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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Time-dependent effects of *Pochonia chlamydosporia* endophytism on gene expression profiles of colonized tomato roots

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Abstract

A transcriptome analysis was produced from tomato roots inoculated with the hyphomycete *Pochonia chlamydosporia* at three different times. Gene expression data were also yielded from fungus grown in vitro or endophytic. A next-generation sequencing (NGS) and network analysis approach were applied. We identified 3.676 differentially expressed tomato genes (DEG), highlighting a core of 93 transcripts commonly down- or upregulated at every time point, shedding light on endophytism process. Functional categories related to plant information-processing system, which recognizes, percepts, and transmits signals, were associated with gene upregulated early in time, with higher representations in processes such as plant defense regulation later in time. Network analysis of a DEG subset showed dominance of MAP kinase hubs in the uninoculated control samples, replaced by an increased centrality of WRKY transcription factor and ETR—ethylene response factor genes in the colonized roots. Fungus genes expressed during progression of plant colonization, therefore related to the host colonization process or endophytism persistence, were also identified. Data provided a high-resolution insight on tomato transcriptome changes as induced by endophytism, highlighting a specific modulation of stress-responsive transcripts, related to a selective activation of defense pathways, likely required by the fungus to establish a persistent endophytic lifestyle.

Keywords Colonization · Root endophytism · *Pochonia chlamydosporia* · Tomato · Transcriptome · Signaling · Plant defense · Transcription factor

Introduction

Soil microbial communities sustain plant nutrition and roots' health, underpinning soil fertility, plant growth, and regulation of many pests and pathogens. Their study is encouraged by the enormous number of species occurring in soil, of which only a small fraction has been characterized thus far. Consequently, many services provided by the rhizosphere microbiome are yet largely unexplored (Zuccaro et al. 2011; Hirsch and Mauchline 2012). Identifying molecular mechanisms of one or more components of the root microbiome may allow exploitation of species to sustain crops by improving rhizosphere conditions or regulating noxious organisms.

The hyphomycete *Pochonia chlamydosporia* has been isolated worldwide from soil or rhizosphere of cultivated plants, and from eggs of cyst and root-knot nematodes (RKN) (Kerry 2000; Manzanilla-López et al. 2013). The fungus plays a useful, functional role in the rhizosphere of plants, and is considered a beneficial species also susceptible of industrial exploitation. It has a tri-trophic behavior, including RKN parasitism, root endophytism, and saprotrophic metabolism, with a role in nematode biocontrol and plant growth promotion (Manzanilla-López et al. 2013; Larriba et al. 2014, 2015; Rosso et al. 2014; Zavala-Gonzalez et al. 2017). As a root endophyte, *P. chlamydosporia* has a broad range of colonized plants, including both mono- and dicotyledons (Kerry 2000;

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Manzanilla-López et al. 2013). Data on genes involved in nematode egg degradation by *P. chlamydosporia* and root interactions on healthy or nematode-parasitized plants have been made available (Escudero and Lopez-Llorca 2013; Larriba et al. 2014; Rosso et al. 2014).

The mechanisms of *P. chlamydosporia* transition among the various lifestyles, however, are still partially understood. Isolates exposed to different nutritional stresses (emulating saprophytism or nematode egg parasitism) showed marked transcriptional reprogramming between treatments, suggesting that special gene families and signal transduction events may be involved in multi-trophic lifestyle transitions (Rosso et al. 2011; Manzanilla-López et al. 2013; Larriba et al. 2014; Zavala-Gonzalez et al. 2017).

A number of secreted proteins is involved in the *P. chlamydosporia* responses to nutrient stress in artificial media, mainly comprised of proteases and glycoside hydrolases (Lin et al. 2018). Particularly, secreted glycosyl hydrolases appeared related to the endophytic processes (Lin et al. 2018). Data recently made available on *P. chlamydosporia–Arabidopsis* interactions showed that jasmonate signaling is an important factor to modulate progression of plant colonization by the fungus (Zavala-Gonzalez et al. 2017). During barley colonization, the fungus showed production of several proteases, hydrolases, and other transport proteins (Larriba et al. 2014). Moreover, *P. chlamydosporia* endophytism in tomato roots affected the expression of several host miRNA and their target genes involved in the plant information-processing system, whose products recognize, percept, and transmit signals, playing an important role in the fungus recognition as an endosymbiont (Pentimone et al. 2018).

The data on *P. chlamydosporia* metabolism made thus far available suggest an adaptation to the rhizosphere environment though multilateral relationships, which need, however, to be better deciphered at the molecular level. In this study, we analyzed the transcriptomic changes along time in tomato (*Solanum lycopersicum* L.) colonized roots, identifying genes differentially expressed for both organisms, to provide insights on the molecular interactions of the endophyte within the roots, and the associated metabolic changes.

Materials and methods

Test setup

Tomato (*S. lycopersicum* L.) cv. Regina seeds were washed in 5 mL of 0.1% Tween 20 for 20 min and surface sterilized in 5 mL of 2.5% NaOCl, rinsed 4–5 times in sterile distilled water (SDW) by gently shaking for 5 min each. The seeds were dried on autoclaved filter paper and then placed, for germination, on 1.5% water agar (WA) in dark at 26 °C. Assays were carried out in 750-mL glass bottles with 200-mL vermiculite and 150 mL SDW. The bottles were plugged with cotton and autoclaved twice for 20 min at 121 °C, prior to receiving a 10-day germinated plantlet each. Subsequently, 6 agar plugs (0.5 cm^2) from 1-week-old corn meal agar cultures of *P. chlamydosporia* isolate DSM 26985, were added for the colonization treatment (I) in the bottles, and were mixed up before transplanting. Plants without fungus (treatment PM) were used as controls. All plants were maintained up to 21 days at 26 °C with 16-h illumination in a growth chamber (Sanyo Electric MLR-351, Osaka, Japan). At assay termination, part of roots was surface sterilized with 1% NaOCl for 5 min under agitation, washed in SDW and cut in 1-cm fragments, and kept at 26 °C on WA for a week in that dark, to check emergence of *P. chlamydosporia* hyphae with light microscopy at × 50.

The *P. chlamydosporia* strain DSM 26985 used in this study is deposited at the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures) and also preserved at Mycotheca Universitatis Taurinensis (MUT, www.mut.unito.it) of the Department of Life Sciences and Systems Biology, University of Turin (Italy), with accession number MUT 6232.

RNA extraction, RNA-Seq library preparation, and sequencing

The roots were sampled during a time course at 4, 7, and 21 days post *P. chlamydosporia* inoculation (dpi). Similarly, extraction was performed from equivalent developmental stages of uninoculated plants, at the same sampling times. Each root sample, used for RNA-Seq analyses, proceeded from two plants. The tissues (100 mg) were powdered in liquid nitrogen. Total RNA was extracted and purified by using RNeasy Plant Mini Kit (Qiagen, Milan, Italy), following the manufacturer's protocol. RNA quantity and quality were determined with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). cDNA libraries were prepared from 4 μg total RNA using TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, California, USA) and validated according to Illumina's low-throughput protocol. After normalization, cDNA libraries were pooled for multiplexing before loading onto a flow cell (8–9 samples per lane). The hybridization and cluster generation were performed on a cBot System using TruSeq SR Cluster Kit v3. Sequencing was performed on Illumina HiScanSQ or HiSeq 2500 platforms by IGA Technology Services (Udine, Italy, www.igatechnology.com), using TruSeq SBS kit v3 (Illumina, Inc.) to obtain single reads 50–100 nt in length. Indexed raw sequencing reads from each library were de-multiplexed using the CASAVA v1.8 software (Illumina, Inc.). The experiment was repeated four times for the 21-dpi sampling time and twice for the 4- and 7-dpi assays.

RNA-Seq data analysis and differential gene expression quantification

The sequences produced have been deposited in the NCBI Sequence Read Archive (SRA), with accession number PRJNA531604. Row sequences were processed for quality check eliminating adapters and indexes as well as genomic sequences added during the sequencing process, using the "RNA-seq analysis" functions included in CLC Genomics Workbench software v. 10.1 (QIAGEN, Aarhus, Denmark, http://www.clcbio.com). Filtered reads from each sample were then separately aligned to the reference genome of *S. lycopersicum* (SL2.50.35, Sol Genomics Network, ftp://ftp.solgenomics.net) using CLC (similarity parameter = 0.8; identity parameter = 0.8; mismatch/insertion/deletion penalties = 2/3/3; multi-position matches allowed) and employed to quantify the abundance of all tomato transcripts, measured as the reads per kilobase per million mapped reads (RPKM) (Mortazavi et al. 2008), with RPKM ≥ 0.05 as cutoff for gene expression.

