent with all the above-mentioned results; as previously reported for other plant–pathogen interactions (Camilios-Neto et al. 2014; Gao et al. 2013), our qPCR data showed a good correlation with the RNA-seq data. The Pearson's correlation coefficients between qPCR and RNA-seq data were 0.88 and 0.96 ($P < 0.0001$) for 4 and 10 DAI samples, respectively. Interestingly, the qPCR was more sensitive in detecting the expression level of these genes (Fig. 2). For instance, for the BRN gene, the FC values (P value ≤ 0.05) for RNA-seq and qPCR were 12.87 and 22.44, respectively.

Functional annotation. The BLASTx results showed that 27,043 unigenes (99.4%) were similar to plant proteins. As expected, the vast majority of the top-hits made by our sequences were to *P. vulgaris* (91.4%) and to other related plant species, including *Glycine max* (3.4%). Altogether 14,099 unigenes were assigned to ontological terms using the Blast2GO tool. Restricting the analysis to level 2, 43 GO terms were annotated: within the category biological process, the most frequent GO terms were metabolic process and cellular process; in the molecular function category, the most frequent terms were catalytic activity and binding; and in the cellular component category, cell, organelle, and membrane were the most abundant terms (Fig. 3).

The singular enrichment analysis results showed that 23 functional categories were relevant to the *P. vulgaris*–*M. incognita* interaction, including five common to both time points. At 4 DAI, we identified 14 functional categories, belonging to eight clusters according to REVIGO (Fig. 4A). The most gene-rich categories were metabolism (116 up-regulated and 79 down-regulated genes) and oxidation-reduction process (56 up-regulated and 15 down-regulated genes). One of the clusters exclusive to 4 DAI (shown in blue, Fig. 4A) and composed of four categories is related to cell wall metabolism, reflecting the phase of nematode penetration. At 10 DAI, we also identified 14 functional categories, but they were grouped into five clusters (Fig. 4B). The oxidation-reduction process was the most

gene-enriched category (7 up-regulated and 35 down-regulated). Only one of the clusters, composed of seven categories, is exclusive to 10 DAI (shown in purple, Fig. 4B), and is related to DNA conformation change, probably reflecting the phase of host cell endo-reduplication initiated by nematode feeding site establishment (de Almeida Engler and Gheysen 2012).

Based on the results of gene classification into MapMan hierarchical categories (BINs), 797 genes were assigned to 29 BINs, including 199 genes classified as unknown proteins.

At 4 DAI, the genes were assigned to 26 BINs (Fig. 5A). Five BINs contained only genes that were up-regulated (fermentation, major carbohydrates, glycolysis, metal handling, and redox) and seven BINs contained only down-regulated genes (photosynthesis, minor carbohydrates, OPP cycle, TCA/organic acid transformations, cofactor and vitamin metabolism, biodegradation of xenobiotics and cell). Focusing on plant defense, 22% of the genes (175/797) were assigned to 10 BINs, chiefly hormone signaling (27/175), transcription factors (21/175), cell wall (29/175), and oxidative stress (11/175) (Fig. 6, Supplementary Table S2). In regard to hormone signaling, we observed a predominance of genes related to ethylene (ET) (five up- and eight down-regulated) and jasmonic acid (JA) biosynthesis (six up- and two down-regulated). Four auxin-related genes were differentially expressed, three of them (Phvul.006G101300, Phvul.008G001500, and Phvul.003G139600) were up- and one (Phvul.006G116100) was down-regulated. Moreover, a brassinosteroid-related gene, cytochrome P450 (Phvul.010G053100), was found to be up-regulated. Of the transcription factors, 17 (10 MYB, 5 WRKY, and 2 AP2/EREBP) were down-regulated and four (1 MYB, 2 WRKY, and 1 AP2/EREBP) up-regulated. In regard to cell wall, a cellulose synthase (Phvul.005G001000), an alpha-1,4-glucan-protein synthase (Phvul.004G041600), four expansins (Phvul.001G003700, Phvul.009G019000, Phvul.007G207600, and Phvul.002G083600), and a xyloglucan endotransglycosylase-related protein (Phvul.011G085200) were up-regulated. Conversely, a cellulose synthase-like (Phvul.006G058700), an expansin (Phvul.008G232200), an expansin-like B1 precursor (Phvul.002G004100), and four xyloglucan endotransglycosylase/hydrolases (Phvul.003G147500, Phvul.009G233200, Phvul.003G147400, and Phvul.003G052400) were down-regulated. In addition, a glycosyl hydrolase (Phvul.008G081200) and pectin lyase-like (Phvul.008G123000) were induced and a rhamnogalacturonate lyase (Phvul.003G287200) and a polygalacturonase precursor (Phvul.005G022500) were repressed. Six genes encoding pectinesterases were differentially expressed, four of them (Phvul.007G206300, Phvul.010G079900, Phvul.011G188900, and Phvul.002G270900) were up-regulated and two (Phvul.003G015600 and Phvul.005G011900) were down-regulated. Finally, concerning oxidative stress, we identified eight genes encoding peroxidases (seven up- and one down-regulated), as well as a thioredoxin-like (Phvul.009G186300) up-regulated and a glutathione-S-transferase gene (Phvul.006G079600) down-regulated. It is interesting to note that at this time point, there were transcriptional changes in defense- and cell wall-related pathways, which could lead to cell wall alterations, probably activated by the host response. Furthermore, an up-regulated gene, TIR-NBS-LRR (Phvul.008G195000), was recognized and is similar to the R genes that provide resistance to *Tobacco mosaic virus* (Whitham et al. 1994).

