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ORIGINAL PAPER

Arbuscular Mycorrhizal Symbiosis-Induced Expression Changes in *Solanum lycopersicum* Leaves Revealed by RNA-seq Analysis

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Abstract Arbuscular mycorrhizal symbiosis is a beneficial association between plant roots and fungi that occurs in approximately 80 % of terrestrial plants and which confers different benefits including mineral nutrient acquisition and enhanced defense capacity. Although mycorrhizal colonization takes place in roots, the symbiosis establishment has systemic effects in other parts of the plant, in processes such as nutrient translocation and systemic resistance. In order to understand the transcriptional changes that occur in leaves of mycorrhizal plants, we used RNA-seq technology to obtain the transcriptomes of leaves from mycorrhizal and non-mycorrhizal tomato plants (*Solanum lycopersicum*). Four weeks after inoculation with the fungus *Rhizophagus irregularis*, leaves from mycorrhizal and non-mycorrhizal tomato plants were used for transcriptome sequencing. Of the 21,113 genes expressed in tomato leaves, 742 genes displayed differential expression between the mycorrhizal and non-mycorrhizal conditions. Most of the transcriptional changes occurred in the "protein," "RNA," "signaling," "transport," "biotic and abiotic stresses," and "hormone metabolism" categories. Some transcriptional changes also occurred in P, N, and sugar transporters, as would be expected for mycorrhizal colonization. Finally, several differentially expressed genes may be related to systemic defense priming, in agreement with our demonstration that symbiotic plants exhibited mycorrhiza-induced resistance against the foliar pathogen *Xanthomonas campestris* pv. *vesicatoria*. This is the first study to take on a genome-wide analysis aimed at understanding the expression changes in leaves of mycorrhiza-colonized plants. The results will therefore be valuable to future analyses focused on specific genes, as well as detailed studies of the expression profiles of certain gene families.

Keywords *Rhizophagus irregularis* · Transcriptome sequencing · Mycorrhiza-induced defense · RNA-seq technology

Introduction

Arbuscular mycorrhizal symbiosis (AMS) is a mutualistic association between plant roots and arbuscular mycorrhizal fungi (AMF). In this mycorrhizal association, the primary benefit for both symbionts is nutrient exchange (Smith and Read 2008). The fungus mainly supplies phosphorous, nitrogen, and other mineral nutrients to the plant; in return, the plant provides carbohydrates to the fungus (Smith and Read 2008). In addition to facilitating host plant nutrient absorption, AMF induce an increased resistance against biotic and abiotic stresses (Campos-Soriano et al. 2012; Hause and Fester 2005; Kapoor et al. 2013; Pozo and Azcon-Aguilar 2007; Ruiz-Lozano et al. 2006; Whipps 2004).

Molecular approaches have been applied to investigate several processes involved in AMS, allowing the identification

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and characterization of many genes that are important for this interaction. Examples of these roles include the following: establishing the symbiosis in the root (Harrison 2012), nutrient acquisition from the soil to the fungus and then to the root (Govindarajulu et al. 2005; Javot et al. 2007), and an increased resistance to root and foliar pathogens, as well as abiotic stresses (Campos-Soriano et al. 2012; Kapoor et al. 2013; Mora-Romero et al. 2015). In line with the occurrence of mycorrhizal colonization in roots, the differential expression of genes in roots of mycorrhizal vs. non-mycorrhizal plants (as observed by microarray analysis) has been reported for *Medicago truncatula* (Liu et al. 2007), *Lotus japonicus* (Guether et al. 2009), tomato (*Solanum lycopersicum*) (Fiorilli et al. 2009), and rice (*Oryza sativa*) (Güimil et al. 2005).

Several systemic effects on shoots have also been reported in response to mycorrhizal colonization of roots, including an increase in resistance to shoot pathogens and abiotic stresses (Campos-Soriano et al. 2012; Mora-Romero et al. 2015; Ouziad et al. 2006; Pozo et al. 2010). Interestingly, far fewer studies have reported the differential expression of genes in shoots of mycorrhizal plants. One such study in *M. truncatula* identified 599 genes that were differentially expressed in the shoots of plants colonized by the AMF *Glomus intraradices*, consisting of 468 up-regulated and 131 down-regulated genes (Liu et al. 2007). A significant number of the differentially expressed genes were associated with defense responses, which is consistent with the observed increase in resistance of *M. truncatula* mycorrhizal plants against a foliar pathogen. Separately, 422 genes were reported as modulated in the shoots of mycorrhiza-colonized tomato using a microarray strategy, including 85 up-regulated and 337 down-regulated genes (Fiorilli et al. 2009).

These two studies clearly demonstrate the ability of microarrays to provide useful information on differentially expressed genes. Nevertheless, microarrays are limited to the annotated genes available at the moment, and thus, the results from these studies are not comprehensive for all the transcriptional changes induced by AMS in shoots.

This lack of coverage can be circumvented with next-generation sequencing technologies. In this approach, RNA-seq can be utilized with the tomato genome and transcriptome sequence (The Tomato Genome Consortium 2012) to identify other important genes involved in the systemic response of plants following mycorrhizal colonization. Massive RNA sequencing presents other advantages in comparison to microarrays, including the elimination of cross-hybridization artifacts from microarrays and the identification of a greater number of differentially expressed genes (Marioni et al. 2008; Xu et al. 2013). Recently, RNA-seq technology was used to demonstrate that AMS can induce changes in the expression of genes related to tomato ripening (Zouari et al. 2014). Here, we present the first study of the transcriptional changes that occur in leaves of mycorrhizal tomato plants, which we have used to investigate the systemic changes induced by AMS in shoots. Furthermore, we report that leaves of colonized plants display increased resistance in response to a foliar pathogen, consistent with the altered regulation of genes related to biotic stress occurring prior to pathogen infection.

Materials and Methods

Plant Growth and Tissue Collection

Mycorrhizal and non-mycorrhizal tomato plants (*S. lycopersicum* var. Missouri) were generated. Seeds were surface-sterilized in 70 % ethanol (5 min) and 5 % sodium hypochlorite (30 min) and rinsed five times in sterile distilled water. Seeds were planted in germination trays with a mix of sterilized vermiculite/sand (3:1 *v*/*v*) and maintained at 25 °C. Four 4-week-old tomato plants per treatment were transplanted individually to 1-L pots with the same substrate. One half of the plants were inoculated with 0.1 g of *Rhizophagus irregularis* (previously *G. intraradices*) colonized transformed carrot roots grown in M media (Chabot et al. 1992) and maintained in a growth room with a 16:8-h photoperiod (light/dark) at 25 °C (MYC treatment). Control plants (non-MYC treatment) were inoculated with non-mycorrhizal carrot roots and grown under the same conditions.

Plants were watered once per week with distilled water and twice per week with a modified Hoagland's solution containing the following: $(Ca(NO_3)_2{\cdot}4H_2O$, 2.5 mM; KNO_3, 2.5 mM; $MgSO_4{\cdot}7H_2O$, 1 mM; NaFe EDTA, 0.05 mM; H_3BO_3, 10 μM; $Na_2MoO_4{\cdot}2H_2O$, 0.2 μM; $ZnSO_4{\cdot}7H_2O$, 1 μM; $MnCl_2{\cdot}4H_2O$, 2.0 μM; $CuSO_4{\cdot}5H_2O$, 0.5 μM; $CoCl_2{\cdot}6H_2O$, 0.2 μM; HCL, 25 μM; MES buffer, 0.5 mM) (Hoagland and Arnon 1950). Phosphate concentration of the solution was adjusted to 0.05 mM by lowering the content of KH_2PO_4 to favor mycorrhizal colonization. The *Xanthomonas campestris* infection experiment included a set of MYC and non-MYC plants fertilized with 0.2 mM KH_2PO_4 (Liu et al. 2007).

Plants were harvested 4 weeks after AMF inoculation, and the fresh weight of shoots was measured. The shoot and one half of the root system of each plant were immediately frozen in liquid nitrogen and stored at −70 °C. All leaves pooled from each plant were ground to a fine powder in liquid nitrogen.