A multiple correlation test (Pearson's correlation) on RPKM values for all pairwise combinations was performed for preliminary batch comparisons of replicates and experimental conditions. Principal component analysis (PCA) was also performed to analyze the variation sources in the dataset.

Differential expression analysis was performed with the CLC statistical tools, applying "Empirical analysis of Digital Gene Expression in R" (EDGE), implemented in the EdgeR Bioconductor package (Robinson et al. 2010). The analysis estimates the gene-wise dispersions from a linear combination of the likelihood for the gene of interest and neighboring genes, with similar average expression levels. Fold changes (FCs) were calculated comparing the RPKM expression values for each sample group against a reference group (equivalent developmental stages of uninoculated roots) with the generalized linear model (GLM), which corrects for differences in library size between the samples, over-dispersion caused by biological variability, and the effects of confounding factors. Differential gene expressions (DEGs) were calculated at each time points between "treated" and "reference" conditions and considered significant when displayed at least a 2-fold FC (FC threshold ≥ 2 or ≤ − 2), with a p value ≤ 0.05. The differentially expressed genes were then submitted to functional analysis. The terms "upregulation" and "downregulation" were employed to indicate transcript expression levels for inoculated roots that were higher or lower than those observed in the non-inoculated controls (PM), respectively.

Functional analysis of tomato DEGs

Enrichment analysis detects functional categories of biological processes, molecular functions, and cellular component that are over-represented, with a statistical significance (Fisher's Exact Test: p value ≤ 0.05; FDR ≤ 0.05), in a gene subset (DEGs), using annotations for that gene set as compared with the remaining genes of the reference organism. Enrichment analysis was performed using the AgriGO device (http://bioinfo.cau.edu.cn/agriGO) (Du et al. 2010) in order to identify, in each DEG selected set, over-represented GO categories related to the endophyte–plant interactions. We used as a reference the *S. lycopersicum* cDNA library ver. 2.4 (ftp://ftp.solgenomics.net). Web-based tools provided by the KEGG Automatic Annotation Server (KAAS: http://www.genome.jp/kaas-bin/kaas_main) were used to predict involved biosynthetic pathways.

Network analysis

The transcripts per kilobase million (TPM) of subset (all times, TPM sum per transcript > 5) of gene (see below), mostly including transcription factors (TFs), were used to calculate Spearman's correlations among transcripts, in both conditions tested, using RStudio ver. 1.1.453 with package Hmisc (RStudio Team 2015; Harrell 2018). Two matrices were built using only positive coefficients ($p \leq 0.01$) including 419 transcript pairs for the samples inoculated with *P. chlamydosporia* and 436 for those not inoculated. Undirected network graphs were produced from both matrices with Gephi ver. 0.9.2 (Bastian et al. 2009), with transcripts as nodes and the correlation coefficient values as edge weights. The nodes' degree and betweenness were used for construction of two networks, applying the Fructherman Fruchterman and Reingold 1991 layout, with straight edges. The online tool InteractiVenn (http://www.interactivenn.net/index.html) (Heberle et al. 2015) was then used for counting and identifying transcripts in common or unique, for the two networks.

Validation of RNA-Seq analysis by RT-qPCR

RT-qPCR analysis was carried out to validate RNA-Seq results for six selected DEGs, at the three sampling times 4, 7, and 21 dpi. Total RNA extracted from two biological replicates was used. All primer pairs were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/) with the forward ones designed on the exon-junction sites of the target gene to amplify only cDNA and not possible contaminant genomic DNA (Supplementary Table S1). First-strand cDNA was synthesized from 0.1 μg of RNA using reverse transcriptase Super Script III (Invitrogen, Carlsbad, CA, USA) and random examers in a volume of 20 μL, according to the manufacturer's instructions. qPCR was performed in a Mx3000P Real-Time PCR Detection System (Stratagene, La Jolla, CA, USA) in a volume of 25 μL containing 12.5 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μM of each primer, and 1 μL of the reverse transcription reaction. The conditions for

amplification were as follows: 3-min denaturation at 95 °C followed by 35 cycles of 95 °C for 10 s, 52 °C for 30 s, and 72 °C for 10 s. The absence of unwanted products was assessed through melting curve from 60 to 95 °C with 10-s steps of 0.5 °C increments. All samples were analyzed in duplicate. Relative gene expression was calculated using MXpro QPCR Software (Stratagene, La Jolla, CA) and the 2-ΔΔCT method (Livak and Schmittgen 2001; Pfaffl et al. 2002) that uses Ct values to estimate the initial target DNA amount compared to the normalizer transcript (GenBank XM_004229002) and primers coding for a ubiquitin-40S ribosomal protein S27a, with a constant rate of expression.

Identification of *P. chlamydosporia* transcripts in roots

To discover *P. chlamydosporia* genes expressed during endophytism, the fungus reads were separated from those of *S. lycopersicum* by mapping the complete RNA-Seq data set of inoculated roots onto the sequence of SL.2.50.35 draft genome. Unmapped reads, potentially fungus-derived, were isolated and mapped to the *P. chlamydosporia* genome accession LSBJ02000000 (Lin et al. 2018) retrieved from DDBJ/ENA/GenBank, using the CLC utility as previously described. The RPKM were determined and used to estimate the *P. chlamydosporia* transcripts' abundance at every time point. To assess differential *P. chlamydosporia* gene expression in roots over time, RPKM cutoff values ≥ 10 were applied before comparing expressed genes, between time course conditions (4, 7, 21 days post inoculation—dpi).

Assembling *P. chlamydosporia* transcriptome

P. chlamydosporia was inoculated in 250-mL flasks with Czapek Dox broth (Oxoid, Basingstoke, UK) for production of the mycelium. The culture was incubated for 7 days at 26 °C in the dark with agitation, collecting the resulting hyphal matter on sterile cellulose filters. The mycelium was then frozen in 2-mL vials until extraction of RNAs. RNA-Seq library was prepared from 1-week-old *P. chlamydosporia* mycelium (100 mg) likewise previously described for roots. Fungus reads were assembled to yield contigs, by applying the "de novo assembly" CLC utility (word length = 22; bubble size = 50; minimum contig size = 200 bp), using de Bruijn graphs, with word length automatically chosen based on input data amounts. Blast2GO ver. 2.4.6 (http://www.blast2go.com/b2ghome) (Conesa et al. 2005) was applied (default parameters) to find functional motifs and related GO terms in assembled contigs. Homologies were checked in the non-redundant GenBank protein database with Blastx algorithm (*E*-value = 1.0E−3; high-scoring segment pairs' length cutoff fixed to 33). The "Augment annotation by ANNEX" function was used to improve annotation profile information. To investigate the genetic differentiation among the *P. chlamydosporia* biotypes, fungus reads were mapped onto two *P. chlamydosporia* available genome: *P. chlamydosporia* 170, accession LSBJ02000000 (Lin et al. 2018), and *P. chlamydosporia* 123, accession AOSW00000000 (Larriba et al. 2014).

Results

RNA-Seq analysis

A total of 449.5 million high-quality (QS ≥ 30), short-sequence single reads (100 bp) were obtained from all the samples, yielding approx. 32 GB of transcriptomic data. Around 98% of short reads (ranging from 56 to 85 million qualified reads per sample) were successfully mapped on annotated transcripts of the *S. lycopersicum* reference SL2.50.35 genome. No significant difference was observed in terms of total number of aligned reads between conditions (*P. chlamydosporia* inoculated or not) or among sampling times (4, 7, and 21 dpi). Out of 33810 predicted genes in the *S. lycopersicum* genome, under *P. chlamydosporia*-inoculated and uninoculated conditions, 24050 and 23967 were expressed at 4 dpi, 23656 and 24193 genes were expressed at 7 dpi, and 24265 and 24553 at 21 dpi, respectively. All replicated samples showed significant ($p \leq 0.001$) correlations of the RPKM values (Pearson's r range, 0.28–0.95).

Differential gene expression and functional analysis

Analysis showed a total of 3676 DEGs (minimum absolute FC = |2|; $p \leq 0.05$) between the two conditions tested. Their total number arose with time from 1088 (4 dpi) up to 1865 (21 dpi; Fig. 1), with a higher prevalence of downregulated transcripts (Table 1).

Enrichment analysis for gene ontology (GO) terms was carried out in order to determine processes and functions over-represented by DEGs at each time point, as compared to the reference transcriptome. Data showed 33 and 45 GO terms significantly (Fisher's Exact Test p value ≤ 0.05) enriched in the upregulated and downregulated genes sets, respectively, in at least one sampling time (4-7-21 dpi) (Table 2; Supplementary Tables S2). Functional categories related to recognition, signal transduction, response, and defense were strictly associated with upregulated genes. Terms associated with downregulated transcripts were the following: cell wall structural conformation, those related to transferase activities, peptide and nitrate transport, oxidoreductase and catalytic activities (Table 2; Supplementary Tables S2).

