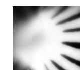

Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in *Lotus japonicus*

Mike Guether[1], Raffaella Balestrini[1], Matthew Hannah[2], Ji He[3], Michael K. Udvardi[3] and Paola Bonfante[1]

[1]Department of Plant Biology, University of Torino and IPP-CNR, Viale Mattioli, 25 – 10125 Torino, Italy; [2]Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Postdam-Golm, Germany; [3]The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

Author for correspondence:
Paola Bonfante
Tel: +390116705965
Fax: +390116705962
Email: p.bonfante@ipp.cnr.it

Received: *5 September 2008*
Accepted: *9 November 2008*

New Phytologist (2009) **182**: 200–212
doi: 10.1111/j.1469-8137.2008.02725.x

Key words: cell wall, *Gigaspora margarita*, laser microdissection, *Lotus japonicus*, mycorrhizal functioning, nutrient transporters, perifungal membrane.

Summary

- Arbuscular mycorrhizas (AMs) contribute significantly to soil nutrient uptake in plants. As a consequence of the fungal colonization and of the deep reorganization shown by arbusculated cells, important impacts on root transcriptome are expected.
- An Affymetrix GeneChip with 50 000 probe-sets and real-time RT-PCR allowed us to detect transcriptional changes triggered in *Lotus japonicus* by the AM fungus *Gigaspora margarita*, when arbuscules are at their maximum (28 d postinoculation (dpi)). An early time (4 dpi) was selected to differentiate genes potentially involved in signaling and/or in colonization of outer tissues.
- A large number (75 out of 558) of mycorrhiza-induced genes code for proteins involved in protein turnover, membrane dynamics and cell wall synthesis, while many others are involved in transport (47) or transcription (24). Induction of a subset (24 genes) of these was tested and confirmed by qRT-PCR, and transcript location in arbusculated cells was demonstrated for seven genes using laser-dissected cells.
- When compared with previously published papers, the transcript profiles indicate the presence of a core set of responsive genes (25) that seem to be conserved irrespective of the symbiotic partner identity.

Introduction

Mycorrhizal fungi are soil microbes that associate with 90% of the land plants and assist them in multiple ways, especially in obtaining nutrients from the soil (Martin *et al.*, 2007). Genetic approaches have uncovered a small number of plant genes that are required for both the arbuscular mycorrhizal (AM) and root nodule symbiosis (RNS) (Parniske, 2008), but it is likely that hundreds if not thousands of genes contribute to the development and functioning of these important partnerships. Transcriptome analysis using DNA arrays is a powerful approach to identify genes that are regulated in any organism for which gene sequence is available. Genome-wide analysis of transcriptional changes that accompany establishment of AM symbioses offers a way of identifying many of the genes that are required for effective symbiosis. Unfortunately, the first plant species to be fully sequenced, *Arabidopsis thaliana*, does not establish symbiosis with mycorrhizal fungi. However, other species that do host AM symbioses have either been sequenced (poplar, grapevine and rice) or are in the process of genome sequencing, as is the case, for example, with *Lotus japonicus* (Sato *et al.*, 2008).

A number of studies have already employed transcriptome analyses to identify genes involved in AM symbiosis, albeit in a manner limited by the availability of DNA sequence. The pioneering study of Liu *et al.* (2003) was based on a macroarray of probes for 2268 genes of *Medicago truncatula* and identified 92 genes that were up-regulated upon fungal colonization. Later, using a microarray with 16 000 probes, Hohnjec *et al.* (2005) identified approx. 200 *Medicago* genes that were activated upon colonization of *Glomus mosseae* and *G. intraradices*, including genes involved in transport, such as the phosphate transporter gene *MtPT4*, and genes involved in signaling. Based on the whole-genome *Oryza sativa* Genechip, Güimil

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

et al. (2005) demonstrated that a set of genes was similarly expressed in symbiotic and pathogenic associations, suggesting the presence of a conserved response to fungal colonization. Many of the genes implicated in mycorrhizal symbiosis in rice were similar to those associated with mycorrhization in dicots. Interestingly, the transcript data indicate responses which were also independent of the fungal identity (Hohnjec *et al.*, 2005; Liu *et al.*, 2007), reflecting substantial evolutionary conservation in plant transcriptional responses to AM colonization. However, recent work by Deguchi *et al.* (2007), which failed to identify some 'common' symbiosis marker genes amongst mycorrhiza-induced genes in *L. japonicus*, may indicate significant divergence in symbiotic programs between species.

In view of the still limited number of transcriptomic studies of AM symbiosis development, and given the recent development of an Affymetrix GeneChip for *L. japonicus* that contains probes for the vast majority of *Lotus* genes (Sanchez *et al.*, 2008), we set out to obtain a genome-wide view of transcriptional changes associated with a key stage of symbiosis development, namely arbuscule formation and activation. Arbuscules are considered to be the key element of the symbiotic nutrient exchange between the plant and the fungus, since they are enveloped by a plant membrane with unique transport specificities and functions (Harrison, 2005). *In vivo* confocal imaging studies making use of fluorescent cellular markers have suggested that the formation of such perifungal membrane is preceded by the formation of cytoplasmic assemblies consisting of cytoskeletal, endoplasmic reticulum, vesicular and organelle components which involve both epidermal and cortical cells (Genre *et al.*, 2005, 2008). Because mycorrhization is not a synchronous process, and plant gene expression depends on the timing of the colonization events (Liu *et al.*, 2003; Deguchi *et al.*, 2007; Siciliano *et al.*, 2007), we selected two different time points during mycorrhizal establishment, in addition to an uninoculated root control, to identify genes associated with arbuscule formation. Twenty-eight days post-inoculation (dpi) was chosen as a time point at which mycorrhization in *Lotus* has reached a plateau, arbuscules are fully developed and active, and senescence events are not yet significant (Bonfante *et al.*, 2000). For comparison, an early time (4 dpi) was selected in order to distinguish genes that are potentially involved in signaling and/or colonization of outer tissues from those involved in arbuscule development and mycorrhizal functioning. The predicted location of expression of some genes in arbusculated cells was confirmed using laser microdissected cells.