The other half of each plant's root system was fixed in 50 % (*v*/*v*) ethanol for 24 h, clarified for 2 days in 20 % (*w*/*v*) KOH, and stained for 1 day in a solution containing trypan blue 0.05 % (*w*/*v*) (Phillips and Hayman 1970). Roots were maintained in lactoglycerol 1:1:1 (water/lactic acid/glycerol), and the percentage of colonization was determined according to the "line intersection" method (Giovannetti and Mosse 1980).

RNA Extraction and Sequencing

Total RNA from leaves of three biological replicates (individual plants) of MYC and non-MYC plants fertilized with 0.05 mM phosphate was obtained using TRIzol® reagent (Ambion, Carlsbad, CA). Total RNA was purified again with an in-column purification step (Qiagen; Hilden, Germany). The concentration of total RNA was estimated using a NanoDrop 2000c Spectrometer (Thermo; USA), as well as A260/280 and A260/230 ratios. RNA samples were sent to the Unidad de Biotecnología Genómica (LANGEBIO, CINVESTAV-IPN Irapuato, México) for sequencing. Before processing, RNA quality was verified using the Agilent RNA 6000 Nano Kit. Six independent complementary DNA (cDNA) libraries, corresponding to three MYC and three non-MYC biological replicates, were constructed using the TruSeq RNA Sample Preparation V2 (Illumina) according to the manufacturer's instructions. cDNA library quality and fragment length were verified using an Agilent DNA 1000 Kit. Fragment length distributions were within the expected range according to the CLC software, and the average read length was 133 bp. The resulting cDNA libraries were sequenced in a paired-end system (2×150 bp) with the Illumina MiSeq system. Samples were individually labeled for library construction and were simultaneously run in one lane. The raw sequences were deposited as a Sequence Read Archive at the National Center for Biotechnology Information [GenBank: PRJNA263841].

Bioinformatics Analyses

Sequences obtained for each of the six libraries were imported and individually processed using the CLC Genomics Workbench, version 4.9 (CLC bio; Aarhus, Denmark). Sequencing coverage was estimated according to the Lander–Waterman statistics (Lander and Waterman 1988), by dividing the total number of acquired sequence bases per condition by the number of reference bases. For quality filtering, a trimming step was performed on raw reads with the following parameters: Phred quality score ≥20, Q value ≤5 %, ambiguous nucleotides ≤2, and read length ≥75 bp. High quality reads were then mapped against the tomato reference transcriptome "ITAG2.4_cdna" consisting of 34,725 protein-coding genes (http://solgenomics.net/), using the following mapping parameters: insertion cost=3, deletion cost=3, mismatch cost=2, mismatches ≤2, minimum length fraction ≥0.9, and minimum similarity fraction ≥0.8. The distance for paired reads was set from 1 to 350 bp according to the size of the libraries, which for CLC mapper must include the length of the reads plus the distance between them. The number of uniquely mapped reads per gene was used as expression values and represented as unique gene reads (UGR). The resulting UGR were subjected to normalization by the Scaling method (Bolstad et al. 2003), which is part of the CLC Genomics Workbench for removing the bias of sequencing depth across samples. Resulting expression values were then expressed as normalized UGR. Differentially expressed genes (DEGs) were obtained by Baggerly's test (Baggerly et al. 2003), which is included in the CLC Genomics Workbench, and multiple testing corrections were performed by controlling the false discovery rate (FDR) (Benjamini and Hochberg 1995) of p value. To be considered as differentially expressed or mycorrhiza-regulated, genes had to have at least 10 UGR, an FDR ≤0.05, and a Log 2 fold change (Log2FC) of ≥1 and ≤−1. To interpret the results, the DEGs were arranged into two classes: "up-DEG," genes that were up-regulated in MYC plants in comparison to the expression in non-MYC plants and "down-DEG," genes that were down-regulated in MYC plants in comparison to the expression in non-MYC plants. A principal component analysis was performed on the DEGs of the three replicates from each condition using the STATGRAPHICS Centurion XVI software (version 16.1.03). This analysis indicated that biological replicates from each condition grouped together and segregated from the other conditions (data not shown).

Functional Classification

All mycorrhiza-responsive genes were visualized using the MAPMAN tool (software version 3.5.1) (Thimm et al. 2004; Usadel et al. 2006). Lists of gene identifiers and DEGs Log2FC values were imported into the MAPMAN software. Mapping files were created using the Mercator tool (http://mapman.gabipd.org/web/guest/mercator), which bins all transcripts according to hierarchical ontologies. We used default parameters and the ITAG2.4 tomato transcriptome for annotation. Functional categories were tested for significance using the Wilcoxon rank sum test included in MAPMAN software, and significant (P<0.05) BINS were displayed.

Validation by qPCR

The quantitative RT-PCR experiments were carried out on the same RNA used for the RNA-seq procedure. All RNA samples were treated with the Turbo DNA-free™ kit (Ambion; Austin, TX, USA) to remove any genomic DNA contaminant before qRT-PCR analyses, according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA with the Superscript III reverse transcriptase kit (Invitrogen), following the manufacturer's instructions.

Quantitative RT-PCR reactions were performed in triplicate for each of the three biological replicates, using a Rotor Gene-Q Real time PCR system (Qiagen). PCR reactions were run in a total reaction volume of 10 μL, which included 5 μL of SYBR Green master mix (Qiagen, cat. no. 204074), 200 nM

of each primer, and 50 ng of cDNA. The PCR program included a preheating step at 95 °C (5 min), followed by 40 cycles of a 95 °C (5 s) and another step of at 60 °C (10 s). Dissociation curves were performed at the end of each run.

The genes glyceraldehyde-3-phosphate dehydrogenase and 60S ribosomal protein were used for normalization. The set of primers used in this work are listed in Supplementary Table 1. PCR efficiency was determined from standard curves constructed from serial dilutions of cDNA (from 1 to 100 ng) taken from non-MYC samples. The comparative threshold cycle method $2^{-\Delta\Delta Ct}$ was used as the analysis method for the relative RNA expression, as previously described (Salvioli et al. 2012).

Infection with *X. campestris* pv. *vesicatoria*

Bacteria were grown in nutritive agar and resuspended in 4 L of sterilized water at a final concentration of 1.6×10^5 CFU/mL for use in the dipping infection assays. Four weeks after mycorrhizal inoculation, shoots of MYC and non-MYC plants fertilized with 0.05 mM PO_4 and 0.20 mM PO_4 were infected with *X. campestris* pv. *vesicatoria*. Plants were surfaced-rubbed to induce slight trichome damage, and shoots were submerged in the bacterial suspension. Pots were individually placed in plastic bags and maintained at 28 °C for 24 h and then transferred to 25 °C, where bags were perforated to allow gas exchange. Finally, the total number of lesions per square centimeter of leaf was counted 10 days after infection.

Statistical Analysis

X. campestris pv. *vesicatoria* infection experiments were analyzed using a completely randomized design. Multifactorial ANOVA was calculated using SAS system 9.0 (Windows version 6.2.9200). Comparisons of means were made using Tukey's test at a 95 % confidence level.

Results

Mycorrhizal Colonization

To investigate changes in gene expression induced by AMS in tomato leaves, 4-week-old plants were inoculated with in vitro cultured carrot roots colonized by the AMF *Rhizophagus irregularis* (MYC condition); control plants were inoculated with the corresponding non-mycorrhizal carrot roots (non-MYC condition). Mycorrhizal colonization was 84.48 %±12 4 weeks post-inoculation, whereas no fungal colonization was observed in non-MYC plants. RNA from shoots of these plants was then purified and sequenced.

Bioinformatics Analysis of RNA-seq Data

After trimming and applying quality filters, high-quality reads ranged from 813,704 to 1,084,964 reads for individually sequenced libraries. Overall, approximately 76 % of the reads mapped to one specific locus of the reference transcriptome and were used for subsequent analysis. Of sequenced reads, 88.3 % mapped as proper pair reads, whereas the other 11.7 % mapped as single (broken) reads and were also included in the analysis (Table 1). It is possible that some of the non-matching reads (24 % of the total high-quality reads) corresponded to sequences that map to more than one locus and were discarded by the program before further analysis or as a sequencing error.

Taking into account the overall number of reads in the three biological replicates per condition (MYC and non-MYC) and the average read length, the overall coverage in the MYC condition was 5.7X. In contrast, the overall coverage in the non-MYC condition was 6X.