TABLE 2. Total and average number of reads captured in the early (4 days after inoculation [DAI]) and late (10 DAI) time points and number of reads mapped to the respective reference genomes^a

		Total number of reads	Average number of reads	<i>Phaseolus vulgaris</i>		<i>Meloidogyne incognita</i>	
				Mapped reads	%	Mapped reads	%
Control	4 DAI	157,260,977	52,420,326 (9,633,585)	87,020,000	55.33	91	0.00
	10 DAI	168,804,531	56,268,177 (7,973,533)	80,190,000	47.50	123	0.00
Inoculated	4 DAI	131,562,681	43,854,227 (8,264,915)	79,160,000	60.18	2,482	0.00
	10 DAI	137,476,872	45,825,624 (15,072,813)	81,130,000	59.02	98,074	0.07

^a Data refer to three biological replicates with standard deviations shown in parenthesis.

Fig. 2. Relative expression levels of 15 common bean genes in response to nematode infection at **A**, 4 days after inoculation (DAI) and **B**, 10 DAI obtained from RNA-seq (dark gray/blue bars) and quantitative polymerase chain reaction (qPCR) (light gray/orange bars) data. At each time point, only the significantly changed genes are shown. Genes coding for Wuschel-related homeobox, transcription elongation factor, ferredoxin, and 26S protease regulatory subunit were used to normalize the expression values for each target gene. BRN, Bearskin1; UGT, UDP-glycosyltransferase; MYB, MYB transcription factor; EXPA, Expansin; WRP_a and WRP_b, wound-responsive proteins; WRKY, WRKY transcription factor; Stig1, stigma-specific gene family; HSP, heat shock protein; N, *Tobacco mosaic virus* resistance protein; POX, peroxidase; ERF, ethylene-responsive transcription factor; PR, pathogenesis-related protein; Chi, acidic endochitinase; and CLV3, Clavata3 esr-related protein. Note the consistency between RNA-seq and qPCR data.