Most DEGs were modulated only at one sampling time, whereas relatively few genes were affected in common at four and 7 dpi (7.3%), or 7 and 21 dpi (10.8%), with only 104 transcripts (2.8 %) always differentially (up or down)

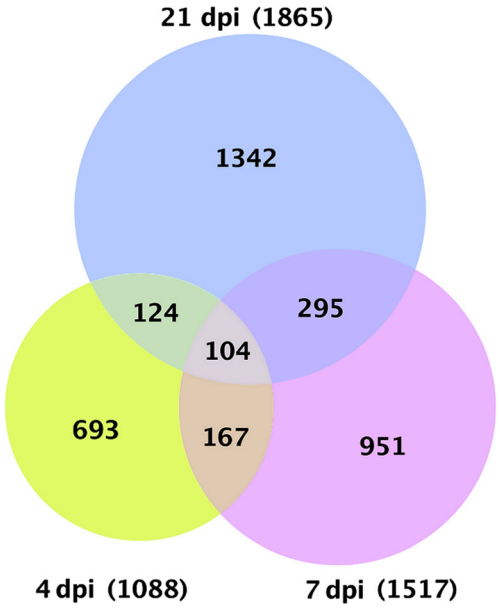


Fig. 1 Venn diagram of differentially expressed genes (DEGs, min absolute fold change (FC) = |2|; max p value ≤ 0.05) observed comparing *P. chlamydosporia*-inoculated (I) vs uninoculated (C) tomato roots, at different times (4, 7, and 21 dpi)

modulated (Fig. 1). Most of them play important roles in pathogen-mediated defense responses, involving physiochemical processes, such as cell wall regulation and modification, and biochemical responses such as biosynthesis and regulation of compounds associated with fatty acids and phenylpropanoid-signaling pathways (Supplementary Tables S3-A). The 40 genes upregulated at all times are implicated in plant defense responses such as the peroxidases and coumaroylquinate (coumaroylshikimate) 3′-monooxygenases, involved in the phenylpropanoid biosynthesis, pathogenesis-related proteins (PR), germin-like proteins (one of them over expressed 58-fold at 4 dpi and up to 101-fold at 21 dpi), NAC (NAM, ATAF1,2, CUC2) transcription factor, chitinases, and glucanases (Supplementary Tables S3-A). Other genes upregulated all times are involved in lipid metabolism, therefore associated with defense responses, such as cytochrome P450 or encoding lipid-modification enzymes (desaturases). Concerning the latter group, the most abundant one, the major FC values were observed at the early stage of fungus–plant interaction (4 dpi) (Supplementary Fig. S1-A). Moreover, in the *P. chlamydosporia*-inoculated roots, a group of genes related to hormone signal, including auxin-related and gibberellin receptor genes, was also overexpressed (Supplementary Tables S3-A). The most significant (p = 2.5e−05) enriched biological process term to which common upregulated genes belonged to was the "response to stress" (GO:0006950).

Table 1 Number of differentially expressed genes (DEGs) between *P. chlamydosporia*-inoculated vs uninoculated tomato roots at different sampling times, and annotated reads scored on the *S. lycopersicum* SL2.50.35 reference transcript database

dpi	DEGs*		% of total transcripts
	Downregulated (annotated)	Upregulated (annotated)	
4	786 (616)	302 (175)	3.21
7	782 (560)	735 (446)	4.48
21	1355 (903)	510 (372)	5.51

*Differentially expressed genes (min. absolute FC = |2|; max. $p \leq 0.05$)

An additional group of transcripts that might shed light on endophytism regulation of gene expression was given by 53 genes downregulated in inoculated roots, at every time point (Supplementary Tables S3-B). This group included genes involved in cell wall modification and remodeling, such as those encoding expansin-like proteins (downregulated up to 136 times at 21 dpi) and cell wall protein (CWP), hormone-related genes such as those encoding gibberellin synthesis and IAA-amino acid hydrolase, and genes of the phenylpropanoid pathway and of the cellular carbohydrate metabolic process. Moreover, a MYB family transcription factor showed 91-fold repression at 21 dpi (Supplementary Tables S3-B). The most significant (p = 1.2e−03) enriched biological process term to which common downregulated genes belonged to was the "cellular carbohydrate metabolic process" (GO:0044262).

To elucidate the endophyte role in the plant crosstalk, we analyzed also the genes uniquely expressed in uninoculated control samples, as their function could have been inhibited or replaced by some fungus products in inoculated plants. The genes not expressed in presence of *P. chlamydosporia* were 772, 1259, and 1033 (at 4, 7, and 21 dpi, respectively). They included several transcription factors and intramembrane transporter channel (Supplementary Tables S4). Between GO terms enriched in the 4-dpi subset, the most significant one was oxidoreductase activity (GO:0030613) and its child GO terms GO:0030614 and GO:0008794, with six genes annotated as glutaredoxin (GRX) (Solyc04g011780.1; Solyc04g011790.1; Solyc04g011840.1; Solyc04g011830.1; Solyc04g011800.1 and Solyc04g053110.1). Lipid transport (GO:00068699) and carboxylesterase activity (GO:0004091) were the most significant enriched GO terms of the 21-dpi subset, with 9 and 21 members, respectively.

The genes uniquely expressed in *P. chlamydosporia*-inoculated control samples were 855, 722, and 745 (at 4, 7, and 21 dpi, respectively). Between GO terms enriched in the 4-dpi subset, the most significant term was "membrane part" (GO:0044425) and its child GO terms "integral to membrane" (GO:0016021) and "intrinsic to membrane" (GO:0031224).

Table 2 Enrichment analysis for gene ontology (GO) terms in each time point (4, 7, and 21 dpi) DEG sets as compared to reference transcriptome. F_ and P_ indicates function and process respectively. Down- and upregulated GO are indicated with symbols − and +

GO term	Description	4	7	21	GO term	Description	4	7	21
GO:0016209	F_antioxidant activity		−		GO:0051082	F_unfolded protein binding		−	
GO:0005516	F_calmodulin binding			−	GO:0016722	F_oxidoreductase activity, oxidizing metal ions		−	−
GO:0004091	F_carboxylesterase activity			−	GO:0070279	F_vitamin B6 binding			
GO:0003824	F_catalytic activity	−	+	+	GO:0016724	F_oxidoreductase activity, oxidizing metal ions, oxygen as acceptor	−	−	
GO:0071555	P_cell wall organization		−		GO:0019825	F_oxygen binding	−	+	+
GO:0016759	F_cellulose synthase activity	−			GO:0030414	F_peptidase inhibitor activity		+	−
GO:0006952	P_defense response			+	GO:0042277	F_peptide binding	−		
GO:0042936	F_dipeptide transporter activity			−	GO:0015197	F_peptide transporter activity			−
GO:0003677	F_DNA binding	−	+		GO:0004601	F_peroxidase activity		−	
GO:0004866	F_endopeptidase inhibitor activity		+	+	GO:0016773	F_phosphotransferase activity, alcohol group as acceptor			+
GO:0004857	F_enzyme inhibitor activity		+		GO:0009664	P_plant-type cell wall organization		−	
GO:0000293	F_ferric-chelate reductase activity			−	GO:0071669	P_plant-type cell wall organization or biogenesis		−	
GO:0004322	F_ferroxidase activity	−	−		GO:0004672	F_protein kinase activity			+
GO:0046527	F_glucosyltransferase activity	−			GO:0004674	F_protein serine/threonine kinase activity			+
GO:0043295	F_glutathione binding	−			GO:0080044	F_quercetin 7-O-glucosyltransferase activity	−		
GO:0004364	glutathione transferase activity	−			GO:0004872	F_receptor activity			+
GO:0016798	F_hydrolase activity, acting on glycosyl bonds			−	GO:0009607	P_response to biotic stimulus	+		+
GO:0004553	F_hydrolase activity, hydrolyzing O-glycosyl compounds			−	GO:0050896	P_response to stimulus	+		+
GO:0016301	F_kinase activity			+	GO:0006950	P_response to stress			+
GO:0008289	F_lipid binding			−	GO:0004867	F_serine-type endopeptidase inhibitor activity		+	+
GO:0080054	F_low affinity nitrate transmembrane transporter activity			−	GO:0004871	F_signal transducer activity			+
GO:0046873	F_metal ion transmembrane transporter activity			+	GO:0003700	F_transcription factor activity	−	+	
GO:0003777	F_microtubule motor activity		−		GO:0030528	F_transcription regulator activity	−	+	
GO:0007018	P_microtubule-based movement		+		GO:0016740	F_transferase activity	−		+
GO:0007017	P_microtubule-based process		+		GO:0016765	F_transferase activity, transferring alkyl or aryl (other than methyl) groups	−		
GO:0060089	F_molecular transducer activity			+	GO:0016757	F_transferase activity, transferring glycosyl groups	−	−	−
GO:0003774	F_motor activity		−		GO:0016758	F_transferase activity, transferring hexosyl groups	−	−	−
GO:0015112	F_nitrate transmembrane transporter activity			−	GO:0016772	F_transferase activity, transferring phosphorus-containing groups			+
GO:0045735	F_nutrient reservoir activity			+	GO:0004888	F_transmembrane receptor activity			+
GO:0015198	F_oligopeptide transporter activity			−	GO:0019199	F_transmembrane receptor protein kinase activity			+
GO:0016491	F_oxidoreductase activity	−		+	−	GO:0035251			
F_UDP-glucosyltransferase activity	−								