Differentially Expressed Genes

The published tomato transcriptome includes 34,725 genes, which is composed of both experimentally and theoretically

Table 1 Read numbers obtained during the bioinformatics analysis

Sample replicates	Total reads per sample (raw data)	Quality filter	Mapping to the reference transcriptome			
		Reads after trim step	Total matching reads	Proper pairs	Broken reads	Non-matching reads
Non-MYC 1	931,518	923,153	699,958	620,582	79,376	223,195
Non-MYC 2	1,095,022	1,084,964	821,502	727,920	93,582	263,462
Non-MYC 3	903,842	896,728	694,667	606,730	87,937	202,061
MYC 1	973,020	964,384	732,180	648,924	83,256	232,204
MYC 2	820,798	813,704	608,237	536,642	71,595	205,467
MYC 3	1,017,126	1,007,876	758,493	669,912	88,581	249,383

A quality filter (trim step) was applied on raw reads to obtain high-quality reads, which were mapped back to the reference transcriptome

described genes (The Tomato Genome Consortium 2012). In the present work, we found 21,113 genes expressed in tomato leaves, including both MYC and non-MYC samples.

DEGs were identified by comparing their expression levels under MYC and non-MYC conditions, using the CLC Genomics Workbench. This resulted in the identification of 742 loci as MYC-responsive genes.

Among the 742 DEGs, 506 were up-DEG, and 236 were down-DEG (Supplementary Table 2). Regulation of expression of most of the differential genes in AMS was modest, in that 41 % of the MYC-regulated genes showed an increase in expression that was only 2- to 3-fold (Log2FC 1.0 to 1.57), while 25 % of the genes presented a Log2FC between −1.0 and −1.57. These two expression groups represent more than 60 % of the DEG genes presented in this work. Less than 5 % of the genes increased their expression by 7-fold or more (Log2FC ≥2.8), and only 0.1 % drastically decreased their expression in response to AMS (Log2FC ≤−2.8) (Fig. 1).

As stated in the "Materials and Methods" section, only those genes with at least 10 UGR were considered for the differential expression analysis. This strategy increased the stringency of the analysis by eliminating genes with a low expression. Nevertheless, the genes that were only expressed in the MYC condition (IND, or completely induced) but not in the non-MYC condition, and vice versa (REP, or completely repressed), were inevitably discarded by this analysis despite the fact that they passed the FDR ≤0.05 and Log2FC ≥1 and ≤−1 filters. A total of 58 genes were completely induced, and 31 were completely repressed. Since it is possible that some of these discarded genes have biological relevance, they are presented in Supplementary Table 3.

Functional Categorization of Differentially Expressed Genes

Analyses of the 742 myc-responsive genes, including annotation and functional classification, were performed with the MAPMAN software (Table 2, Supplementary Table 2). This program sorted 23 genes into more than one category, resulting in 768 outputs after categorization (Table 2). Genes that were categorized more than once are presented in Supplementary Table 4. MAPMAN classified the MYC-responsive genes in 26 out of the 35 functional categories (Table 2). The six categories containing the highest number of DEGs (excluding "not-assigned or unknown" and "miscellaneous enzyme") include the following: "protein" with 126 genes, "signaling" with 91 genes, "RNA" with 80 genes, "transport" with 42 genes, "biotic and abiotic stress" with 34 genes, and "hormone metabolism" with 32 genes. Differentially expressed genes in these six categories represent approximately 53 % of the total MYC-responsive genes identified in this work. The remaining genes were distributed among the other 20 categories, including not-assigned or unknown with 161 genes (approximately comprising 21 % of all DEG) and miscellaneous enzymes with 54 DEGs (Table 2).

In addition, we used the Wilcoxon rank sum test to select genes in functional categories or sub-categories that show consistent expression changes, which could be indicative of a given response. The significant functional categories identified by this test are shown in Fig. 2. The majority of the subcategories identified by the Wilcoxon test contained either up- or down-regulated genes.

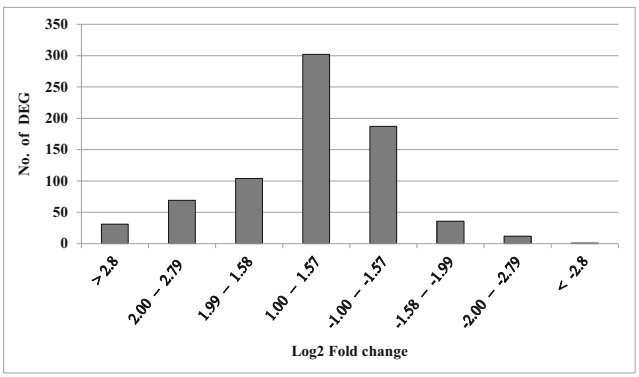

Fig. 1 Number of mycorrhizal DEGs grouped by range of Log 2 fold change

Genes Involved in Posttranslational Regulation

Except for the not-assigned or unknown category, the protein category BIN 29 includes the highest number of MYC-responsive genes which were significant by the Wilcoxon test. In this category, 86 genes were up-DEG and 40 genes were down-DEG (Table 2, Supplementary Table 2). Two subcategories were mainly represented: "protein posttranslational modifications" (BIN 29.4; 53 genes) and "protein degradation" (BIN 29.5; 57 genes). Posttranslational modification of proteins has been proposed as one of the main regulatory mechanisms that could modulate responses such as defense priming in leaves of mycorrhizal plants (Pozo and Azcon-Aguilar 2007). Notably, within the "protein posttranslational modifications subcategory" (BIN 29.4), different kinase-like genes displayed a MYC-regulated expression. Twenty such genes were annotated as "receptor-like cytoplasmic kinase VII," including 14 up-DEG and six down-DEG genes (Supplementary Table 2). Receptor kinases are key enzymes that regulate diverse and important biological processes. Additionally, a group of 13 phosphatases was identified as MYC-responsive in this subcategory, indicating roles for protein phosphorylation and dephosphorylation mechanisms in leaves of mycorrhizal plants. Thirty-five of the 57 genes listed in the protein degradation subcategory were ubiquitin-related, possibly reflecting the importance of ubiquitin degradation mechanisms in leaves of mycorrhizal plants.

Table 2 Mycorrhizal-responsive genes grouped into functional categories according to the MAPMAN software program

BIN	Category	Total no. of genes per category	% of total mycorrhiza-responsive genes	No. of up-DEG	No. of down-DEG
35	Not assigned or unknown	161	20.99	95	66
29	Protein	126	16.43	86	40
30	Signaling	91	11.86	70	21
27	RNA	80	10.43	46	34
26	Miscellaneous enzyme families	54	7.04	37	17
34	Transport	42	5.48	38	4
20	Biotic/abiotic stress	34	4.43	28	6
17	Hormone metabolism	32	4.17	24	8
31	Cell	25	3.26	19	6
16	Secondary metabolism	21	2.74	17	4
33	Development	21	2.74	15	6
11	Lipid metabolism	19	2.48	17	2
10	Cell wall	18	2.35	11	7
28	DNA	9	1.04	1	8
13	Amino acid metabolism	7	0.91	6	1
2	Major CHO metabolism	5	0.65	2	3
23	Nucleotide metabolism	4	0.52	1	3
1	Photosynthesis	3	0.39	2	1
3	Minor CHO metabolism	3	0.39	3	0
4	Glycolysis	2	0.26	2	0
8	Tricarboxylic acid (TCA cycle)/org. transformation	2	0.26	1	1
15	Metal handling	2	0.26	0	2
18	Co-factor and vitamin metabolism	2	0.26	2	0
19	Tetrapyrrole synthesis	2	0.26	2	0
22	Polyamine metabolism	2	0.26	2	0
7	Oxidative pentose phosphate (OPP)	1	0.13	1	0
	Total	768	100.00	528	240

No DEGs were identified in the following nine software categories: 5 (fermentation), 6 (gluconeogenesis/glycolate cycle), 9 (mitocondrial electron transporter), 12 (N metabolism), 14 (S assimilation), 21 (redox), 24 (biodegradation of xenobiotics), 25 (C1 metabolism), and 32 (microRNAs, natural antisense)