At the time that corresponds to giant cell development (10 DAI), the genes were assigned to 22 BINs (Fig. 5B). Six BINs contained only up-regulated genes (signaling, nucleotide metabolism, cell, TCA/organic acid transformations, glycolysis, and minor carbohydrates) and two BINs down-regulated genes (biodegradation of xenobiotics and nitrogen assimilation). Two BINs (nucleotide metabolism and DNA) were found to be exclusive to this time point, reflecting host cell endo-reduplication. Focusing on plant defense, 11% (92/797) of the genes were assigned to the above-mentioned BINs, except beta glucanase (Fig. 6, Supplementary Table S3). In regard to hormone signaling, a predominance of down-regulated genes was observed (11/12). Expression levels were reduced for all genes related to ET biosynthesis and signaling, i.e., aminocyclopropane-1-carboxylic acid (ACC) oxidases (Phvul.007G135600, Phvul.008G213900, Phvul.008G213800, and Phvul.002G326600) and ethylene responsive factors (Phvul.001G160100 and Phvul.008G046400). Moreover, the genes for JA biosynthesis, three lipoxigenases (Phvul.010G135600, Phvul.010G135800, and Phvul.005G156800) and one allene oxide cyclase (Phvul.003G111500) were down-regulated. A brassinosteroid-related gene (Phvul.011G011800) was up-regulated and one abscisic acid-related gene (Phvul.001G193400) was down-regulated. Expression of WRKY transcription factors (Phvul.009G087400 and Phvul.002G160100) was induced and an MYB transcription factor (Phvul.004G116500) was down-regulated. In regard to the cell wall, an expansin-like B1 precursor (Phvul.002G004100), a xyloglucan endotransglycosylase/hydrolase (Phvul.003G147700), a rhamnogalacturonate lyase (Phvul.003G287200), a polygalacturonase precursor (Phvul.005G022500), and a pectin lyase-like (Phvul.003G015600) were up-regulated and two expansins (Phvul.006G077200 and Phvul.008G120700), a pectin methyl-esterase (Phvul.002G048700) and a polygalacturonase (Phvul.003G052000) were down-regulated. Related to oxidative stress, we found two up-regulated genes, a glutathione-synthase (Phvul.006G094500) and a glutathione-S-transferase gene (Phvul.006G079600) but also detected a reduction in the expression levels of five peroxidases, a peroxiredoxin (Phvul.001G186000), L-ascorbate oxidase precursor (Phvul.006G011700), and GDP-mannose 3,5-epimerase (Phvul.006G101600). Overall, a reduction in gene expression levels of certain categories was observed (Fig. 6), namely hormone signaling, oxidative stress, biotic stress, and secondary metabolism.

In regard to the set of genes differentially expressed in both time points (79), 15 showed continuous differential expression (Fig. 7), 14 were up-regulated and one of unknown function (Phvul.009G132200) was down-regulated. Most of the up-regulated genes (6/14) were wounding-responsive genes, reflecting host responses to nematode penetration. The remaining genes (64) showed an opposite expression pattern depending on the time point of the interaction. This applies in particular to genes assigned to the cell wall degradation and modification (down-regulated at 4 DAI, but up-regulated at 10 DAI) and hormone metabolism (up-regulated at 4 DAI, but down-regulated at 10 DAI).

DISCUSSION

The dynamic of the compatible interaction between *P. vulgaris* and *M. incognita* is illustrated in Figure 7 using gene expression profiles. In particular, modulated expression was noted for wounding-responsive genes that were induced at both time points, and genes involved in the hormonal signaling pathway that were induced at nematode penetration and movement into root cells (4 DAI) but inhibited at the giant cell development step (10 DAI). Furthermore, genes involved in cell wall degradations were induced at 10 DAI. All this evidence indicates that the pathogen was efficient at subverting the plant's defense mechanisms.

Modifications in plant cell structure and root development result in the generation of nematode feeding cells (giant cells), possibly as a result of an imbalance of hormones, including auxins (Gheysen

and Mitchum 2011; Gutierrez et al. 2009; Saini et al. 2013). We detected overexpression of auxin-related genes at the early stage of parasitism (4 DAI) but not at 10 DAI. Note that the first J₂s appeared in root tissues at 3 DAI (data not shown), thus our RNA-seq library contains transcripts accumulated over a period of 24 h. Conversely, Hutangura et al. (1999) reported an accumulation and transportation of auxin at the beginning of gall formation during the first hours of the interaction between *Trifolium repens* and *M. javanica*. One of the up-regulated transcripts we were able to identify at the nematode penetration stage was the auxin-induced PCNT115, a protein implicated in plant defense (Li et al. 2013; Margaria et al. 2013). A PCNT115 up-regulation was also detected in banana infected with *Fusarium oxysporum* (Li et al. 2013) and its expression increased according to the resistance level of the genotypes under evaluation.