Materials and Methods

Plant materials, growth conditions and inoculation methods

Lotus japonicus (Regel) K. Larsen seeds (Gifu, WT) were scarified and surface-sterilized for 5 min in concentrated sulfuric acid and washed three times with sterile water. In a second step the seeds were incubated for 5 min in 1 : 3 diluted commercial bleach with 1 : 1000 Triton-X. After washing three times with sterile water, the seeds were germinated on water agar in Petri dishes. Mycorrhization was performed by inoculation with *Gigaspora margarita* Becker and Hall (strain deposited in the Bank of European Glomales as BEG 34) by a modified millipore sandwich method (Giovannetti *et al.*, 1993). Three seedlings were placed between a nitrocellulose and a transparent membrane (pore diameter 0.45 µm; Sartorius, Goettingen, Germany), either with 10–15 fungal spores or without any spores. The assembled sandwiches were attached with the transparent side against the wall of Magenta GA-7 filter-lid vessels (Sigma Aldrich, St. Louis, MO, USA). The vessels were filled with sterile acid-washed quartz sand and then soaked up with half-strength Long-Ashton nutrient solution (Hewitt, 1966) with 20 µM PO_4^{3-}. Plants were grown in a climatic chamber at 20°C, 60% humidity, with 14 h of light per day. In the case of the 4 dpi experiment, plants were pre-grown for 14 d before spores were injected between the two membranes. The lower part of the vessels was covered with light-impermeable plastic foil in order to prevent greening of the roots. Spore germination was followed by removing the foil and observation under a stereo microscope. After 4 and 28 dpi, samples from roots were cut after observation under a stereo microscope. Some segments were stained with 0.1% cotton blue in lactic acid and the infection was quantified as described by Trouvelot *et al.* (1986); other fragments were processed for cellular and molecular analysis. Root segments from three independent experiments and a total of 12 sandwich cultures were analysed.

RNA isolation, dye labelling of cDNA probes and microarray hybridization

Plant organs were harvested and immediately frozen in liquid nitrogen in a 2 ml reaction tube. Two clean metal balls were added into every tube and frozen again. Plant material was then ground using a Retsch® ball mill for 2 min. Owing to the difficult root material, RNA was extracted using the 'pine-tree-method' (Chang *et al.*, 1993). Integrity of RNA samples was checked using an Agilent 2100 Bioanalyzer. RNA purity was determined by ensuring spectrophotometric ratios of A260nm/A280nm ~ 2 and A260nm/A230nm ≥ 2. Removal of genomic DNA was done using the Turbo DNA-free™ reagent (Ambion, Austin, TX, USA) following the manufacturer's instructions. Absence of genomic DNA was verified by quantitative RT-PCR (qRT-PCR) with intron-specific primer for tubulin β-5 (TM0371b.4/TC18284).

For each sample, 100 ng of total RNA was used to synthesize double-stranded cDNA by using two successive reverse transcription reactions according to standard Affymetrix protocols (GeneChip® Two-Cycle Target Labeling; Affymetrix; http://www.affymetrix.com). Linear amplification with

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

T7-RNA polymerase and biotin labelling were performed by *in vitro* transcription after standard Affymetrix procedures. The resulting biotin-labelled cRNA was fragmented and hybridized to the Genechip® Lotus1a520343 and scanned, according to the manufacturer's instructions.

Data analysis

Microarray data were analysed using the bioconductor softwarepackage for the R programming language (Gentleman *et al.*, 2004). Data quality was assessed using the affy (Gautier *et al.*, 2004) and AffyPLM packages, and expression estimates were obtained using the RMA algorithm (Irizarry *et al.*, 2003). Control and bacterial probe-sets were removed, and only genes assigned as present ($P < 0.05$) using the MAS5 present/absent algorithm were retained. Statistical testing for differential expression was performed using mixed models with the LIMMA bioconductor package (Smyth, 2004).

Comparison of the obtained data sets to the previous published microarray studies were based on TBLASTX. For the *L. japonicus* data sets of Deguchi *et al.* (2007) an e-value threshold of 1e–50 was applied. For the *M. truncatula* mycorrhiza-induced 'core-set' an e-value threshold of 1e–5 was used.

cDNA synthesis and real time RT-PCR

Real-time experiments were carried out on material derived from root. cDNA synthesis was performed using SuperScriptII® Reverse Transcriptase and 1 µg of total RNA, following the protocol of the supplier (Invitrogen Ltd, Paisley, UK). Quantitative RT-PCR primers were designed using PerlPrimer, a free, open-source GUI application (http://perlprimer.sourceforge.net/). Amplicons were analysed for potential secondary structures using the Mfold web server for nucleic acid folding and hybridization prediction (Zuker, 2003). Before qRT-PCR, all primers were tested on genomic DNA and cDNA for amplification.

Oligonucleotide sequences of all the primers are listed in Supporting Information, Table S6. Quantitative RT-PCR was carried out with an iCycler apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR reaction was carried out in a total volume of 20 µl containing 1 µl diluted cDNA (1 : 5), 10 µl 5 × SYBR Green Reaction Mix and 3 µl of each primer (3 µM) using a 96-well plate. The following PCR programme was used: 95°C for 90 s, 40 cycles of 95°C for 15 s, 60–68°C for 30 s.