Table 2 (continued)

GO term	Description	4	7	21	GO term	Description	4	7	21
GO:0016705	F_oxidoreductase activity, acting on paired donors, with incorporation/reduction of mol. O_2	+			GO:0008194	F_UDP-glycosyltransferase activity		–	
GO:0016706	F_oxidoreductase activity, acting on paired donors, with incorporation/reduction of mol O_2.	+			GO:0016702	F_oxidoreductase activity, acting on single donors with incorporation of molecular O_2, incorporation of two atoms of oxygen		+	
GO:0016684	F_oxidoreductase activity, acting on peroxide as acceptor	–			GO:0016723	F_oxidoreductase activity, oxidizing metal ions, NAD or NADP as acceptor		–	+

Metabolic process (GO:0008152) was the most significant enriched GO term for the 7- and 21-dpi subsets, with 111 and 88 members, respectively.

Ethylene, salicylic, and jasmonic acid pathways

Genes generally involved in the plant–fungus interaction belonging to the jasmonic (JA) and salicylic (SA) acids and ethylene (ET) pathways, signaling, phenylpropanoids, and other antimicrobial molecules showed a significant differential expression (Supplementary Table S5). Their behavior appeared to change during the time course, as showed by the corresponding heat maps (Figs. 2 and 3).

The expression of 15 phenylalanine ammonia lyase (PAL) genes was detected in roots (Supplementary Tables S5). PAL is the first enzyme in the phenylpropanoid pathway leading to the biosynthesis of SA and different plant defense–related metabolites, such as lignin precursors, flavonoids, and hydroxycinnamic acid esters (Vogt 2010). Solyc03g071870.1 showed the highest values at every time point, being 4 (4 dpi) to 43 (7 dpi)-fold downregulated, and 15-fold upregulated at 21 dpi. The majority of other PAL genes were slightly affected at 4 dpi, with only one displaying a 3-fold induction. At later times (7 dpi), four PAL genes were 2- to 6-fold upregulated, only one showed about 3-fold downregulation. Finally, at 21 dpi, 5 genes were 2- to 8-fold upregulated, whereas all the others were not affected.

SA can be synthesized also via the isochorismate pathway (involving isochorismate synthase (ICS)), a route alternative to the phenylpropanoid pathway (Lee et al. 1995). Therefore, expression of ICS was examined, but showed that FC values were not significantly affected, during endophytism.

The fungus endophytism affected several SA-response WRKY genes, DNA-binding proteins containing WRKY domains. The largest number of downregulated transcripts was observed during the early endophytism stage (Fig. 2). Out of the 84 tomato WRKY, 10 were > 2-fold (up to − 13) downregulated at 4 dpi, with five overexpressed (of which two up to 18-fold), the resting being not affected or not expressed. At 7 dpi, six WRKY genes were > 2-fold (up to 11) downregulated, and 12 overexpressed (of which 2- up to 12-fold), the resting were not affected nor expressed. Finally, at 21 dpi, five WRKY genes were 2- to 9-fold downregulated, nine were at least 2-fold overexpressed, with all the remaining being not expressed or very slightly influenced (Supplementary Table S5).

The presence of *P. chlamydosporia* affected a number of genes coding for enzymes involved in JA biosynthesis (lipoxygenase (LOX), allene oxide synthase (AOS), 12-oxophytodienoate reductases) (Supplementary Tables S5). In some cases, gene behavior was reverted during time. The highest induction was observed at 7 dpi for LOX Solyc01g006560.2 and Solyc01g009680.2 (17- and 12-fold

overexpressed, respectively) and for a 12-oxophytodienoate reductase (Solyc11g032230.1) that was 18-fold upregulated. In contrast, other genes involved in the JA response were significantly downregulated, such as two encoding for a LOX (Solyc01g099180.2, FC − 10 at 7 dpi) or a 12-oxophytodienoate reductase (Solyc04g039920.1, FC − 12.6 at 21 dpi).

In the ethylene synthesis pathway, the key, rate-limiting genes in ethylene synthesis coding for the 1-aminocyclopropane-1-carboxylate (ACC) synthases were most affected at 7 dpi, with Solyc12g008740.1, Solyc04g077410.2, and Solyc12g056180.1 that were 50-, 40-, and 29-fold down-expressed, respectively. Three ACC oxidase genes were the most affected at 21 dpi, being Solyc09g089820.1, Solyc03g095900.2, and Solyc09g089760.1 about 24-, 10-, and 7-fold down-expressed, respectively (Fig. 3).

Out of the 93 tomato ethylene response factor (ERF), 22 were more than 2-fold (up to − 7.78) downregulated at 4 dpi, with 10 overexpressed (2- up to 5-fold), the resting being not affected. At 7 dpi, 15 ERF genes were more than 2-fold (up to 57-fold) downregulated, 13 were overexpressed (2- up to 9-fold), the resting being not affected. Finally, at 21 dpi, 27 ERF genes were 2- to 12-fold downregulated, 10 were at least 2-fold overexpressed, with all the others very slightly influenced (Supplementary Tables S5).

Flavonoids

Chalcone synthase (CHS) is the first enzyme recruited in the production of flavonoids. Remarkably, nine of the 22 detected CHS genes were significantly overexpressed early at 4 dpi (with FC up to 34) to subsequently return at basal (4 genes) to moderate overexpression value (5 genes) at 7 dpi (Supplementary Tables S5). Eight CHS genes were not expressed at any endophytism stage. Other genes involved in flavonoid production (flavonols, dihydroflavonols, isoflavonoids) showed variable levels of expression, being under-expressed (with FC up to 44) at the first stage of plant–fungus interaction. Solyc10g009520.2, encoding for a dihydroflavonol reductase, was markedly activated (10-fold) at 4 dpi (Supplementary Tables S5).

Terpenoids

A high number of genes encoding enzymes of the terpenoid pathway (18 out of 60) were activated at 4 dpi (FC = 2–30), whereas nine were repressed (FC = − 2 to − 13). Notably, one terpene synthase gene (Solyc01g101190.2), 23-fold overexpressed at 4 dpi, was very strongly induced (FC = 564) at 7 dpi (Supplementary Tables S5).

Fig. 2 Differentially expressed genes from the SA, JA, and signaling pathways, in the *P. chlamydosporia*-inoculated tomato roots at 4, 7, or 21 dpi. Average linkage hierarchical clustering (Pearson's distance) of the relative transcript abundance profiles (FC) was calculated based on two (4 and 7 dpi) or four (21 dpi) biological replicate expression values. Block colors (see legend) show decreased (red) or increased (green) transcript accumulation relative to the corresponding control (genes not expressed are black colored). For every pathway, some genes with a Fc ≥ |2| at least at a single observation time are showed. For further details (genes id. and Fc values), see Supplementary Tables S5