Up-DEG up-regulated genes, *Down-DEG* down-regulated genes

Genes Involved in RNA Processing and Transcription Regulation

The RNA category (BIN 27) was also abundantly represented by MYC-responsive genes. Among the 80 DEGs listed in this category, seven genes were categorized with RNA processing roles (BIN 27.1), whereas 71 (89 %) were related to transcription regulation (BIN 27.3 and subsequent levels). Twenty-eight different types of transcription factors were MYC-regulated (Fig. 3). The two most numerous groups of genes in this subcategory were as follows: the WRKY transcription factors (BIN 27.3.32) with seven MYC-responsive genes, all of which were up-DEG, and the MYB transcription factors (BIN 27.3.25) with three up-DEG and four down-DEG genes. Other identified MYC-regulated transcription factors include ethylene-responsive transcription factors (AP2/EREBP, APETALA2/ethylene-responsive element-binding protein family; BIN 27.3.3), which composed one up-DEG and four down-DEG genes, and basic helix-loop-helix (bHLH) transcription factors (BIN 27.3.6), with six DEGs (comprising four up-DEG and two down-DEG genes) (Fig. 3, Supplementary Table 2). The three genes categorized in BIN 27.3.35 and annotated as bZIP transcription factors exhibited statistical significance, according to the Wilcoxon test (Fig. 2), suggesting that they may have a coordinated response regarding mycorrhizal colonization.

Genes Involved in Signaling

The signaling category (BIN 30) contains 91 genes that were MYC-responsive. Forty-seven such genes (approximately 52 % of the genes in this category) were identified as

Category name	Category Bin	Up-DEG		Dn-DEG		Total Genes	Log2 FC mean
		Genes	Log2 FC	Genes	Log2 FC		
cell wall.cellulose synthesis.cellulose synthase	10.2.1	2		-		2	
cell wall.degradation	10.6	-		3		3	
lipid metabolism	11	17		2		19	
HM.auxin.induced-regulated-responsive-activated	17.2.3	7		-		7	
HM.jasmonate.synthesis-degradation	17.7.1	2		-		2	
HM.jasmonate.signal transduction	17.7.2	3		-		3	
misc.oxidases - copper, flavone etc.	26.7	4		-		4	
misc.peroxidases	26.12	2		-		2	
misc.GDSL-motif lipase	26.28	-		3		3	
RNA.RT.bZIP transcription factor family	27.3.35	-		3		3	
DNA.synthesis/chromatin structure.histone	28.1.3	-		6		6	
protein.degradation.aspartate protease	29.5.4	4		-		4	
signalling.receptor kinases.leucine rich repeat XI	30.2.11	19		4		23	
signalling.receptor kinases.DUF 26	30.2.17	18		2		20	
transport	34	39		4		42	

Log2 FC: 3, 1, 0, -1, -3

Fig. 2 Categories and subcategories identified by the Wilcoxon rank sum test as significant ($P<0.05$). The average expression changes (Log2FC) of all the genes per subcategory are represented on a color scale: *Up-DEG* up-regulated genes (*blue*), *Dn-DEG* down-regulated genes (*red*). *HM* hormone metabolism, *misc* miscellaneous enzyme, *RT* regulation of transcription

different receptor kinases including DUF26 receptor kinases (20 genes) and receptor kinases with a leucine-rich repeat XI (23 genes) (Supplementary Table 2). Notably, 19 genes related to calcium signaling were MYC-responsive, including 17 up-DEG genes. This category also contained 10 genes involved in sugar and nutrient physiology (four up-DEG and six down-DEG) and seven genes that were classified as G-proteins (six up-DEG and one down-DEG genes). Subcategories 30.2.11 (signaling.receptor kinases.leucine-rich repeat XI) and 30.2.17 (signaling.receptor kinases.DUF 26), containing 19 and 18 genes, respectively, were significant according to the Wilcoxon test (Fig. 2).

Genes Involved in Transport Processes

The transport category (BIN 34) contains 42 genes that were differentially expressed in leaves of mycorrhizal plants, including 38 up-DEG and four down-DEG genes. Two of these transport genes were annotated as ABC transporters and multidrug resistance systems. Five sugar transporters were listed as MYC-responsive genes, all of which were up-DEG (Supplementary Table 2). Four amino acid transporter genes were MYC-responsive and were all up-DEG. Two ammonium transporter genes were up-DEG, as well as two calcium transporter genes. Two MYC-responsive peptide and oligopeptide

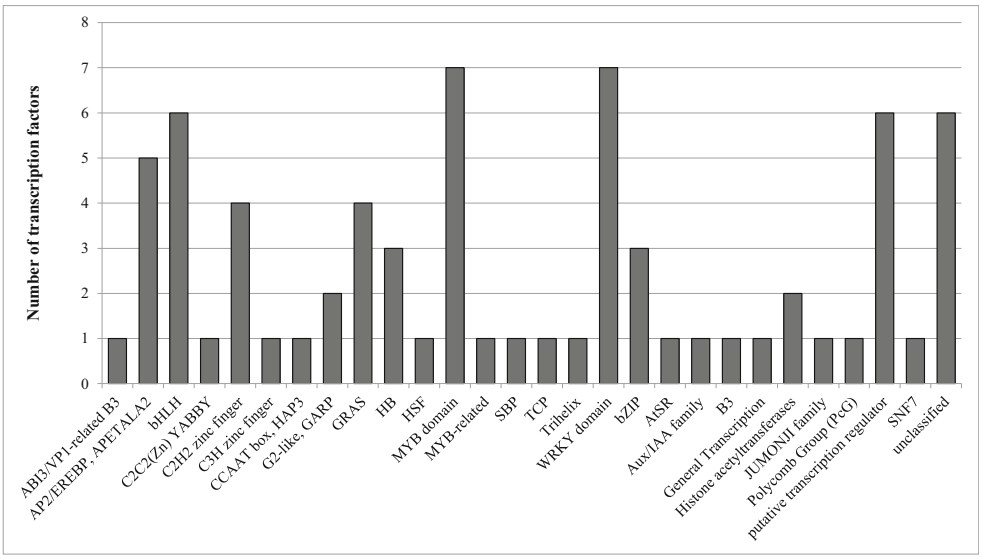

Fig. 3 Number of transcription factors identified in different gene families that are regulated by mycorrhizal colonization

transporter genes were up-DEG, and one was down-DEG (Supplementary Table 2). The transport category was statistically significant, as determined by the Wilcoxon test (Fig. 2).

Genes Involved in Abiotic and Biotic Stresses

The "abiotic and biotic stress" category (BIN 20) contains 34 genes that were identified as MYC-responsive, 18 of which were related to biotic stress and 16 to abiotic stress (Supplementary Table 2). Notably, the jasmonate ZIP-domain proteins solyc03g122190.2.1 and solyc12g009220.1.1 were strongly up-DEG (displaying 10- and 7.8-fold changes, respectively; BIN 20.1.3) (Supplementary Table 2). Eight nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance proteins were also listed in this category (BIN 20.1); only one such gene was down-DEG, whereas the rest were up-DEG. One chitinase gene classified in this category was up-DEG. The abiotic stress subcategory (BIN 20.2 and sublevels) contains 16 genes, including seven related to heat stress and three related to drought and salt stress.

Genes Involved in Hormone Metabolism

The hormone metabolism category (BIN 17) contains 32 genes that were MYC-responsive, 24 of which were up-DEG (75 %). Nine genes in the auxin metabolism subcategory (BIN 17.2) were differentially expressed and were all up-DEG. One gene was associated with auxin signal transduction (solyc02g088010.2.1, BIN 17.2.2), one gene was involved in the release of active indole-3-acetic acid from conjugates (BIN 17.2.1), and eight genes were classified as being either induced by, regulated by, or responsive to auxins (Supplementary Table 2). One cytokinin dehydrogenase (BIN 17.4.1) was up-DEG and categorized as a MYC-responsive gene. The subcategory with the highest number of genes within the hormone metabolism category contains 15 genes and is related to the hormone ethylene. Eleven of these genes are related to ethylene signal transduction (BIN 17.5.2), including five up-DEG and six down-DEG genes. Regarding the synthesis and/or degradation of ethylene (BIN 17.5.1), four genes were annotated as MYC-responsive, all of which were up-DEG. In BIN 17.7, a group of five genes related to the metabolism and signal transduction of the hormone jasmonic acid was notably up-DEG (Supplementary Table 2). Three subcategories within the hormone metabolism category were statistically significant, as determined by the Wilcoxon test (Fig. 2), indicating their potential relevance in the mycorrhizal response.