A melting curve (54–70 steps with a heating rate of 0.5°C per 10 s and a continuous fluorescence measurement) was recorded at the end of every run to exclude primers generating nonspecific PCR products (Ririe *et al.*, 1997). All reactions were performed for three biological and three technical replicates. Baseline range and C_T values were automatically calculated using the iCycler software. In order to compare data from different PCR runs or cDNA samples, C_T values of all genes were normalized to the C_T value of UBQ10 (chr1.TM0487.4), which was the most constant of the three tested housekeeping genes (*UBQ10*, *GAPDH*, *EF1α*) with an average C_T value of 19.79 (± 0.24). Candidate gene expression was normalized to that of UBQ10 by subtracting the C_T value of UBQ10 from the C_T value of the candidate gene resulting in ΔC_T. Because the slopes of the different samples and replicates in the exponential phase were the same, the S/R expression ratios were calculated without PCR efficiency correction from the equation $2^{\Delta\Delta C_T}$. At this, $\Delta\Delta C_T$ represents $\Delta C_{T\text{sample}} - \Delta C_{T\text{control}}$. Quantitative RT-PCR products were separated on 2% agarose gels run at $V\ cm^{-1}$ in TAE tris-acetate-EDTA buffer, along with the 100 bp DNA standard (Invitrogen).

DNA extraction and PCR amplifications

Lotus genomic DNA was extracted from 200–300 mg of plant material. Samples were ground in liquid nitrogen before 300 µl of CTAB buffer were added and incubated for 30 min at 60°C, mixing once during incubation. Three hundred microlitres of chloroform : isoamylalcohol (24 : 1) were then added and the sample was carefully mixed followed by 10 min centrifugation at 6000 ***g*** at room temperature. The upper phase was transferred to a new tube, and 2.5 µl RNase (10 mg ml^{-1}) was added and incubated at 37°C for 30 min. Afterwards, samples were stored on ice for 5 min before 0.6 volumes of isopropanol were added and DNA was precipitated 1 h to overnight at –20°C. The DNA was precipitated by centrifugation at 10.600 ***g*** for 10 min at 4°C and the pellet washed with 70% EtOH. After 10 min centrifugation at 4°C, supernatant was removed and DNA pellets were air-dried at room temperature. DNA was resuspended in 50 µl TE buffer and stored at –20°C for further analysis. Fungal genomic DNA was extracted from *c.* 200 *Gi. margarita* spores using a DNA Plant Mini Kit (Qiagen).

The PCR reactions were carried out in a final volume of 30 µl containing 3 µl of 10X buffer, 2.5 µM, 200 µM each dNTP, 0,2 µM of each primer, 50–100 ng of genomic DNA, and 2 units of GoTaq DNA polymerase (Promega). The PCR program was as follows: 95°C for 3 min (1 cycle), 94°C for 45 s, 60–68°C 45 s, 72°C for 45 s (40 cycles), and 72°C for 5 min (1 cycle).

Laser microdissection

Mycorrhizal and nonmycorrhizal roots (dissected into 5- to 10-mm segments with a razor blade in the freshly prepared fixative) were fixed in freshly prepared Methacarn (absolute methanol/chloroform/glacial acetic acid 6:3:1) at 4°C overnight for paraffin embedding (Balestrini *et al.*, 2007). Leica RNase-free PEN-foil slides were used for LMD. Sections of 15-µm thickness were cut using a rotary microtome, and the ribbons were placed and stretched out on the slides with double-distilled (dd)H_2O on a 40°C warming plate. The sections were dried in a 40°C oven, stored at 4°C, and used within 2 d. A Leica AS Laser Microdissection system (Leica Microsystems, Inc., Bensheim, Germany) was used to isolate

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

cells from the prepared tissue sections. Just before use, the paraffin sections were deparaffinized by xylene treatment for 10 min, 100% ethanol for 2 min, and then air dried. The deparaffinized slides were placed face down on the microscope. The tissues were visualized on a computer monitor through a video camera and the selected cells were dissected as described in (Balestrini *et al.*, 2007). The dissection conditions were optimized to obtain a clean, narrow excision of the selected cells: 40-XT objective at power 35 to 45 and speed 3 to 4. The cells subsequently fell into the 0.5-ml RNase-free PCR tube caps located beneath the visualized tissue section. On average, *c.* 500–1000 cell sections were collected in a tube. After collection, *c.* 15–25 μl of RNA extraction buffer from the PicoPure kit (Arcturus Engineering, Mountain View, CA, USA) was added. Samples were incubated at 42°C for 30 min, centrifuged at 800 ***g*** for 2 min, and stored at –80°C. Then, for the following steps of RNA extraction, cells were pooled in a single tube with a final volume of 50 μl.

RNA extraction and RT-PCR on microdissected samples

RNA extractions were performed with a slightly modified PicoPure kit protocol (Arcturus Engineering) as described in (Balestrini *et al.*, 2007). RNA quantification was obtained using the NanoDrop 1000 spectrophotometer. A One-Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used for the nonquantitative RT-PCR experiments conducted on the RNA extracted from the several samples. Reactions were carried out in a final volume of 20 μl containing 4 μl of 5× buffer, 1 μl of 10 mM dNTPs, 0.5 μl of each primer (1 μg μl^{-1}, diluted 1 : 10), 1 μl of One-Step RT-PCR enzyme mix, and 1.5 μl of total RNA diluted 1 : 5. A comparable RNA amount has been used for the three different samples considered. The samples were incubated for 30 min at 50°C, followed by a 15 min incubation at 95°C. Amplification reactions (the primers in Table S6) were run for 40 cycles. The RT-PCR experiments were conducted on at least two independent biological and technical replicates. All the RNA samples were checked for DNA contamination through RT-PCR analyses conducted with *Lotus EF1α* and ubiquitin10-specific primer pairs. The PCR products were separated by agarose gel electrophoresis in a Trisacetate-EDTA 0.5X buffer, stained with ethidium bromide, and visualized using a VersaDoc Imaging System (Bio-Rad Laboratories).