The genes involved in ET biosynthesis and signaling were clearly affected by changes in the expression levels of genes encoding aminocyclopropane-1-carboxylic acid (ACC) synthase, ACC oxidase, and ERF. This hormone production in plant tissues is the result of the conversion of methionine to S-adenosylmethionine to ACC and finally to ET. At the early stage of infection (4 DAI), the repression of ACC synthase transcript affected the first step of ET production, and as time progressed, repression of biosynthesis was confirmed by the down-regulation of all ET-related transcripts (Fig. 6). Changes in the expression of ET-related genes have been frequently detected in plant-pathogen interaction analyses, though there is a lack of consensus on the association between the compatibility of the interaction and the level of expression of these genes (Van Loon et al. 2006). A transient increase in the expression level of ACC synthase and ACC oxidase was observed in both susceptible and resistant tomato plants 12 h after inoculation (HAI) under *M. incognita* attack (Mantelin et al. 2013). Herein, ERFs were found to be down-regulated at both time points. These transcription factors act as downstream regulators of ET-responsive genes, suggesting that a decrease in ethylene sensitivity and basal resistance occurred in the pathosystem under analysis. The reduction in sensitivity to this hormone did not affect tomatoes whose resistance to *M. incognita* was conferred by the *Mi-1* gene. However, basal resistance was affected when the interaction was compatible (Mantelin et al. 2013). Furthermore, ERFs are involved in the ET and JA pathway connection, which play an important role in determining whether an interaction is compatible (Grennan 2008; Lorenzo et al. 2003).

Changes in the JA pathway were evidenced by the differential expression of lipoxigenases, 12-oxophytodienoic acid reductases (OPR2 and OPR3-like), and allene oxide cyclase. In the initial stages of the interaction, the injuries caused by nematode infection induced the production of LOX, activating the primary step of JA biosynthesis, which was repressed as time progressed. The same phenomenon was detected for ET biosynthesis. The down-regulation of genes encoding enzymes OPR2 and OPR3-like that act in the second step of JA biosynthesis resulted in the subsequent down-regulation of all JA-related genes in the final stages of the interaction (Fig. 6).

We would speculate that ET/JA-dependent defenses against *M. incognita* are active in common bean, but the nematode is able to subvert the defense mechanisms in the course of the interaction. In rice, analyses involving JA mutants, ET-insensitive transgenics and JA-pharmacological inhibition have shown that the JA-pathway is essential to systemically induced defense against *M. graminicola* (Nahar et al. 2011). Despite the evidence that JA activates a succession of signaling pathways resulting in the activation of genes required for diverse functions (reviewed in Liu et al. 2015), including plant defense, intact JA-regulated signaling was identified as required for root-knot nematode (RKN) susceptibility in tomato (Bhattarai et al. 2008). Moreover, lipoxigenase family members have shown distinct functions in *Arabidopsis* response to *M. javanica*; LOX3 expression seems to increase susceptibility to this nematode, but LOX4 presents the opposite effect (Ozalvo et al. 2014). ET/JA and salicylic acid (SA) defense pathways were

activated in tomato infected by RKN in both, compatible and incompatible reaction, though these pathways were more induced in the incompatible one (Bhattarai et al. 2008). Conversely, JA may interact antagonistically with other hormones, such as SA. In this context, the activation of ET/JA pathways we detected at the earlier time point of interaction could have prevented SA pathway activation, leading to a compatible host response to *M. incognita*.

We detected several genes associated with the response to abiotic stress, namely the wounding-responsive genes that reached high levels of expression. At the onset of the interaction, the induction of these genes is the result of root penetration by the nematodes (wounding agents), possibly due to the fact that damage-inducible defenses are controlled by jasmonates that start to accumulate rapidly after a plant tissue is wounded. Consistent with this, Chauvin et al. (2013) sought to identify lipoxygenases that initiate wound-induced jasmonate synthesis within a short timeframe, especially LOX6 in response to long-distance wound signals in *A. thaliana*.

In turn, the induction of wounding-responsive genes altered the expression of hormone signaling pathway associating genes, as well as the expression of the transcriptional factors MYB and WRKY, genes that produce PR proteins and genes involved in generating reactive oxygen species (ROS) (Cao et al. 2006; Casalougué et al. 2012; Cheong 2002).