Other Processes Regulated by Mycorrhizal Colonization

The six categories described above comprise 405 genes, representing approximately 53 % of the MYC-responsive genes identified in this work. The remaining genes were classified within the 20 additional categories. Some of these latter categories include the "cell" category (BIN 31); 14 of the 25 genes are involved in cell organization (BIN 31.1), 11 of which were up-DEG. The second largest subcategory in the cell category was "vesicle transport" (BIN 31.4); notably, all five genes listed in this group were up-DEG (Supplementary Table 2). In the "secondary metabolism" category (BIN 16), 21 genes were myc-responsive. Fifteen genes in this category were related to phenylpropanoid metabolism (BIN 16.2); eight of these genes were related to lignin biosynthesis (BIN 16.2.1), including seven up-DEG and one down-DEG. Three genes related to flavonoid metabolism (BIN 16.8) were also listed as mycorrhizal-responsive, and three other genes were related to isoprenoid metabolism (BIN 16.1). In the "lipid metabolism" category (BIN 11), 19 genes were identified as mycorrhizal-responsive; several genes in this group are involved in different types of lipid degradation (9), fatty acid synthesis and elongation (2), and phospholipid synthesis (2) (Supplementary Table 2).

Three genes associated with the "photosynthesis" category (BIN 1) are mycorrhizal-responsive; two of these genes have roles in the light reactions, whereas the other gene was related to the Calvin cycle. The ten categories with the lowest number of MYC-responsive genes contain less than 25 genes (Supplementary Table 2).

Validation of Sequencing Results by qPCR

A subset of genes was randomly selected for validation by quantitative RT-PCR, using RNA from biological samples employed for the sequencing analysis. Primers for the selected sequences were designed to specifically amplify gene family sequences (Supplementary Table 1). The glyceraldehyde-3-phosphate dehydrogenase (Solyc04g009030.2.1) gene and the 60S ribosomal protein L21-like protein (Solyc10g078960.1.1) gene were used as normalizers. Eighty percent (12 out of 15) of the genes examined by qPCR displayed the same expression trend as observed in the RNA-seq experiment (Table 3).

Mycorrhizal Tomato Leaves Display Induced Resistance Against *X. campestris* pv. *vesicatoria*.

Eighteen myc-responsive genes from this study were categorized in the "biotic stress" subcategory (BIN 20.1) (Supplementary Table 2), suggesting that mycorrhiza-colonized plants may be able to activate a resistance mechanism prior to a potential pathogen attack. To test whether mycorrhizal plants can manifest resistance to a foliar pathogen, non-mycorrhizal and mycorrhiza-colonized tomato plants fertilized with Hoagland's solution containing 0.05 mM phosphate were challenged with the foliar pathogen *X. campestris* pv. *vesicatoria*. To investigate whether the induction of resistance could be due to mycorrhizal colonization and not phosphate

Table 3 Validation of RNA-seq results by quantitative PCR

ID	Gene	Log2FC	
		RNA-seq	q-PCR
Solyc03g116890.2.1	WRKY transcription factor 2	4.41	2.92
Solyc03g122190.2.1	Jasmonate ZIM domain 2	3.33	1.22
Solyc10g076480.1.1	Ammonium transporter (ch10)	3.12	2.36
Solyc03g122340.2.1	Lipoxygenase (ch03)	2.63	1.36
Solyc07g049530.2.1	1-Aminocyclopropane-1-carboxylate oxidase	1.98	1.98
Solyc06g068990.2.1	Mitogen-activated protein kinase	−0.37	−0.36
Solyc03g045070.1.1	Ammonium transporter (Ch03)	−0.76	−0.54
Solyc06g051860.1.1	Inorganic phosphate transporter 6	−2.55	−0.69
Solyc02g069060.2.1	Phloem lectin	IND	1.40
Solyc08g029000.2.1	Lipoxygenase (Myc ch8)	IND	5.63
Solyc04g081860.2.1	Peroxidase	NE	NE
Solyc06g051850.1.1	Inorganic phosphate transporter 6 (PT4)	NE	NE
Solyc08g062620.2.1	TP53-regulated inhibitor of apoptosis 1	3.04	0.38[a]
Solyc02g062000.2.1	RUN and FYVE domain-containing protein 1	1.20	0.23[a]
Solyc05g050340.2.1	WRKY transcription factor 6	0.86	1.11[a]

Glyceraldehyde-3-phosphate dehydrogenase and 60S ribosomal protein were used for normalization. Genes with a Log2FC value ≥1 were considered as up-regulated, whereas genes with a value ≤−1 were considered down-regulated

Ind completely induced gene in mycorrhizal plants, *NE* gene with no expression in leaves and used as negative control for q-PCR

[a] No validated gene expression

nutritional status, mycorrhizal and non-mycorrhizal plants fertilized with 0.20 mM phosphate were obtained and challenged with the pathogen. Colonization percentage and shoot and root growth were determined right before *X. campestris* pv. *vesicatoria* infection, as well as 10 days after infection. Although an increase in shoot growth was observed for plants fertilized with 0.2 mM phosphate, no differences in colonization percentage were found between the two plants in either fertilizing regime (Table 4). When MYC and non-MYC tomato plants were challenged with *X. campestris* pv. *vesicatoria*, mycorrhizal plants displayed fewer necrotic lesions/per leaf area than non-mycorrhizal plants, for both 0.05 and 0.20 mM phosphate fertilization conditions (Table 5). This indicates that mycorrhizal colonization triggers a priming state that prepares the plant for an increased resistance response, which occurs irrespective of the phosphate nutritional status. These findings suggest a role for the differential regulation of some biotic stress genes (and possibly other DEGs identified in this work) in preparing the plants for a faster and stronger response to a subsequent pathogen attack.

Discussion

Mycorrhizal colonization induces important changes in plant roots, many of which are necessary to physically

Table 4 Colonization percentage and fresh weight (g) growth in *R. irregularis* mycorrhizal and non-mycorrhizal tomato plants

Factor	Level	Fresh weight (g)		Colonization percentage in mycorrhizal plants	
		Shoot	Root	0.05 PO_4	0.20 PO_4
Mycorrhiza	+ *Ri*	5.30 a	2.28 a	84.48±12	85.06±9
	– *Ri*	5.89 a	2.40 a		
Phosphate (PO_4)	0.05	4.95 b	2.40 a		
	0.20	6.24 a	2.28 a		

Plants were fertilized with Hoagland's solution containing low (0.05 mM) and normal (0.20 mM) phosphate, and results were analyzed by a multifactorial ANOVA. Reported results are the means of four biological replicates per level. A similar letter indicates no significant difference in levels according to Tukey's test. ± Standard deviation is indicated

+*Ri* mycorrhizal plants, − *Ri* non-mycorrhizal plants, PO_4 phosphate nutrition level in micromolar

Table 5 Mycorrhiza-induced resistance in mycorrhizal tomato plants

Factor	Level	Severity (no. of lesions/cm^2 on leaf)
Mycorrhiza	+ *Ri*	4.88 b
	– *Ri*	7.50 a
Phosphate (PO_4)	0.05	6.32 a
	0.20	6.06 a

Severity of *X. campestris* pv. *vesicatoria* infection on tomato leaves estimated as the number of lesions per square centimeter on leaf. Statistical analysis per level of two factors: mycorrhiza and phosphate (PO_4). Different letters indicate significant differences per level according to a multifactorial ANOVA and Tukey's test (α=0.05). These results indicate that induced resistance is related to mycorrhizal symbiosis and independent of phosphate nutrition

accommodate the fungal symbiont within this organ. Several studies have reported on the extensive transcriptional modulation that occurs in mycorrhizal roots in different plant–AMF systems (Fiorilli et al. 2009; Guether et al. 2009; Güimil et al. 2005; Liu et al. 2007). Massive transcriptional modulation has also been reported in shoots, with the use of high-throughput transcriptional profiling analysis by microarrays (Fiorilli et al. 2009; Liu et al. 2007), and more recently in fruit tissue by RNA-seq (Zouari et al. 2014).