Results

Transcript profiles of *Lotus japonicus* roots at 4 and 28 dpi

The goal of this investigation was to characterize the transcriptome of a model legume, *Lotus japonicus* at a key stage of symbiosis with *G. margarita*, namely arbuscule development. On the basis of previous experiments (Novero *et al.*, 2002), 28 dpi was selected as the most appropriate time to sample the root system. Morphological and quantitative investigation of stained root samples at 28 dpi revealed an average mycorrhization of 69% with an arbuscule presence of 75% in the colonized segments (Table S1, Fig. S1).

Owing to the asynchronous nature of mycorrhizal colonization, 28 dpi roots were expected to include some new colonization events, starting from appressoria developed at the epidermal surface. To avoid an overlapping of transcripts related to early events with those mostly involved in arbuscule development, an earlier time point was included. Roots 4 dpi harboured *G. margarita* germinating spores, developing appressoria, and contained the first-colonized epidermal cells. However, no arbuscules were detected in these roots (Fig. S1).

At 4 dpi, development of *L. japonicus*/*G. margarita* plants was indistinguishable from those of uninoculated controls in terms of root growth, while at 28 dpi, roots from inoculated plants weighed 62% more than the uninoculated roots (Table S2). Three biological replicates were established, and for each experiment RNA from the three root samples was isolated, labelled and used to probe *a Lotus1a520343 Genechip®*.

Data sets were analysed by a multiple testing and a significance-based comparison of inoculated and control plants, applying a *P*-value < 0.05 and a twofold change threshold. For roots harvested at 4 dpi, only 33 genes met these criteria, and 23 of these were up-regulated compared with control levels in uninoculated control roots. At 28 dpi, 565 genes were found to be differentially expressed, 435 up- and 130 down-regulated compared with control levels (Tables S3–S5). The two data sets showed an overlap of only seven genes (Table S3). Because of the distinct profile of root gene expression at 4 vs 28 dpi, we reasoned that these seven genes were probably involved in early signaling events or in the process of fungal colonization of the epidermis or outer cortex before reaching the inner cortex (Genre *et al.*, 2008). Therefore, these were removed from the list of 565 differentially regulated genes, leaving 558 genes that were considered to be strictly related to inner cortex colonization. Eighty per cent of these genes were up-regulated sequences (Table S4). Annotation of mycorrhiza-regulated genes was performed by comparing all translated sequences to the *Arabidopsis thaliana* proteome. Subsequent visualization of genes/proteins in a functional context, using MapMan software (Usadel *et al.*, 2005) revealed substantial numbers of genes/proteins involved in protein turnover, lipid metabolism, transport and other cell functions (Figs 1, S3–S6).

Annotation of mycorrhiza-responsive genes which are common to other AM hosts

Annotation of mycorrhiza-regulated genes in *Lotus* revealed putative orthologs which have already been described as differentially expressed genes of other mycorrhizal hosts. The largest subset of genes in the protein turnover category contained two dozen genes belonging to the cysteine proteinases,

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

1 Major/minor CHO metabolism
2 Glycolysis/TCA/ATP synthesis
3 Cell wall
4 Lipid metabolism
5 Amino acid metabolism
6 Secondary metabolism
7 Hormone metabolism
8 Stress-related
9 Large enzyme families (miscellaneous)
10 Transcription and RNA processing
11 Protein synthesis/modification/degradation
12 Signaling
13 Cell organization/vesicle transport
14 Development
15 Transport
16 Not assigned
17 others (metal handling, tetrapyrrole synthesis, nucleotide metabolism, etc.)

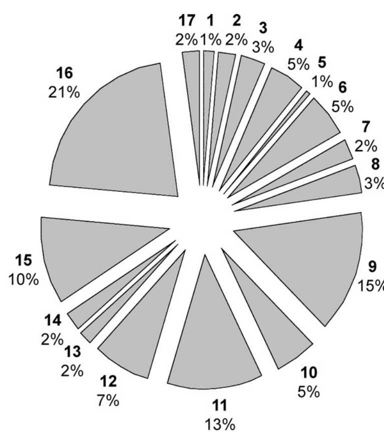

Fig. 1 Functional categories of 28 d post-inoculation mycorrhizal-regulated genes/gene products.

subtilisin-like proteinases or serine carboxypeptidases. Many of these were among the most highly regulated genes in mycorrhizal roots, with up to 3100-fold induction compared with uninoculated roots. While protease genes are known to be induced in AMs (Liu *et al.*, 2003; Hohnjec *et al.*, 2005; Kistner *et al.*, 2005) and root nodules (Kistner *et al.*, 2005), the detection of protease inhibitors as mycorrhiza-responsive genes has been more rarely detected (Hohnjec *et al.*, 2005). Here a gene (Ljwgs_009761.2) encoding a protein with 89% identity to a subtilisin inhibitor, CLSI-I from the legume *Canavalia lineata*, was found to be up-regulated. The predicted protein is also highly homologous to *M. truncatula* BF003324, encoded by an EST of seedling roots 24 h after inoculation with *Sinorhizobium meliloti*.