The WRKY transcription factors herein identified were previously reported in plant response to nematode infection (Ali et al. 2014; Bhattarai et al. 2008; Ibrahim et al. 2011). Exemplifying, the WRKY33 and WRKY70 were among the transcription factors repressed during root penetration by the nematode (4 DAI). Similarly, WRKY33 was repressed in *Arabidopsis*, favoring the

development of syncytia caused by *Heterodera schachtii* (Ali et al. 2014). However, the up-regulation of WRKY33 was reported in the tomato response to RKN, in compatible and incompatible reactions (Bhattarai et al. 2008). This transcriptional factor is involved in the synthesis of the phytoalexin camalexin as well as in the positive regulation of ET/JA-related genes (Alves et al. 2014; Seo et al. 2015). Transcription factor WRKY70, also involved in crosstalking between SA and ET/JA pathways (Derksen et al. 2013), has been identified as a key player in tomato resistance to potato aphid (*Macrosiphum euphorbiae*) and *M. javanica*, mediated by *Mi-1* gene (Atamian et al. 2012). In contrast, an up-regulation of WRKY70 was observed in soybean 10 weeks after *M. incognita* infection (Ibrahim et al. 2011).

An attempt by the common bean defense system to suppress the *M. incognita* attack was noted in terms of a TIR-NBS-LRR gene overexpression at 4 DAI (*N* gene, Fig. 2A). It has been suggested that this gene, homologous to the *N* gene, plays a role in the hypersensitive response to *Tobacco mosaic virus* (Whitham et al. 1994) and RKN in pepper (Brito et al. 2007; Kiewnick et al. 2009). We are aware that HSP90 suppression undoes the resistance to *Tobacco mosaic virus* conferred by the *N* gene, and to RKN conferred by the *Mi-1* gene (Bhattarai et al. 2007; Liu et al. 2004), a cloned *R* gene for RKN (Milligan et al. 1998; Vos et al. 1998). Changes in HSP expression could explain the loss of resistance in pepper against some RKN species under high temperatures (Brito et al. 2007; Kiewnick et al. 2009).

In regard to HSPs, we found contrasting gene expression responses for the two time points studied. All 14 HSP genes identified in early response were down-regulated, and only HSP70 and HSP83 genes were up-regulated at the later response. Differential expression profiles of HSPs were also detected in other plant–nematode

Fig. 3. Distribution of common bean unigenes herein identified, based on their functional annotations to the level 2 GO terms. The functional annotation was performed using Blast2GO software, in accordance with BLASTx results (e -value 1×10^{-6}).

interaction studies (Hirao et al. 2012; Szakasits et al. 2009). The HSP70 was up-regulated in incompatible reactions between soybean and *H. glycines*, suggesting its involvement in the cellular function maintenance under stress response (Klink et al. 2007). Furthermore, as already mentioned, HSPs have been identified as components of resistance mechanisms, such as the Hsp90-SGT1-Rar1 complex required for *M. incognita* resistance mediated by the *Mi-1* gene (Bhattarai et al. 2007). Similarly, we can assume that the *N* gene-mediated nematode resistance was suppressed in common bean by decreasing the rate of expression of HSPs, as confirmed by qPCR results (Fig. 2A).

Thereafter, a compatible reaction was established as time progressed, also leading to down-regulation of pathogenesis related proteins, e.g., dirigent-like proteins, a protease inhibitor, and an

acidic endochitinase, which in particular had its down-regulation confirmed by qPCR results. Note the bar graph relative to Chi gene in Figure 2B.

Several cell wall-related genes were found to have their expression profiles clearly modulated by nematode infection (Fig. 6). This was found for genes involved in the synthesis, degradation, and modification of the cell wall, triggered by nematode penetration at 4 DAI, as well as genes related to cell wall modification that were differentially expressed as a result of giant cell development and maintenance, i.e., expansins (EXP) and xyloglucan endo-transglycosylases (XTH) at 10 DAI.

Four expansin genes were up-regulated during J₂ migration in root tissues and auxin concentration heightening. Both J₂ migration and feeding site establishment are dependent on loosening and

Fig. 4. TreeMap visualization obtained from REVIGO clustering analysis based on semantic similarity measurement (SimRel) of the significant GO terms found in singular enrichment analysis (SEA) performed in AgriGO. SEA was done using the Fisher exact test (false discovery rate ≤ 0.1). Only the biological process category was considered and the list of common bean genes implemented on the AgriGO platform was used as reference. The two sets correspond to two time points at **A**, 4 days after inoculation (DAI) and **B**, 10 DAI. Rectangles were adjusted proportionally according to the clustering significances in $\log_{10}(P \text{ value})$. The number of genes is indicated in each of the clusters (distinctly colored) and the expression gene trends are shown by arrows pointing up and down.