Signaling

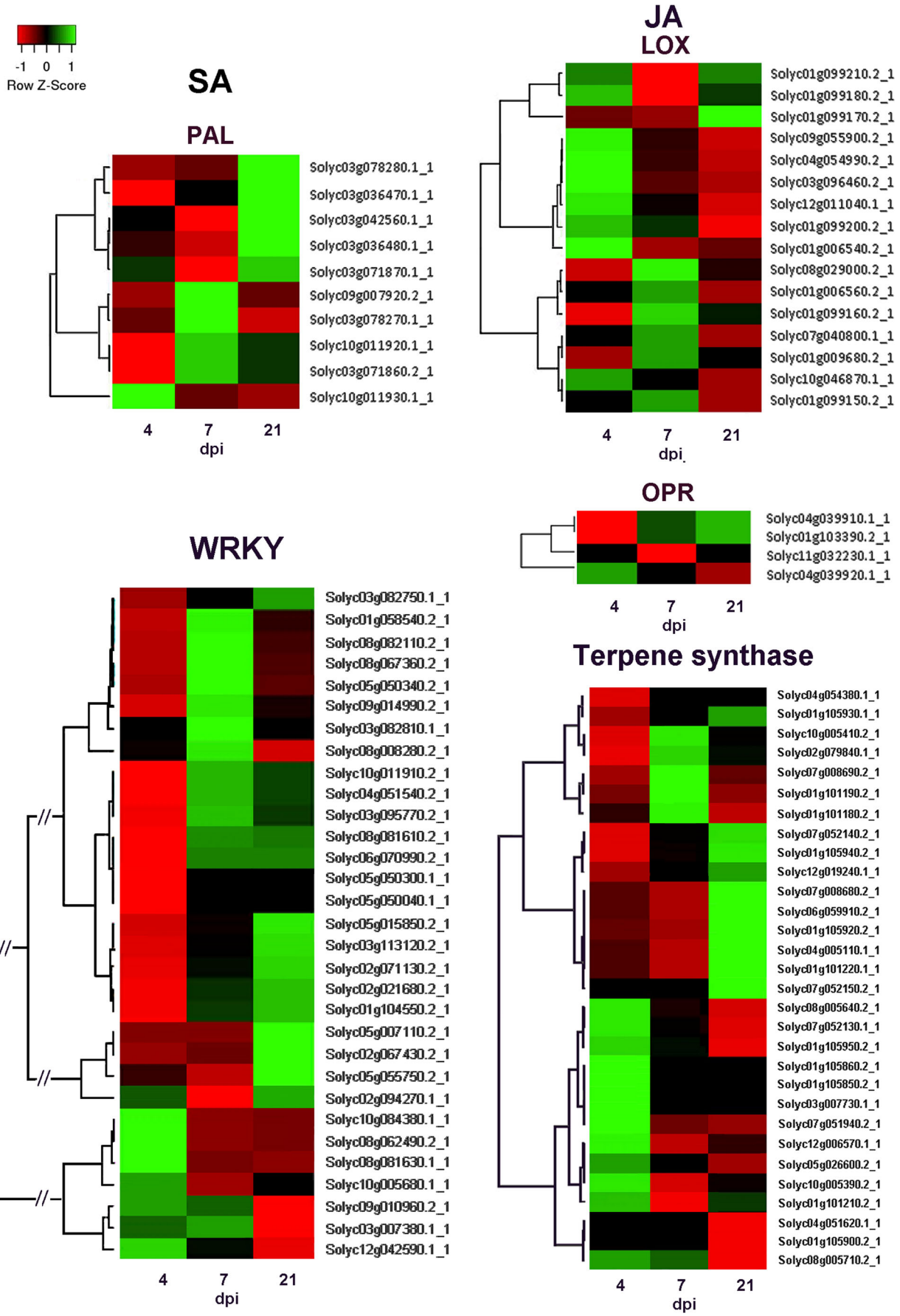
We examined the expression of U-box-type ubiquitin ligases (SlPUB) E3 and the ubiquitin-conjugating (UBC) E2 enzymes, recently found to play a significant role in tomato pattern-triggered immunity (PTI) (Zhou et al. 2017). With exception of UBC7, about 10-fold downregulated at 21 dpi, no further tomato E2 enzymes (of the 39 remaining) were significantly affected (Supplementary Tables S5). However, two other genes annotated as ubiquitin were significantly overexpressed at every time point (Supplementary Table S3).

Mitogen-associated protein (MAP) kinase expression was minimally affected by *P. chlamydosporia* endophytism, at all time points. The same behavior was observed for the tomato genes known to be involved in respiratory burst oxidase (RBO), except for Solyc05g025690.1 that showed a significant (about 28-fold) downregulation at 7 dpi.

Network analysis

Structural differences were found between the two networks constructed with the Spearman's correlations, for both conditions. The control samples network included 132 nodes and 382 edges, whereas that for the inoculated one showed 124 nodes with 368 edges. The β-connectivity (no. of edges/no. of nodes) showed close values for both networks (2.89, control, and 2.96, inoculated). Venn counting of nodes showed 93 transcripts that were in common between the two networks, with 39 unique for the control and 31 present only in the inoculated samples. Unique transcripts for both networks were mostly ETR and WRKY (> 64% in total), with a higher frequency of terpene synthase and a lower number of ETR transcripts in the inoculated samples network (Supplementary Table S6). Unique correlations between transcripts TPM were 378 and 361 in the control and the inoculated data sets, respectively, with 58 correlated transcript pairs present in both conditions, mostly WRKY and ETR (Supplementary Table S6).

In control samples, the network based on the nodes' degree (number of correlations) showed three major communities of highly correlated transcripts including WRKY and ETR transcripts (Supplementary Fig. S2). Betweenness showed as main hub a MAP kinase (Solyc02g078140.2_1) that was more expressed at all times in the inoculated samples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dpi
JA
LOX
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Solyc10g046870.1_1
Solyc01g099150.2_1
4 7 21
dpi
OPR
Solyc04g039910.1_1
Solyc01g103390.2_1
Solyc11g032230.1_1
Solyc04g039920.1_1
4 7 21
dpi
WRKY
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Solyc05g050340.2_1
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dpi
Terpene synthase
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Solyc01g101210.2_1
Solyc04g051620.1_1
Solyc01g105900.2_1
Solyc08g005710.2_1
4 7 21
dpi

(Supplementary Fig. S3), with three further MAP kinase hubs (Solyc10g009060.1_1, Solyc03g123800.1_1, and Solyc01g094960.2_1), one LOX (lipoxygenase) (Solyc03g96540.2_1) and one ETR (Solyc10g0786110.1_1) (Fig. 4).

In the inoculated samples, the network based on the nodes' degree showed only two major communities of correlated transcripts, with two WRKYs (Solyc02g94270.1_1 and Solyc08g067360.2_1) as main hubs (Supplementary Fig. S4). Betweenness showed a number of WRKY and ETR hubs such as Solyc04g071770.2_1, with a single MAP kinase (Solyc07g042890.2_1), also observed in control as mostly correlated to several WRKYs (Fig. 5). Of the MAP kinase hubs present in the control network, only Solyc02g078140.2_1 was found in the inoculated one, however as a marginal node.

Validation of RNA-Seq results by RT-qPCR

RT-qPCR was performed on selected DEGs involved in different signaling pathways and defense response: two LRR receptor-like serine/threonine-protein kinase, RLP (Solyc01g005720.2, Solyc01g005730.2), two SQUAMOSA promoter binding protein-like, SBP (Solyc02g077920.2, Solyc05g015840.2), a coiled coil-nucleotide binding-leucine-rich repeat domain (CC-NBS-LRR) (Solyc09g092300.2), and a pyruvate kinase (Solyc01g106780.2), DEGs. Gene expression patterns of the selected DEGs, assessed at 4, 7, and 21 dpi by RT-qPCR, were consistent with those obtained by RNA-Seq (Supplementary Fig. S5), confirming the reliability and accuracy of the NGS analysis.

Identification of *P. chlamydosporia* transcripts in inoculated roots

The high-quality reads proceeding from inoculated samples, which could not be mapped on the tomato transcriptome, were aligned onto the *P. chlamydosporia* reference genome (Table 3). More than 1 million reads of each dataset were mapped on the Pc170 genome (1.33–1.55% of total cleaned reads). Out of the 14205 *P. chlamydosporia* putative genes, 65% were expressed during endophytism in tomato roots (RPKM cutoff ≥ 10). Their number decreased to 8584, 6796, and 6456 at 4, 7, and 21 dpi, respectively. Out of them, 5368 genes were expressed at every time point (data not showed). Due to the exiguous fungus recovered reads, to assess differential *P. chlamydosporia* genes expression in roots over time, a cut off RPKM ≥ 1000 was applied. We identified 246 highly expressed genes, including ribosomal proteins (61), secreted protein (35), proteases (11), heat shock proteins (4), and others, putatively involved in multiple lifestyle transitions of the fungus (Supplementary Tables S6). Among them, 66 were expressed at every time step. Out of 154, 168, and 123 expressed genes at 4, 7, and 21 dpi, respectively, 24, 14, and 17 were secreted proteins, including peptidases such as serine peptidases (S08A, S09X, S01E, S33), metalloproteinase (M35, M43B, M80), and cysteine peptidase (C14B). The higher number of proteases (8) was observed at 4 dpi. Among them, two peptidases of cluster S08A, subtilisin serine proteases (VFPPC_14262 and VFPPC_03048) (corresponding to NCBI accessions XM_018292031.1 and XM_018282599.1) were found (Supplementary Tables S7).