Here, we report the first massive transcriptome sequencing of leaves from mycorrhizal tomato plants. This methodology has generated 742 MYC-responsive genes, which represents a greater number of regulated genes than described in similar studies using microarrays. As a comparison, 599 genes were differentially expressed in leaves of *M. truncatula* mycorrhizal plants (Liu et al. 2007), while 422 genes were identified from shoots of mycorrhiza-colonized tomato (Fiorilli et al. 2009). Recently, the RNA-seq approach was used to obtain the tomato fruit transcriptome, which yielded 713 DEG (Zouari et al. 2014), similar to our results.

We used the MiSeq (Ilumina) sequencer, and the number of reads obtained was not as abundant as that normally obtained by other sequencers such as the HiSeq. However, taking into account the overall high-quality reads of all the biological replicates per condition, acceptable coverages were obtained: 5.7X for MYC samples and 6X for non-MYC samples. Furthermore, using three biological replicates per condition reinforces our results, since replication has a better impact on the RNA-seq outcome than the depth (Liu et al. 2014). We had defined stringent criteria in order to increase the certainty of our results regarding differential expressed genes, even though this could have resulted in the omission of actual differential genes, particularly DEGs with low expression. Nevertheless, a deeper sequencing analysis will be needed in order to complement this set of genes. The mycorrhiza-responsive genes identified in this work thus represent a base for further investigations into how aerial plant parts respond to mycorrhizal colonization.

Transport of Nutrients

Mycorrhizal colonization is known to increase the absorption of P and N in plants (Smith and Read 2008). Although mineral nutrient uptake primarily happens in the roots, minerals must also reach other plant organs (Smith et al. 2011). Pi loading from root cells to the xylem vessels, and its transfer from roots to shoots, is regulated by *AtPHO1* in Arabidopsis and *OsPHO1;2* in rice (Secco et al. 2010). Here, we report an up-DEG gene (solyc02g088230.2.1) in the "transport.phosphate" subcategory (Supplementary Table 2) that is annotated as a xenotropic and polytropic retrovirus receptor. Interestingly, this gene is also annotated as a *PHO1*-predicted protein when searched against the GenBank database using the BLAST program (100 % identity). This gene may therefore have a role in favoring phosphate movement in shoots of mycorrhizal plants. Translocation of phosphate from roots to shoots is required for shoot growth, and the regulation of this process may impact plant productivity (Secco et al. 2010). Therefore, a more detailed characterization of this myc-responsive gene may help understand some of the benefits that this mycorrhizal symbiosis confers to plants.

The differential regulation of ammonium transporters in the roots of mycorrhizal plants has previously been reported for several species (Kobae et al. 2010; Pérez-Tienda et al. 2014; Breuillin-Sessomsa et al. 2015). In contrast, there is currently no available information on the regulation of ammonium transporters in leaves of mycorrhizal plants. Our RNA-seq results indicate that two AMT are up-DEG in leaves of mycorrhizal plants (Supplementary Table 2). These data suggest that the nitrogen acquired by specific transporters in mycorrhizal roots is moved to the leaves by a regulated process, which possibly utilizes a specific set of transporters.

Five sugar transporters, including one high-affinity glucose transporter and four solute carrier family 2 transporters, were identified as differentially expressed in this study. Notably, all sugar transporters were up-DEG, which confirms the importance of sugar transport in shoots of mycorrhizal plants. Specifically, carbon assimilated into leaves (source cells) is transported to the sink organs. Before its utilization as a source of carbon and energy, sucrose must be hydrolyzed into glucose and fructose by either an invertase or a sucrose synthase (Fotopoulos 2005; Kruger 1990). In the present study, two up-DEG invertases were identified as differentially expressed, including the vacuolar invertase *TIV1* (Solyc03g083910.2.1) and the cell wall invertase *LIN6* (solyc10g083290.1.1) (Supplementary Table 2), which both cleave sucrose into hexoses. The precise role of each gene in leaves within the context of mycorrhizal symbiosis needs to be investigated in greater detail. The entire transport category was statistically significant

according to the Wilcoxon test (Fig. 2). This strongly suggests that these genes are relevant to how leaves respond to mycorrhizal colonization and are thus worthy of further study.

Modulators of the Resistance to Biotic Stress

In addition to their ability to increase mineral nutrient uptake, mycorrhizal colonization is well known to provide plants with resistance to abiotic and biotic stresses (Aroca et al. 2007; Porcel et al. 2006; Whipps 2004). Regarding biotic stresses, it has previously been documented that local and systemic defense responses are triggered by AMS, in a mechanism referred to as mycorrhiza-induced resistance (MIR) (Pozo and Azcon-Aguilar 2007). Mycorrhizal colonization prepares the plant for a faster and stronger response to a posterior pathogen attack through a mechanism known as priming, which precedes the manifestation of resistance and occurs both locally and systemically (Jung et al. 2012).

In the present study, we used RNA-seq to detect 742 genes that were differentially expressed in leaves of mycorrhizal tomato plants, some of which may be candidates for modulators of the systemic defense priming mechanism. Interestingly, a great number of DEGs were involved in posttranslational modification process (Supplementary Table 2). This is consistent with the hypothesis that posttransductional and posttranslational modifications mainly regulate the priming response (Cartieaux et al. 2008; Verhagen et al. 2004). Thus, it is possible that key proteins are transcribed and accumulated in plant tissues in an inactive form prior to any stress, which will then be ready for modification in response to pathogen attack. Upon attack, such "activated" proteins would act by themselves or with other proteins to trigger an effective and rapid defense response.

Specific transcription factors are likely to have a central role in the regulation of the mycorrhiza-induced response in leaves, as they are subject to discrete transcriptional regulation and can amplify a cellular signal following specific stimuli. Notably, 71 transcription factors were detected as MYC-responsive genes in leaves of tomato mycorrhizal plants. Seven WRKY transcription factors displayed differential expression in this study (Fig. 3) (Supplementary Table 2). These transcription factors are activated as a final step in amplification cascades induced by MPK genes, and they play a role in plant immunity (Conrath 2011). The up-regulation of a WRKY transcription factor in potato mycorrhizal roots was previously reported in response to pathogen challenge (Gallou et al. 2012), suggesting a role in the mechanism of systemic priming. A relevant aim for future study could therefore be to determine which transcription factors affected in leaves by mycorrhizal colonization are related to the different mycorrhizal-responsive pathways. Interestingly, three bZIP transcription factors belonging to the "bZIP transcription factor family" (BIN 27.3.35) were all down-regulated and identified as statistically significant by the Wilcoxon test (Fig. 2). This might indicate that coordinated negative regulation of transcription factors is a response mechanism of the leaves to mycorrhizal colonization in roots.

Ethylene has been implicated in plant defense responses (van Loon et al. 2006), and it has been suggested to participate in mycorrhizal priming (López-Ráez et al. 2010; Pozo et al. 2010); however, its roles are still not fully understood in these processes. In the present study, ethylene-related genes were identified as differentially expressed, confirming the importance of this plant hormone in the systemic response of plants to mycorrhizal colonization. Among the MYC-responsive ethylene-related genes, five ethylene-responsive transcription factors and three 1-aminocyclopropane-1-carboxylate oxidases (ACC oxidase) were identified in this study. The enzyme ACC oxidase is responsible for the final stage in the production of ethylene by higher plants (Prescott and John 1996). We found that the Solyc07g049530.2.1 gene, which shares 99 % identity with ACC-oxidase-1, was slightly up-regulated in MYC plants. Previous work in tomato has shown that ACC-oxidase-1 expression is barely detectable in green leaves but accumulates transcripts in leaves during senescence and in response to mechanical wounding (Barry et al. 1996; Davies and Grierson 1989; John et al. 1995). Another MYC-regulated (as well as up-regulated) gene that we identified was solyc09g010020.2.1. Indeed, its presence was somewhat anticipated in that it has been reported in libraries constructed from leaves of cv. Micro-Tom from different sources, including leaves treated with different pathogens such as tomato mosaic virus, *Pythium oligandrum*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Pseudomonas syringae* pv. tomato, as well as in leaves treated with salicylic acid and jasmonic acid (Aoki et al. 2010). Although no increased expression of ACC oxidase has been reported in response to pathogen attack in these previous studies, our results suggest that these genes could be involved in the mycorrhizal defense response.