A comparison of our 4–28 dpi *Lotus* data set to the 7 dpi data set reported by Deguchi *et al.* (2007) showed no significant overlap (Fig. S2A). Even the comparison of the later stages (28 dpi–4 dpi versus the 56 dpi) revealed only a small overlapping pool of regulated genes. By contrast, the *M. truncatula* genes reported as the mycorrhiza core-set (Liu *et al.*, 2007) showed a significant overlap to our 28 dpi–4 dpi data pool: in fact 64% of this *M. truncatula* gene set has homologous-responsive genes in *L. japonicus* (Fig. S2B). When the e-value was lowered from 1e–5 to 1e–50, still 36% of the *M. truncatula* core-set genes were overlapping with *L. japonicus*-regulated genes (data not shown).

Gene validation

Because *P*-values < 0.05 and a twofold change threshold were used as an indication of gene regulation we validated a subset of the Affymetrix data by quantitative RT-PCR. Twenty-five regulated genes, representing a range of biological functions, were selected and the differential-expression pattern was confirmed for 24 of these, using RNA from independent biological replicates (Table 1). Linear regression analysis of qRT-PCR and Affymetrix data for these genes (Fig. 2) yielded a value of

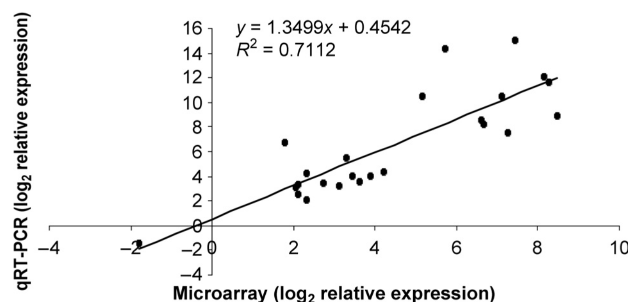

Fig. 2 Comparison of microarray and quantitative real-time RT-PCR of the 24 confirmed probe-sets. Each symbol represents the mean expression level (log_2-transformed) of the 28 d post inoculation (dpi) mycorrhized roots relative to 28 dpi control roots of *Lotus japonicus*.

$R^2 = 0.7112$ and a slope of 1.3499, which reflects the greater sensitivity and dynamic range of qRT-PCR.

To verify the output of the automated annotation which was done for the Genechip and for the MapMan software, the 24 selected sequences were analysed with BLASTX in the National Center for Biotechnology (NCBI) database and the Institute for Genomic Research (TIGR) MtGI (*Medicago truncatula* Gene Index) database, respectively. The annotation of 23 out of 24 could be confirmed. By contrast, the sequence of probe-set chr3.CM0460.2_at which was listed as putative protein showed homology to protease inhibitor/seed storage/lipid transfer proteins (LTP).

Transcription factors and genes potentially involved in root morphogenesis

At 28 dpi the whole root system of mycorrhizal *L. japonicus* was more developed and ramified compared with the control plants (Table S2), in line with previous reports that proposed improved nutrition as an explanation for the increased root branching (Berta *et al.*, 1995). However, the molecular basis of this mycorrhizal phenotype has never been elucidated. Our

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

Table 1 Twenty-four genes with differential expression in 28 d post inoculation (dpi) – 4 dpi mycorrhized *Lotus japonicus* roots validated by quantitative real-time PCR

		Array data	Quantitative RT-PCR data	
Lotus Affymetrix ID	Putative annotation	$\log_2$ mean relative expression	$\log_2$ mean relative expression	Standard deviation
Ljwgs_009761.2_at	Subtilisin inhibitor	2.74	3.41	± 0.67
Ljwgs_014433.2_at	Phosphate transporter	7.12	10.46	± 0.73
Ljwgs_016263.1_at	*Scarecrow 3 like protein A	1.80	6.65	± 0.53
Ljwgs_027761.2_at	*Scarecrow 3 like protein A	2.32	4.13	± 0.09
Ljwgs_023888.1_at	*Scarecrow 1	2.34	2.02	± 0.09
Ljwgs_039859.2_x_at	Cysteine proteinase	8.27	11.60	± 0.88
Ljwgs_068634.1_s_at	Subtilisin like protease	8.50	8.86	± 0.22
chr1.CM0150.1_at	*MLO like protein	4.24	4.34	± 0.52
chr3.CM0127.86_at	*TOM (target of myb) – like protein	3.13	3.13	± 0.22
TM0445.17_at	ABC-transporter like protein (ATP-Binding Casette)	6.69	8.18	± 0.67
TM1490.15_at	Glycosylhydrolase family 1 like protein	2.14	2.51	± 0.24
chr1.CM0113.49_at	GA-2-oxidase (gibberelline oxidase)	3.46	3.96	± 0.74
Ljwgs_024744.1_at	Endo-1,4-betaglucanase CEL1	3.64	3.54	± 0.49
chr2.CM0177.41_at	ABC-transporter like protein	3.89	3.90	± 0.19
TM0674.17_at	Unknown protein	7.27	7.51	± 0.44
chr3.CM0460.2_at	Protease inhibitor/seed storage/ LipidTransfer Protein (LTP)	8.16	11.99	± 0.36
chr1.CM0295.2.1_at	*Peptide transporter	5.18	10.38	± 1.23
Ljwgs_016680.1_at	*Ammonium transporter	7.46	14.96	± 1.21
chr1.CM0064.6_at	*Amino acid permease LHT (Lysine Histidine Transporter)	6.62	8.48	± 0.86
Ljwgs_037698.1_at	*Cellulose synthase catalytic subunit	2.12	3.26	± 0.59
chr1.BM1732.4_at	Myb transcription factor like protein	5.73	14.31	± 1.58
TM0445.37_at	*Potassium transporter	3.31	5.48	± 1.24
Ljwgs_011755.1_at	*Sulfate transporter	2.08	3.02	± 1.18
TC7888_at	Proline-rich cell wall protein	–1.76	–1.5	± 0.16