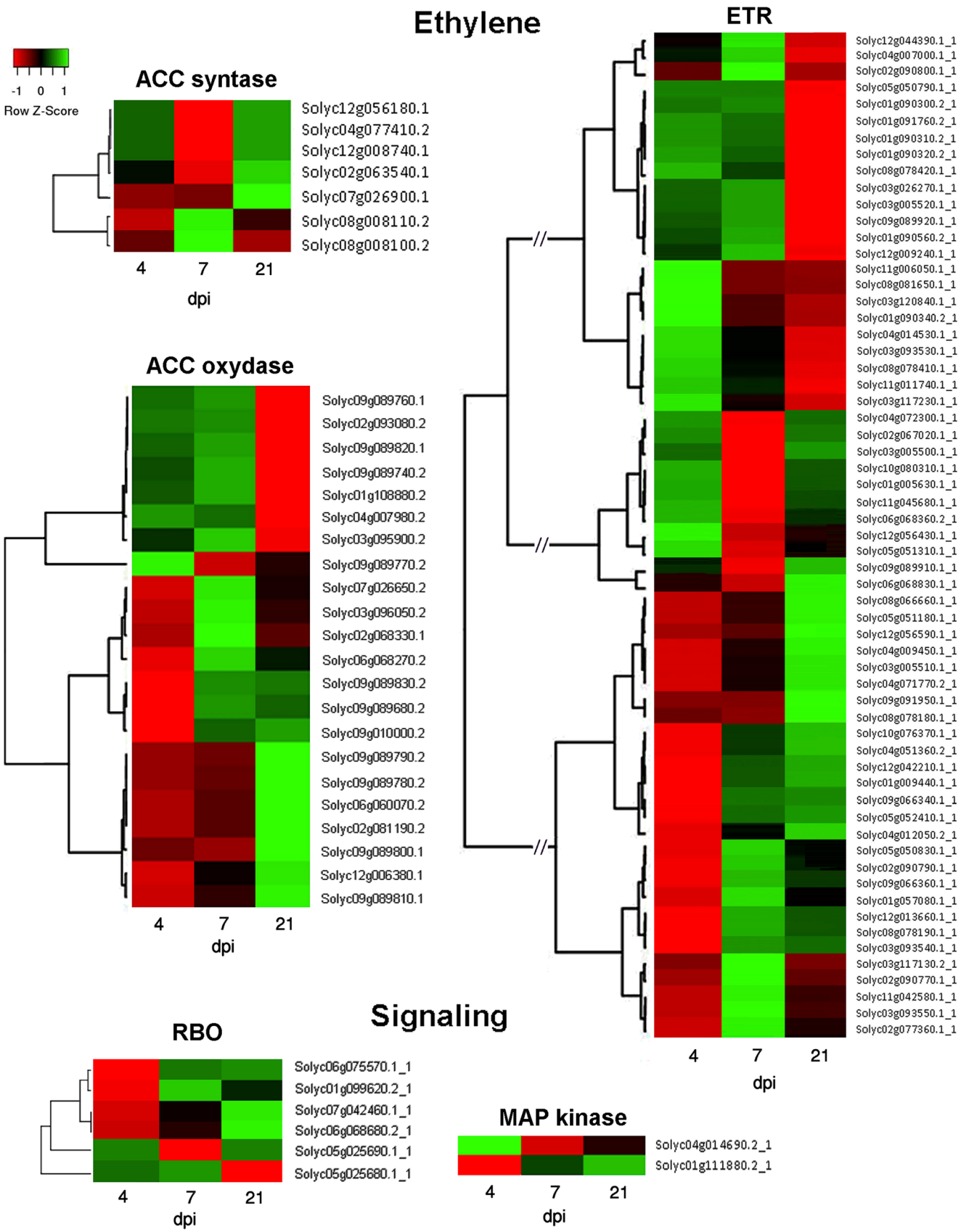
Fig. 3 Differentially expressed genes from the ethylene pathway in *P. chlamydosporia*-inoculated tomato roots at 4, 7, or 21 dpi. Average linkage hierarchical clustering (Pearson's distance) of the relative transcript abundance profiles (FCs) was calculated based on two (4 and 7 dpi) or four (21 dpi) biological replicate expression values. Block colors (see legend) show decreased (red) or increased (green) transcript accumulation relative to the corresponding control (genes not expressed are black colored). For further details (genes id. and Fc values), see Supplementary Tables S5

P. chlamydosporia in vitro transcriptome

To investigate the genetic differentiation among the *P. chlamydosporia* biotypes, we sequenced the RNAs produced by the DSM 26985 mycelium grown in liquid Czapek medium. The fungus sequence have been deposited in the NCBI Sequence Read Archive (SRA), with accession number SAMN11458719. A total of $22 \cdot 10^6$ high-quality un-paired reads (51 bp) were obtained that mapped with higher frequency on the Pc170 (95.38%), than the Pc123 (77.6%) genome sequences. Moreover, the DSM 26985 reads were de novo assembled in 17076 contigs (N50, 1479 bp; N75, 672 bp; average coverage, 42.5%) for a 14.8-Mbp putative transcriptome. Blast2GO analysis showed 12333 contigs (72.2%) with highest homology for *Metarhizium anisopliae* and 11148 (65.2%) for *Metarhizium acridum*, confirming these species as closest taxa. Minor homologies were found for *Fusarium oxysporum* or *Trichoderma virens* (Supplementary Fig. S1-B).

Discussion

Endophytic fungi must overcome the host defense machinery to establish within its tissues and start a mutualistic interaction. Endophytes are capable to evade the plant immune system, colonizing the host without causing visible damage or harm. Therefore, any successful association involves the endophyte capacity to modulate the host plant immunity. The molecular mechanisms active during the fungal endophytic phase, however, are complex and still poorly understood. In this study, we generated whole transcriptome data for a plant host and an endophyte at three time points after the fungus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dpi