We also identified five mycorrhizal-responsive ethylene-responsive element-binding proteins (EREBP) (Supplementary Table 2). EREB genes are differentially regulated in leaves of mycorrhizal *M. truncatula* (Liu et al. 2007) and tomato plants (Fiorilli et al. 2009), although their roles in the leaves of colonized plants remain unknown.

In addition to helping to establish mycorrhizal symbiosis and MIR, several oxylipins (including jasmonic acid and some of its derivatives) participate during the onset of priming, which occurs before the manifestation of this resistance (Campos-Soriano et al. 2012; Hao et al. 2012; Mora-Romero et al. 2015). Our results are consistent with these reports, since several genes related to the jasmonate biosynthetic and signaling pathways were differentially expressed in leaves of mycorrhizal tomato plants. Specifically, we found that two jasmonate ZIM proteins and one lipoxygenase were up-DEG (Supplementary Table 2).

Regulation of lipoxygenase expression in roots has been demonstrated during colonization with *Glomus mosseae* and *G. intraradices* (López-Ráez et al. 2010). Furthermore, previous results from our group indicate a role for the oxylipin pathway in common bean leaves during a priming state induced by mycorrhizal symbiosis (Mora-Romero et al. 2015).

In the current study, the lipoxygenase gene solyc08g029000.2.1 was found to be expressed in leaves of tomato plants colonized with *R. irregularis*, but not in non-colonized plants (Supplementary Table 3). In addition, the gene was found to be statistically significant according to the Wilcoxon test (subcategory 17.7.1) (Fig. 2). This gene was listed as completely induced (IND) in the transcriptome analysis; however, quantitative RT-PCR revealed a basal expression of this gene in non-mycorrhizal plants (Table 3). Although qPCR did not confirm that this gene was completely induced in the MYC condition, it did reveal that the gene is highly differentially expressed (5.63-Log2FC; Table 3). This tomato mycorrhiza-specific lipoxygenase gene shares 93–100 % identity with lipoxygenases from *Nicotiana tabacum*, *Nicotiana attenuata*, and *Adelostemma gracillimum*. The lipoxygenase gene also shares high coverage with the recently reported MYC-responsive *POTLX-3* (Gallou et al. 2012) and *POTLX1* genes from *Solanum tuberosum* and *LOX1* from *Capsicum annuum*, which encodes a protein related to the 9-LOX branch of the oxylipin pathway (Wasternack et al. 2006). Additionally, this lipoxygenase gene shows similarity to *PvLOX2* from *Phaseolus vulgaris*. This novel tomato mycorrhiza-induced lipoxygenase is more similar to tomato *LOXD* than to *LOXA*. Although our identification of this gene suggests that it could have a role in defense priming, further studies are needed to functionally correlate the induction of its expression with defense priming and MIR.

Our results suggest that the activation of signaling pathways is important in the systemic response of plants to mycorrhizal colonization, since a high number of DEGs with this function (including receptor kinases and calcium-related proteins) were up-DEG in mycorrhizal plants. Kinases are important regulatory proteins, and some have a leucine-rich repeat that mediates specific protein–protein interactions (Jones and Jones 1997). Plant receptor-like kinases function in diverse signaling pathways (including the perception of extracellular signals) to control various processes in plant growth and development, as well as responses to microbial signals in symbiosis and defense (Antolín-Llovera et al. 2012; De Smet et al. 2009). Receptor kinase complex formation is the initial step in several signaling pathways, such as PAMP-triggered immunity (Antolín-Llovera et al. 2012). It is therefore likely that processes such as priming and MIR are mediated by some of these proteins in leaves of mycorrhizal plants, including the activation of calcium signaling pathways. The receptor-like kinase SYMRK is one example of this type of protein, since mutants of the corresponding gene were unable to establish symbiosis with either bacterial or fungal symbionts (Stracke et al. 2002).

Eight NBS-LRR proteins were identified as differentially expressed, seven of which were up-regulated. The NBS-LRR proteins are proposed to function as receptors that bind effector molecules secreted by pathogens. Alternatively, they can act as "guard proteins", where they monitor the status of other plant proteins targeted by pathogen effectors (McHale et al. 2006). Some of these proteins that act through signaling pathway networks may be related to the defense priming mechanism in leaves of mycorrhiza-colonized plants, where they could act as receptors of specific mycorrhizal root signals.

Our analysis of the DEGs in leaves of mycorrhizal tomato plants (along with a review of the related literature) suggests that a defense priming mechanism could take place in mycorrhizal plants, indicating that mycorrhizal tomato plants should manifest MIR upon pathogen attack. In our study, we inoculated mycorrhizal and non-mycorrhizal tomato plants with the foliar pathogen *X. campestris* pv. *vesicatoria* and measured the level of infection. The results show that colonized plants display increased resistance in comparison to non-colonized plants, therefore demonstrating MIR onset. These findings also suggest that changes in gene expression in response to mycorrhizal colonization preceding pathogen attack prepare the plant against the pathogen in a more efficient way than in non-mycorrhizal plants.

Specifically, we found that AMS induces important modifications in processes including transcription regulation, nutrient transport, and defense responses. We hypothesize the existence of a priming state that prepares leaves of mycorrhizal plants for biotic (and possibly also abiotic) stress. This hypothesis is consistent with the putative functions of many of the DEGs identified in this study, as well as the evidence for MIR against the pathogen *X. campestris* pv. *vesicatoria*. However, direct confirmation for the participation of some genes in the transport or priming processes is still needed.

Finally, we have provided a list of DEGs that will enable focusing on certain candidates in a more direct functional analysis, which will help determine the actual players in these processes.

Although it is possible that deeper sequencing studies will reveal new mycorrhizal-responsive genes, this work provides essential data upon which further investigations of the responses to mycorrhizal colonization in the aerial parts of plants may be built.

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Author contributions RGCG participated in the experimental design, performed most of the experiments, participated in the sequencing analysis, and drafted the manuscript. MABI, ACM, and CLCV participated in the sequence analysis and drafted the manuscript. CMRD, IEMM, and MAVL participated in the experimental design and drafted the manuscript. AVO assisted in drafting the manuscript. MLM conceived the study and participated in its design and coordination, as well as the manuscript writing. All authors read and approved the final manuscript.

References

Antolín-Llovera M, Ried MK, Binder A, Parniske M (2012) Receptor kinase signaling pathways in plant-microbe interactions. Annu Rev Phytopathol 50:451–473

Aoki K et al (2010) Large-scale analysis of full-length cDNAs from the tomato (Solanum lycopersicum) cultivar Micro-Tom, a reference system for the Solanaceae genomics. BMC Genomics 11:210

Aroca R, Porcel R, Ruiz-Lozano JM (2007) How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in Phaseolus vulgaris under drought, cold or salinity stresses? New Phytol 173:808–816

Baggerly K, Deng L, Morris J, Aldaz C (2003) Differential expression in SAGE: accounting for normal between-library variation. Bioinformatics 19:1477–1483

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J 9:525–535

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57:289

Bolstad B, Irizarry R, Astrand M, Speed T (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185–193

Breuillin-Sessomsa F., Floss DS, Gomez K, Pumplin N, Ding Y, Levesque-Tremblay V, Noar RD, Daniels DA, Bravo A, Eagleshama JB, Beneditob VA, Udvardib MK, Harrison MJ (2015) Suppression of arbuscule degeneration in Medicago truncatula phosphate transporter4 mutants is dependent on the ammonium transporter 2 family protein AMT2;3. Plant Cell tpc.114.131144. doi: 10.1105/tpc.114.131144

Campos-Soriano L, García-Martínez J, Segundo BS (2012) The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. Mol Plant Pathol 13:579–592. doi:10.1111/j.1364-3703.2011.00773.x

Cartieaux F et al (2008) Simultaneous interaction of Arabidopsis thaliana with Bradyrhizobium Sp. strain ORS278 and Pseudomonas syringae pv. tomato DC3000 leads to complex transcriptome changes. Mol Plant-Microbe Interact 21:244–259. doi:10.1094/MPMI-21-2-0244

Chabot S, Becard G, Piche Y (1992) Life cycle of Glomus intraradix in root organ culture. Mycologia 84:315–321