Genes marked with an asterisk have not been reported mycorrhiza-induced before. All quantitative RT-PCR ratios are considered significant (>2-fold or <0.5-fold). For the array hybridisation and the quantitative RT-PCR two independent biological replicate experiments were done.

array analysis revealed up-regulation of several genes that could be involved in root morphogenesis. One group of four mycorrhiza-induced genes belongs to the *SCARECROW (SCR)* family of transcription factor (TF) genes. Members of this family, together with the *SHR* (SHORT ROOT) gene, are genetic determinants of root identity (Dolan, 2007). Three of the *SCR* genes were tested by qRT-PCR and showed an up-regulation between four- and 100-fold compared with control levels. The protein corresponding to the strongest-regulated *SCR* gene (Ljwgs_016263.1) showed 52% identity to *A. thaliana* AtSCL3 (SCARECROW LIKE 3) and 81% identity to the *M. truncatula* protein encoded by TC105118, which comprises ESTs derived from mycorrhizal roots (Wulf *et al.*, 2003).

Twenty other genes encoding TFs or RNA processing enzymes were found to be up-regulated in mycorrhizal roots. Among them, the *LjMyb* TF gene (chr1.BM1732.4) showed the second highest up-regulation of all genes (20 300-fold). Plant MYBs have been implicated in controlling many diverse processes, among them plant organ development (Allan *et al.*, 2008). The homology search for the translated sequence revealed a 79% similarity to an already reported TC101627 in *M. truncatula* (Liu *et al.*, 2003; Hohnjec *et al.*, 2005).

Genes involved in hormone metabolism have been suggested to play a crucial role in root development (Osmont *et al.*, 2007). In this context, it is noteworthy that 11 genes predicted to be involved in hormone metabolism were found to be up-regulated in mycorrhizal roots: one of them, chr1.CM0113.49, encodes a protein sharing 81% identity with Gibberellin 2-oxidase of *Vigna angularis*. Its induction was validated by qRT-PCR, which measured an 11-fold up-regulation. Another protein sequence, (Ljwgs_037161.1), shares 74% identity with Zeatin-O-xylosyltransferase of *Phaseolus vulgaris* and the corresponding gene was induced 10-fold in mycorrhized roots.

Genes involved in membrane proliferation and cell wall synthesis

Transcriptome analysis revealed up-regulation of many genes directly or indirectly involved in cellular reorganization events,

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

which may be required for fungal accommodation (Bonfante, 2001). Among the genes potentially involved in membrane assembly was a member of the pathogenesis-related Mlo protein family, which are integral plasma membrane proteins (Devoto *et al.*, 1999). The gene *LjMlo2* (chr1.CM0150.1) showed a 16-fold increase in expression upon mycorrhization, which was confirmed by qRT-PCR (Table 1). The translated protein shares 58% identity with rice OsMlo2 and 70% identity with *M. truncatula* TC 99328, derived from ESTs of mycorrhizal roots.

Fourteen genes involved in cell wall metabolism were found to be up-regulated in 28 dpi mycorrhized roots compared with the control: two of these were tested further by qRT-PCR and confirmed to be induced *c.* 10-fold (Table 1). The first of these, Ljwgs_024744.1, showed a 78% identity to the *MtCel1* gene, which encodes an endo-1,4-beta-glucanase (Liu *et al.*, 2003). The second gene, Ljwgs_037698.1, encodes a protein with 72% identity to cellulose synthase 6 (CesA6) of *Eucalyptus grandis*. An InterProScan of this protein confirmed the presence of a cellulose_synthase domain (PF03552).

Our data set contained many regulated genes of unknown or poorly defined function. The regulation of two of these was tested and validated by qRT-PCR. Both genes exhibited strong up-regulation upon mycorrhization: 4096-fold for chr3.CM0460.2 and 182-fold for TM0674.17 (Table 1). The translated sequence of chr3.CM460.2 has weak similarity to an *Arabidopsis* protease inhibitor/seed storage/lipid transfer protein (LTP) and a 68% identity to *Medicago* TC11086, derived from ESTs of mycorrhizal roots. LTP proteins may play roles in the endomembrane system and lipid transport. The second sequence, TM0674.17, encodes a 15 kDa protein with high homology to proteins of unknown function in many plants.