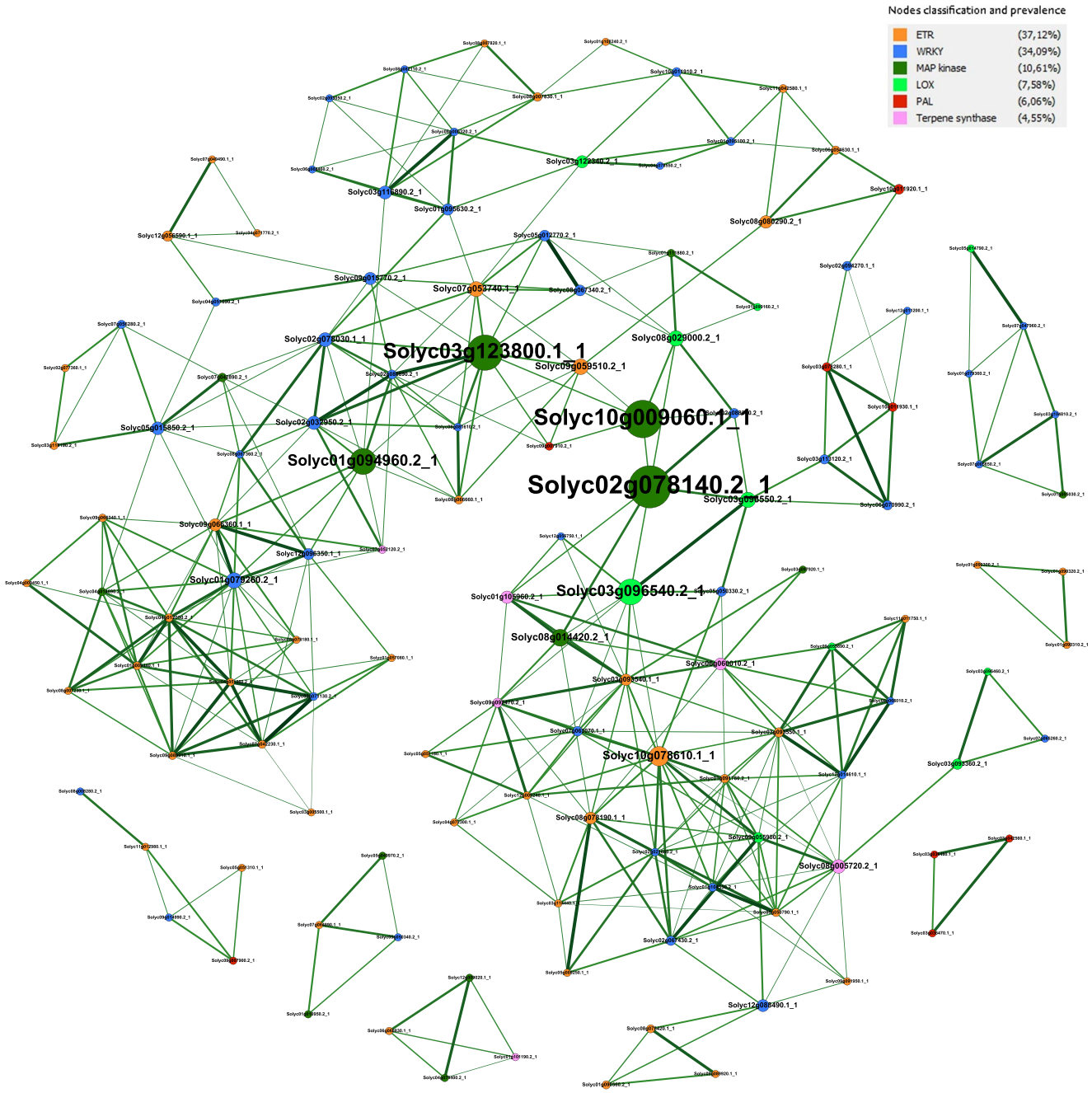

Fig. 4 Network of tomato transcription factors (see Figs. 2 and 3) based on TPM data and Spearman's positive correlation coefficients ($p \leq 0.01$) constructed with data from control, not inoculated samples ($n = 8$). Upper legend shows the color attribution of nodes (family of transcripts), with their repartition. Nodes' diameter is proportional to betweenness. The color depth and thickness of edges are proportional to the value of the corresponding Spearman's correlation coefficient

inoculation, making it possible to chronologically refine the gene expression data.

The molecular analysis was focused on roots at 4, 7, and 21 dpi as, at those times at which *P. chlamydosporia* displays, (i) the minimum incubation period to achieve root endophytism; (ii) an increasing degree of colonization (colonized trichomes, epidermal and cortical root cells, forming appressoria and hyphal coils in cells); and (iii) its maximum degree of root colonization, with production of conidia and chlamydospores in the rhizoplane (Escudero and Lopez-Llorca 2013). Data indicated that *P. chlamydosporia* modified the roots' transcriptome during endophytism, steering their metabolism by alerting defense and stress response pathways, in a complex and concerted way.

As potential key players in the maintenance of the root–endophyte mutualistic interaction, a number of tomato

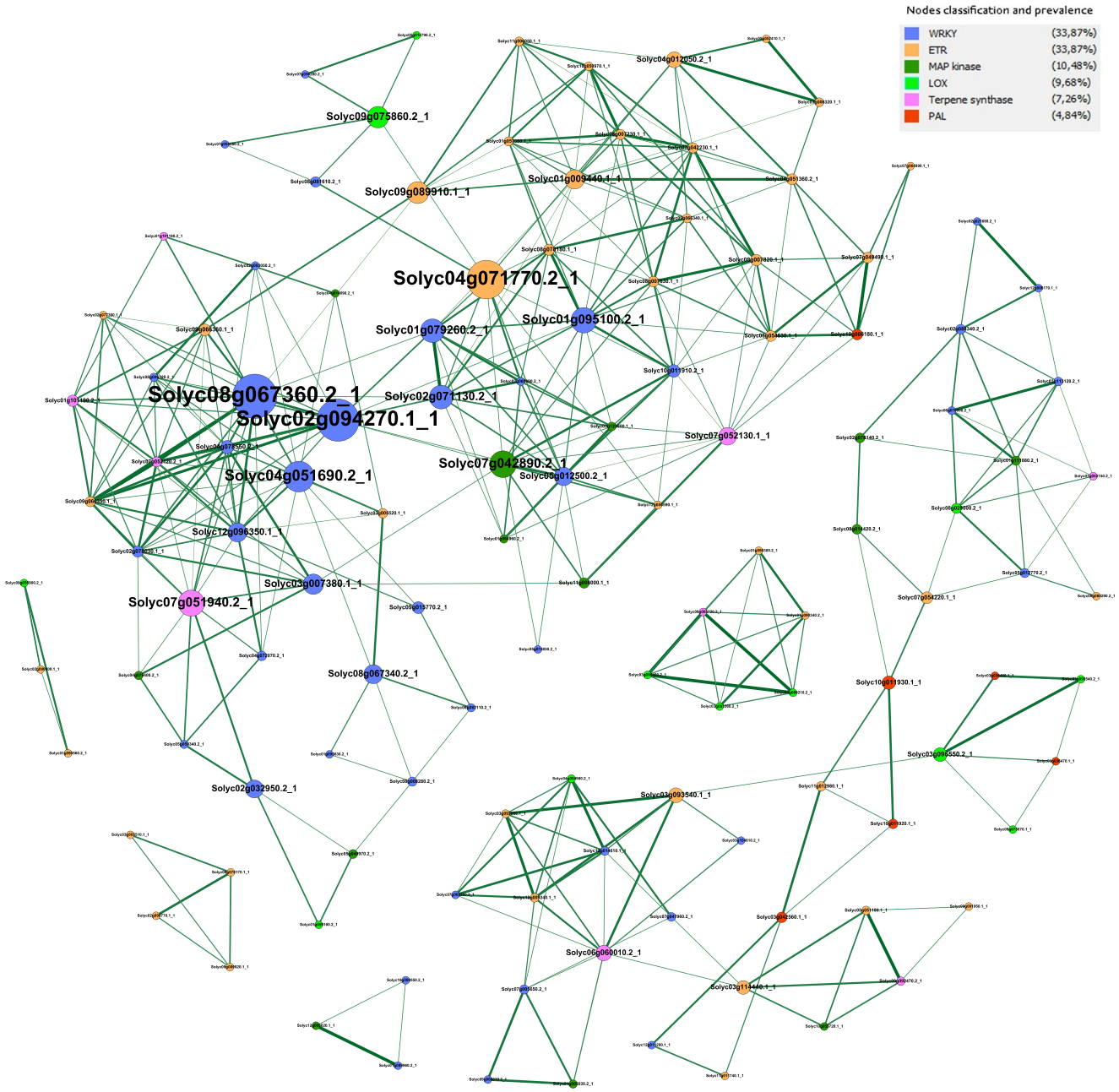

Fig. 5 Network of tomato transcription factors (see Figs. 2 and 3) based on TPM data and Spearman's positive correlation coefficients ($p \leq 0.01$) constructed with data from samples inoculated with *P. chlamydosporia* ($n = 8$). Upper legend shows the color attribution of nodes (family of transcripts), with their repartition. Nodes' diameter is proportional to betweenness. The color depth and thickness of edges are proportional to the value of the corresponding Spearman's correlation coefficients

Table 3 Number of sequenced single reads that were unmapped on tomato, and percentages of those mapped on the *P. chlamydosporia* reference genome, at different sampling times

dpi	Reads unmapped on tomato	Reads mapped on Pc170 genome (%)
4	2099515	54.84
7	2862729	43.66
21	2147259	59.52

transcripts upregulated at every time point were identified, among which are lipid metabolism–related genes and fatty acid desaturases. In tomato, fatty acids per se were reported as messenger and modulator molecules, a key step in the signaling events that activate defense genes (Conconi et al. 1996; Hwang and Rhee 1999). Particularly, fatty acid desaturases are required for JA biosynthesis (Li et al. 2003). Moreover, *P. chlamydosporia* affected the expression of other key genes of the JA pathway, such as lipoxygenases, 12-

oxophytodienoic acid reductases, and allene oxide cyclase, in a mechanism similar to that recently observed in barley and young *Arabidopsis* seedling roots (Larriba et al. 2015; Zavala-Gonzalez et al. 2017). JA is able to control several traits of plant reactions leading to defense and immunity (Wasternack and Hause 2013). This signaling pathway has been reported to mediate recognition and establishment in other mutualistic plant–microbe interactions (Hause and Schaarschmidt 2009). Particularly, JA synthesis was required for the establishment of the mycorrhizal symbiosis in tomato plants, where the effect observed was attributed to the regulation of C partitioning (Tejeda-Sartorius et al. 2008). In this concern, *P. chlamydosporia* RNAseq data showed the down expression of several genes related to sugar transport and sucrose hydrolysis, therefore related to C splitting. However, JA has been also linked to plant protection against excessive root colonization (Vierheilig 2004; Hause and Schaarschmidt 2009; Plett et al. 2014). Our data showed that genes involved in the JA biosynthesis (e.g., lipoxygenases, 12-oxophytodienoic acid reductases, and allene oxide cyclase) were differently modulated at different stages of the interaction, suggesting they could have distinct functions or, in some cases, opposite effects, as reported for LOX family members in *Arabidopsis* (Ozalvo et al. 2014). The JA pathway has also been associated with systemically induced defense against nematode infection (Nahar et al. 2011; Ozalvo et al. 2014) suggesting a *P. chlamydosporia* beneficial effect against nematode invasion.