Conrath U (2011) Molecular aspects of defence priming. Trends Plant Sci 16:524–531

Davies KM, Grierson D (1989) Identification of cDNA clones for tomato (Lycopersicon esculentum Mill.) mRNAs that accumulate during fruit ripening and leaf senescence in response to ethylene. Planta 179:73–80

De Smet I, Voß U, Jürgens G, Beeckman T (2009) Receptor-like kinases shape the plant. Nat Cell Biol 11:1166–1173

Fiorilli V, Catoni M, Miozzi L, Novero M, Accotto GP, Lanfranco L (2009) Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus. New Phytol 184:975–987. doi:10.1111/j.1469-8137.2009.03031.x

Fotopoulos V (2005) Plant invertases: structure, function and regulation of a diverse enzyme family. J Biol Res 4:127–137

Gallou A, Declerck S, Cranenbrouck S (2012) Transcriptional regulation of defence genes and involvement of the WRKY transcription factor in arbuscular mycorrhizal potato root colonization. Funct Integr Genomics 12:183–198

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular–arbuscular Mycorrhizal infection in roots. New Phytol 84:489–500. doi:10.1111/j.1469-8137.1980.tb04556.x

Govindarajulu M et al (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435:819–823. doi:10.1038/nature03610

Guether M, Balestrini R, Hannah M, He J, Udvardi MK, Bonfante P (2009) Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in Lotus japonicus. The New phytologist 182:200–212. doi:10.1111/j.1469-8137.2008.02725.x

Güimil S et al (2005) Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization. Proc Natl Acad Sci U S A 102:8066–8070. doi:10.1073/pnas.0502999102

Hao Z, Fayolle L, van Tuinen D, Chatagnier O, Li X, Gianinazzi S, Gianinazzi-Pearson V (2012) Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode Xiphinema index involves priming of defence gene responses in grapevine. J Exp Bot 63:3657–3672. doi:10.1093/jxb/ers046

Harrison MJ (2012) Cellular programs for arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol 15:691–698. doi:10.1016/j.pbi.2012.08.010

Hause B, Fester T (2005) Molecular and cell biology of arbuscular mycorrhizal symbiosis. Planta 221:184–196. doi:10.1007/s00425-004-1436-x

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agr Expt Sta Circ 347:1–32

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ (2007) A Medicago truncatula phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A 104:1720–1725

John L, Drake R, Farrell A, Cooper W, Lee P, Horton P, Grierson D (1995) Delayed leaf senescence in ethylene-deficient ACC-oxidase antisense tomato plants: molecular and physiological analysis. Plant J 7:483–490

Jones DA, Jones JDG (1997) The role of leucine-rich repeat proteins in plant defences. Adv Bot Res 24:89–167

Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ (2012) Mycorrhiza-induced resistance and priming of plant defenses. J Chem Ecol 38:651–664

Kapoor R, Evelin H, Mathur P, Giri B (2013) Arbuscular mycorrhiza: approaches for abiotic stress tolerance in crop plants for sustainable agriculture. In: Tuteja N, Gill SS (eds) Plant acclimation to environmental stress. Springer Science, New York, pp 359–401

Kobae Y, Tamura Y, Takai S, Banba M, Hata S (2010) Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. Plant Cell Physiol 51:1411–1415

Kruger JN (1990) Carbohydrate synthesis and degradation. In: Dennis DT, Turpin DH (eds) Plant physiology, biochemistry, and molecular biology. Longman Scientific and Technical Publishers, Harlow, pp 59–76

Lander ES, Waterman MS (1988) Genomic mapping by fingerprinting random clones: a mathematical analysis. Genomics 2:231–239

Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD, Harrison MJ (2007) Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. Plant J Cell Mol Biol 50:529–544. doi:10.1111/j.1365-313X.2007.03069.x

Liu Y, Zhou J, White KP (2014) RNA-seq differential expression studies: more sequence or more replication? Bioinformatics 30(3):301–304

López-Ráez JA, Verhage A, Fernández I, García JM, Azcón-Aguilar C, Flors V, Pozo MJ (2010) Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. J Exp Bot 61:2589–2601. doi:10.1093/jxb/erq089

Marioni J, Mason C, Mane S, Stephens M, Gilad Y (2008) RNA-Seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 18:1509–1517

McHale L, Tan X, Koehl P, Michelmore RW (2006) Plant NBS-LRR proteins: adaptable guards. Genome Biol 7:212. doi:10.1186/gb-2006-7-4-212

Mora-Romero GA et al (2015) PvLOX2 silencing in common bean roots impairs arbuscular mycorrhiza-induced resistance without affecting symbiosis establishment. Funct Plant Biol. doi:10.1071/FP14101

Ouziad F, Wilde P, Schmelzer E, Hildebrandt U, Bothe H (2006) Analysis of aquaporins and Na+/H+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Exp Environ Bot 57:177–186

Pérez-Tienda J, Corrêa A, Azcón-Aguilar C, Ferrol N (2014) Transcriptional regulation of host NHþ 4 transporters and GS/GOGAT pathway in arbuscular mycorrhizal rice roots. Plant Physiol Biochem 75:1–8

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Porcel R, Aroca R, Azcón R, Ruiz-Lozano JM (2006) PIP aquaporin gene expression in arbuscular mycorrhizal Glycine max and Lactuca sativa plants in relation to drought stress tolerance. Plant Mol Biol 60:389–404

Pozo MJ, Azcon-Aguilar C (2007) Unraveling mycorrhiza-induced resistance. Curr Opin Plant Biol 10:393–398. doi:10.1016/j.pbi.2007.05.004

Pozo MJ, Jung SC, López-Ráez JA, Azcón-Aguilar C (2010) Impact of arbuscular mycorrhizal symbiosis on plant response to biotic stress: the role of plant defence mechanisms. In: Koltai H, Kapulnik Y (eds) Arbuscular mycorrhizas: physiology and function. Springer, Netherlands, pp 193–207. doi:10.1007/978-90-481-9489-6_9

Prescott AG, John P (1996) Dioxygenases: molecular structure and role in plant metabolism. Annu Rev Plant Physiol Plant Mol Biol 47:245–271. doi:10.1146/annurev.arplant.47.1.245

Ruiz-Lozano JM, Porcel R, Aroca R (2006) Does the enhanced tolerance of arbuscular mycorrhizal plants to water deficit involve modulation of drought-induced plant genes? New Phytol 171:693–698

Salvioli A, Zouari I, Chalot M, Paola B (2012) The arbuscular mycorrhizal status has an impact on the transcriptome profile and amino acid composition of tomato fruit. BMC Plant Biol 12:44

Secco D, Baumann A, Poirier Y (2010) Characterization of the rice PHO1 gene family reveals a key role for OsPHO1;2 in phosphate homeostasis and the evolution of a distinct clade in dicotyledons. Plant Physiol 152:1693–1704

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, Amsterdam

Smith SE, Jakobsen I, Grønlund M, Smith FA (2011) Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiol 156(3):1050–1057

Stracke S et al (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417:959–962

The Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485:635–641

Thimm O et al (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 37:914–939

Usadel B et al (2006) PageMan: an interactive ontology tool to generate, display, and annotate overview graphs for profiling experiments. BMC Bioinformatics 7:535

van Loon LC, Geraats BPJ, Linthorst HJM (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Sci 11:184–191

Verhagen BW, Glazebrook J, Zhu T, Chang HS, van Loon LC, Pieterse CM (2004) The transcriptome of rhizobacteria-induced systemic resistance in arabidopsis. Mol Plant Microbe Interact 17:895–908. doi:10.1094/mpmi.2004.17.8.895

Wasternack C et al (2006) The wound response in tomato—role of jasmonic acid. J Plant Physiol 163:297–306

Whipps JM (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can J Bot 82:1198–1227

Xu X et al (2013) Parallel comparison of Illumina RNA-Seq and Affymetrix microarray platforms on transcriptomic profiles generated from 5-aza-deoxy-cytidine treated HT-29 colon cancer cells and simulated datasets. BMC Bioinforma 14:S1

Zouari I et al (2014) From root to fruit: RNA-Seq analysis shows that arbuscular mycorrhizal symbiosis may affect tomato fruit metabolism. BMC Genomics 15:221