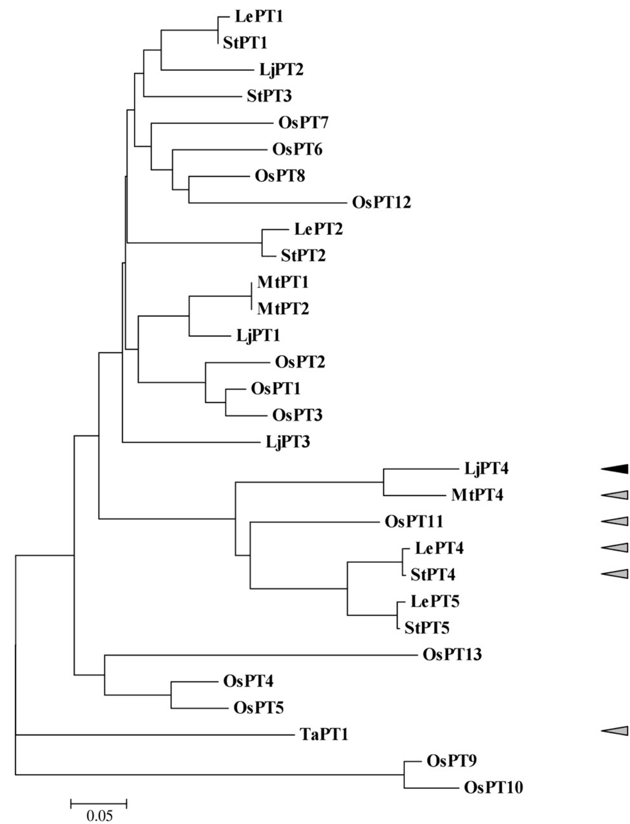

Fig. 3 Phylogenetic tree for the amino acid sequences of plant phosphate (Pi) transporters. The dendrogram was generated by Mega 4.0 software using ClustalW for the alignment and the neighbor-joining method for the construction of phylogeny (Tamura *et al.*, 2007). Abbreviations for plant species: Le, *Lycopersicon esculentum*; Lj, *Lotus japonicus*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; St, *Solanum tuberosum*; Ta, *Triticum aestivum*. Mycorrhiza-specific Pi transporters are marked with gray arrows. The identified mycorrhiza-specific *L. japonicus* phosphate transporter LjPT4 is marked with a black arrow. For the plant Pi-transporters accession numbers, see Javot *et al*. (2007b).

Detection of novel genes involved in nutrient transport

The array analysis performed on *L. japonicus* mycorrhizal roots revealed regulation of 47 genes involved in nutrient transport (Fig. 1 and Fig. S6). These included a phosphate transporter that was induced more than 1400-fold, as confirmed by qRT-PCR (Table 1). The amino acid sequence of the encoded protein shares 94% identity with the mycorrhiza-specific MtPT4 in *M. truncatula*, which is required for arbuscule development and function (Javot *et al.*, 2007a). Both proteins are homologous to the mycorrhiza-specific P transporters of tomato, potato and rice (Fig. 3). By contrast, the *LjPT3* phosphate transporter gene previously described as a mycorrhiza-induced transporter (Maeda *et al.*, 2006) did not show a significant regulation in our array experiments.

The strongest up-regulated gene of the *Lotus* array was a putative ammonium transporter (Ljwgs_016680.1). Using qRT-PCR, a nearly 32 000-fold up-regulation of this gene was confirmed, which is even higher than the known mycorrhiza-specific phosphate transporter. The translated sequence had a 61% identity to the TaAMT2.1 protein from *Triticum aestivum*. In the *M. truncatula* EST databases, only weak similarities were found. To complete the scenario of the mineral N transporters, four different nitrate transporter genes were found, showing four- to 18-fold up-regulation.

Another group of new mycorrhizal-related genes identified via transcriptome analysis consists of putative amino acid transporters (AAP). Three genes of the AAP family were found to be up-regulated between two and 98-fold compared with controls. The strongest up-regulated AAP (chr1.CM0064.6) was shown to be even more highly up-regulated by using qRT-PCR (360-fold). A homology search revealed 80% identity between this AAP and LHT1 (lysine histidine transporter 1) of *A. thaliana*. In the TIGR database, one *M. truncatula* EST (AL368579) derived from roots of N-deprived plants was identified with 89% identity to the *Lotus* AAP. The second

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

putative AAP (TM1440.7) was 15-fold up-regulated in mycorrhizal roots at 28 dpi and had a 65% identity to the AAT1 (cationic amino acid transporter 1) of *A. thaliana*. It also exhibited homology to *Medicago* ESTs derived from N-deprived roots. The third AAP (chr4.CM0025.3) was twofold up-regulated in mycorrhizal roots and shared 79% identity at the amino acid level with *Arabidopsis* AT5G41800 and 85% identity with the *M. truncatula* protein encoded by EST BF635938, which derived from drought-stressed plants.

Ten differentially expressed *Lotus* genes with similarity to PTR-type peptide transporter genes were detected. Nine of them were up-regulated in mycorrhizal roots, while the 10th was down-regulated compared with control roots. Quantitative RT-PCR analysis of the highest-induced peptide transporter gene (chr1.CM0295.2.1) also revealed a higher up-regulation (1333-fold) than seen on the array. The peptide transporter amino-acid sequence was further analysed and showed 60% identity to the AtPTR3 protein sequence. Further homology searches showed 87% identity to a translated EST (AL387494) from *M. truncatula* roots inoculated with *G. intraradices*.

Finally, a potassium transporter (TM0445.37) was confirmed to be 44-fold up-regulated in mycorrhizal roots. Even though there are ESTs of putative potassium transporters from mycorrhizal tissues in databases, only weak identities were found to these. Another macronutrient transporter, which was eightfold up-regulated, is a putative sulfate transporter (Ljwgs_011755.1). The translated sequence shared 82% identity with the *Lycopersicon esculentum* SULTR1;2 sulfate transporter.