Two ubiquitin genes were also significantly over expressed at every time point. U-box-type ubiquitin ligases were recently reported to be recruited to inactivate plant receptors sensing pathogen effectors and initiating the immune response signaling (Chinchilla et al. 2007; Zhou et al. 2017). It is likely that, by affecting those ubiquitins, *P. chlamydosporia* is able to prevent or modulate the activation of the host immune response(s), therefore allowing endophytism, as no other known PTI receptor, such as MAP kinases and reactive oxygen species (ROS), appeared directly influenced by the fungus.

Among the signaling genes strongly repressed at every time point, there was also an expansin-like, suggesting a role for this gene in the endophyte recognition process. An expansin (Ave1) was recognized as responsible for contrasting penetration of the pathogenic fungus *Verticillium dahliae* in roots of tomato resistant lines (de Jonge et al. 2012). According to the RNASeq data, a further DEG that likely plays a crucial role in the recognition mechanism was a member of the MYB family of transcription factors, underexpressed at every time point. MYBs contribute to activate PR proteins and genes involved in generating ROS (Cheong 2002; Cao et al. 2006; Casalongue et al. 2012). Data showed that many antioxidants such as peroxidases that convert ROS into non-reactive molecules, and respiratory burst oxidases, were downregulated. The repression of the oxidative process and the downregulation of the associated genes may lead to a compatibility reaction by avoiding a hypersensitive response. Interestingly, the tomato genes not expressed in the presence of *P. chlamydosporia*, at the early stage of interaction, included several glutaredoxins (GRXs). These antioxidants, involved in SA signaling (Rouhier et al. 2008), play a key role in the plant redox signaling network (Kuźniak et al. 2017) which controls almost all aspects of the plant biology, including defense responses to biotic stress (Foyer and Noctor 2011; Noctor et al. 2017). It has been recently shown that GRXs regulate the activity of basic leucine zipper-type transcription factors which interact with NPR1 and are essential for the regulation of many SA-responsive genes, such as PR1 (Kuźniak et al. 2017). Our data also indicated that other groups of well-known SA-responsive genes, the WRKY transcription factors, were clearly affected by the fungus, although their expressions varied during time.

Time data showed that a general, modulated response to the biotic stimulus was observed in the roots at the early colonization stage, consistent with observations carried out on *P. chlamydosporia* and barley roots interaction (Larriba et al. 2015). We detected several genes involved in the plant general response to stimulus, which reached high levels of expression. At the onset of the interaction, the induction of these genes appears as the result of the endophyte root penetration. Three days later, the majority of upregulated genes appeared implicated in recognition and signal transmission, reflecting a recognition process typical of mutualistic fungi (Lahrmann et al. 2015). Finally, three weeks after endophytism initiation, "defense response" was the prevalent enriched GO term. Upregulated defense categories likely operated to avoid over-colonization, similarly to mechanisms observed for other endophytic microorganism (Lahrmann et al. 2015). This reaction could be responsible for the host pre-immunization, a process coherent with the beneficial property attributed to *P. chlamydosporia*.

In this context, we can consider the increased expression levels observed during time, for Mi-1.2, conferring resistance against some species of root-knot nematodes, specific isolates of potato aphid and white fly (Roberts and Thomason 1986; Rossi et al. 1998; Vos et al. 1998; Nombela et al. 2003). A similar situation was observed for Hsp90-1, required in the *Mi-1*-mediated resistance against pathogens and pests (Bhattarai et al. 2008). Moreover, *P. chlamydosporia* induced the upregulation of several tomato genes related to the biosynthesis of auxins and auxin-modulated transcriptional regulation, as previously observed also in barley roots (Larriba et al. 2015). Auxins, which are involved in apical dominance, root growth, and development, play an important role in the formation of root hairs. This process depends on the auxin biosynthesis, transport, and flow in the root tissues (Takahashi 2013). An increment in lateral root formation was already reported in tomato plants colonized by *P. chlamydosporia*, which suggested to promote an increased tolerance to

nematodes (Escudero and Lopez-Llorca 2013) and to underpin a fertilization-like effect (Maciá-Vicente et al. 2009).

The differences between the two networks confirm a major effect of endophytism in globally re-structuring the root gene expression. Transcripts unique in each network may reflect the effect of endophytism. Higher frequencies of LOX and terpene synthase and a lower occurrence of ETR transcripts in the inoculated network confirm a root defense reaction. Lipoxygenases are involved in several function in plants, including vegetative growth as well as in defense reactions, after wounding or pathogen attack, acting through the metabolism of linolenic acid to jasmonic acid in the induction of a hypersensitive response (Porta and Rocha-Sosa 2002). Solyc09g075860.2_1 and Solyc09g075870.1_1, always expressed in the two conditions, were present only as unique nodes in the inoculated samples network. Both lipoxygenases were downregulated in the response of tomato roots to penetration by *Meloidogyne javanica*, a reaction whose metabolic cascade was associated to the nematode protein Mj-FAR-1 (Iberkleid et al. 2015). This difference suggests a specific and selective effect of endophytism, as also in this case transcripts involved in nematode resistance were not downregulated. Similarly, terpenes act as defense products stored in trichomes, whose biosynthesis is promoted by a number of WRKYs including Solyc07g055280.2.1 (SlWRKY78) (Spyropoulou et al. 2014), uniquely found in the inoculated samples network (Supplementary Tables S6). Their activity appears hence associated to a selective and specific root defense response to *P. chlamydosporia* colonization.

A further difference in the transcript interactions was shown for MAP kinases. The MAP kinase Solyc02g078140.2_1, a main hub in the control network, appeared marginal in the inoculated one, in which the three other MAP kinases were not even present. MAP kinases are conserved and redundant signal transduction proteins involved in development and response to stress, protein phosphorylation, and gene regulation. In roots, they are involved in intra- and extra-cellular signaling, and transducing signals originated from external stimuli responsive sensors, including biotic and abiotic stress sources (Taj et al. 2010). In *Arabidopsis thaliana*, MPK 6 is involved in embryo development and, through the elongation and production of cells, in root architecture, acting as a repressor of primary and lateral root development (López-Bucio et al. 2013). Given their role in orchestrating metabolic pathways in response to biotic and abiotic stress conditions in roots, MAP kinases appear as key players in the gene expression modulation induced by *P. chlamydosporia*, their interactions with other transcripts being likely affected by the fungus during root colonization.

Transcriptome analysis of *P. chlamydosporia* showed that several secreted proteins were expressed in tomato roots during the endophytic process. This behavior is consistent with the gene expression of a different *P. chlamydosporia* isolate reported in barley (Larriba et al. 2014). Their expression suggests an essential role in the *P. chlamydosporia* multiple lifestyles. Moreover, peptidases such as serine peptidases (such as S08A, S09X, S33) and metalloproteinase (M35, M43B, and M80) were identified. They are known to be involved in *P. chlamydosporia* response to nutrient selection pressures (Li et al. 2017). At the initial stage of colonization, the major number of proteases was observed, which included two peptidases of cluster S08A, subtilisin serine proteases (VPC1), previously reported as fungal pathogenic proteins, related to nematode egg infection (Manzanilla-López et al. 2013; Larriba et al. 2014). The *P. chlamydosporia* genes expressed in common at every stage included two transcription factors (C6 and bZIP) and calcipressin, linked to pathogenicity and virulence in other fungi (Pinchai et al. 2009; Li et al. 2017), therefore related to the host colonization process or endophytism persistence.

The dimension of the transcriptome of *P. chlamydosporia* DSM 26985 that was isolated from roots of *Actinidia chinensis* was close to that of PC123, isolated from the cereal cyst nematode *Heterodera avenae* (Larriba et al. 2014), and PC170, proceeding from the root-knot nematode *Meloidogyne incognita* (Li et al. 2017). Obtained reads mapped with higher frequency on Pc170 than Pc123 genome sequences, previously found to differentiate each others', supposedly as a consequence of the fungus evolution for long-term adaptation to diverse hosts and environments (Li et al. 2017). The *P. chlamydosporia* phylogenetic proximity to *Metarhizium* spp. (Larriba et al. 2014) (also herein confirmed) suggests possible metabolic similarities between the two clades. *Metarhizium* spp. complex relationships with roots involve the return, through the endophytic phase, of the N subtracted by parasitic insects feeding on roots that the fungus also attacks in soil (Behie et al. 2012). A similar behavior might be present in the RKN-associated *P. chlamydosporia* supporting a role of the fungus in plant growth apart from nematode biocontrol, linking roots, nematodes, and *P. chlamydosporia* in a complex and plant beneficial, nutrient-based relationship.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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