degradation of the cell wall. On the other hand, a transcript similar to the *Arabidopsis* EXPA6 gene was herein continuously repressed. Though the induction of EXPA6 in *A. thaliana* infected with *M. javanica* was reported previously (Barcala et al. 2010), contrasting expansin expression profiles were identified in the syncytia transcriptome modulated by *H. schachtii* (Wieczorek et al. 2006). XTHs present important roles in plant response to nematode infection acting in cell wall reinforcement once the cell expansion is completed. Several members of the XTH family were identified in this study. XTR6 were down-regulated at 4 DAI as described in soybean tissues infected with *H. glycines* (Puthoff et al. 2007; Tucker et al. 2007), and XTR8 as well as TCH4 were up-regulated at 4 and 10 DAI, respectively. TCH4 was reported to be continuously

induced in *A. thaliana* infected with *H. schachtii* (Szakasits et al. 2009).

In molecular plant–pathogen interactions, ROS act against the pathogen itself or promote plant cell death, leading to a hypersensitive response. To defend themselves against the damage caused by ROS, cells produce antioxidants as peroxidases that act to convert ROS into nonreactive molecules. In our study, a peroxidase down-regulated and seven peroxidases up-regulated as well as a thioredoxin overexpressed were detected in the early stages of interaction, probably as a result of ROS production. Thioredoxins were found to be differentially expressed in *A. thaliana* syncytia modulated by *H. schachtii*, most of them were found to be induced (Szakasits et al. 2009). Interestingly, changes in the expression

Fig. 5. A MapMan display of common bean modulated genes ($\log_2$ fold-change ≥ 1.0 , false discovery rate ≤ 0.05) according to their assignment to functional categories (BINs). The two sets correspond to the time points at **A**, 4 days after inoculation (DAI) and **B**, 10 DAI. BINs colored green are significantly up-regulated, whereas those in red are down-regulated genes.

Fig. 6. Display of transcriptional changes in common bean defense-related genes in response to nematode infection. Differentially expressed genes in response to nematode penetration (4 days after inoculation [DAI]) and giant cell development (10 DAI). Only significantly changed genes ($\log_2$ fold-change ≥ 1.0 , false discovery rate ≤ 0.05) were visualized using MapMan software and organized into functional categories (BINs). Blue indicates an increase and red a decrease in gene expression (see color scale on right). Detailed information on each gene and its expression level can be found in Supplementary Tables S2 and S3.

levels of genes involved in resistance to oxidative stress were detected at 10 DAI, possibly due to a reduction in the presence of ROS typical of a compatible reaction. Similarly, ROS have been detected in tomato resistant and susceptible to *M. incognita*, but higher levels were found only to occur in the compatible reaction during the first 48 HAI (Melillo et al. 2006). In general, the repression of the oxidative process is associated with the mitigation

of cell damage, but the down-regulation of the associated genes may lead to a compatibility reaction to avoid a hypersensitive response. In conclusion, the present data lends weight to the argument that a compatible reaction in *P. vulgaris* to *M. incognita* infection is established early during the interaction. Despite the *N* gene expression triggered by the presence of the nematode, the repression of heat shock protein (required for *N*-mediated host resistance)

Fig. 7. Hierarchically clustered heatmap of common bean genes with differential expression ($\log_2$ fold-change [FC] ≥ 1.0 , false discovery rate ≤ 0.05) identified in both time point evaluations (4 and 10 days after inoculation [DAI]). The $\log_2$ FC values were clustered using the McQuitty hierarchical clustering method, implemented in the R software suite, producing the dendrogram shown on the left. Gradient colors indicate the changes in the expression level. Green indicates an increase and red indicates a decrease in gene expression (see color scale). Sequence IDs are shown on the right, together with their respective functional categories, according to the MapMan software.

results in a lack of ROS accumulation and absence of a hypersensitive response. Furthermore, the host basal response is broken by the repression of the ethylene/jasmonate pathway later in the response, culminating in the establishment of feeding cells, as well as pathogen development within the roots, and a continuous compatible process with consequent plant susceptibility.

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