Detection of transcripts in microdissected arbusculated cells

To confirm that expression of genes was located in arbusculated cortical cells, we subjected RNA isolated from laser microdissected cells to one-step RT-PCR. Three cell types were microdissected: cortical cells from nonmycorrizal roots (C); noncolonized cortical cells from mycorrhizal roots (MNM) and arbuscule-containing cells (ARB). Transcripts corresponding to the following genes were measured: *LjEF1α* (TC14038), *LjUBQ10* (TC14054), *LjPT4* (Ljwgs_014433.2), *LjCesA* (Ljwgs_037698.1), *LjMlo2* (chr1.CM0150.1), *LjCel1* (Ljwgs_024744.1), *LjMyb* (chr1.BM1732.4), *LjLTP* (chr3.CM0460.2) and *LjPTR* (chr1.CM0295.2.1). Primers for these genes were tested first on plant and fungal genomic DNA in order to verify their specificity. All the primers yielded unique PCR products of the expected size from plant DNA and no band from fungal DNA (data not shown). In RT-PCR experiments on microdissected samples, an amplified fragment of the expected size was observed in all cell types tested using specific primers for the housekeeping genes *LjEF1α* and *LjUBQ10* (Fig. 4). Absence of an amplified product in RT minus reactions excluded genomic DNA contamination. In contrast to housekeeping genes, transcripts of *LjPT4*, *LjCesA*, *LjMlo2*, *LjCel1*, *LjMyb*, *LjLTP* and *LjPTR* were found exclusively in arbuscule-containing cells (Fig. 4). RNA preparations were also subjected to RT-PCR using primers specific for *Gi. margarita* to verify the presence of the fungus (Fig. 4). Using primers specific to *Gi. margarita EF1α* gene (Efgig2f: TGAACCTCCAACCAGACCAACTG; Efgigr: CGGTTT-CAACACGACCTACAGGGAC; Salvioli *et al*., 2008), a PCR product of the expected size (103 bp) was found in both samples collected from mycorrhizal roots (Fig. 4). The presence of a slight band also in MNM cell type population could be explained by the presence of fungal structures, probably intercellular fungus, within the 15 m thickness of the section, as already reported by Balestrini *et al.* (2007).

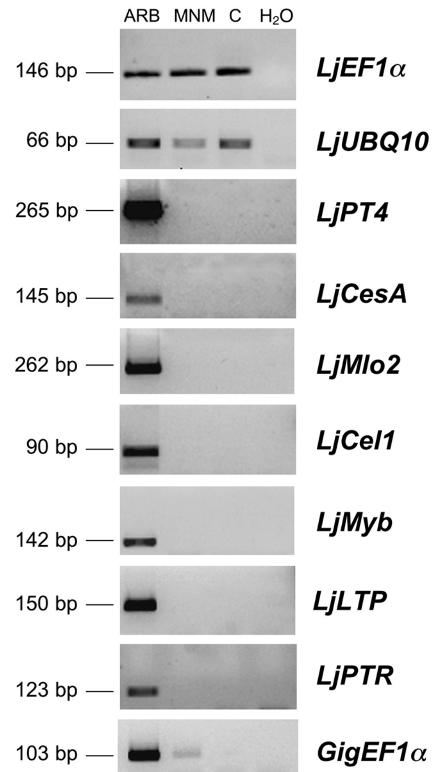

Fig. 4 RT-PCR analysis of microdissected cells with *LotusEF1α* and *Ubi10* primers as 'housekeeping' genes. An amplified fragment of the expected size is present in all the samples. Using specific primers for the mycorrhiza-induced genes *LjPT4*, *LjCesA*, *LjMlo2*, *LjCel1*, *LjMyb*, *LjLTP*, *LjPTR*, a fragment of the expected size is present exclusively in the arbusculated cell population. The fungal 'housekeeping' gene *Gigaspora margarita EF1α* was also amplified with specific primers. C, cortical cells from nonmycorrhizal roots; MNM, noncolonized cortical cells from mycorrhizal roots; ARB, arbusculated cortical cells.

Discussion

Transcriptional profiling and quantitative RT-PCR analyses revealed significant changes in gene expression in roots of *L. japonicus* in response to colonization by *Gi. margarita*, an

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

AM fungus belonging to the Diversisporaceae. The use of a well-controlled experimental mycorrhization system coupled to defined sampling times allowed us to attribute most of the 558 differentially expressed genes to plant processes related to arbuscule accommodation and mycorrhiza functioning. After quantitative RT-PCR validation for 24 selected sequences, transcripts of seven genes were shown to be confined to arbusculated cells using laser-dissected cells.

A high number of mycorrhiza-induced genes in *Lotus* were found to be homologous to genes induced in *Medicago* and rice during AM symbioses, including chitinases, Ser/Thr receptor kinases, glutathione S-transferases, copper-binding proteins and lectins (Liu *et al.*, 2003; Wulf *et al.*, 2003; Brechenmacher *et al.*, 2004; Manthey *et al.*, 2004; Frenzel *et al.*, 2005; Hohnjec *et al.*, 2005). Some of these common genes responded in the same way in *Medicago* mycorrhizal roots regardless of the species of AM fungus used as inoculum (Hohnjec *et al.*, 2005; Liu *et al.*, 2007), highlighting the evolutionary conservation of genetic processes supporting such symbioses (Schußler *et al.*, 2001; Hohnjec *et al.*, 2005; Liu *et al.*, 2007). Interestingly, some of these genes – for example, glycosyltransferases, serine-carboxypeptidases and subtilisin-like protease – have been associated with ectomycorrhizas (Duplessis *et al.*, 2005; Le Quere *et al.*, 2005; Heller *et al.*, 2008). However, ectomycorrhizal studies have so far been limited in scope, and work that takes advantage of the sequenced ectomycorrhizal couple (i.e. poplar and *Laccaria bicolor*) is yet to be performed, as highlighted by Martin *et al.* (2007). Since poplar is also a good host for AM fungi, we might expect a higher number of genetic similarities in host responses, irrespective of the fungal identity and its mycorrhizal strategy.

Mycorrhizal roots of *Lotus japonicus* express transcription factors potentially involved in symbiosis specificity

An important result of our work was the identification of 24 transcription factors or RNA-processing enzymes, whose genes were induced during symbiosis. Extensive sequencing of cDNA and genomic DNA indicates that legumes encode upwards of 2000 TFs per genome, but < 1% of these have been characterized genetically (Udvardi *et al.*, 2007). While the potential role of legume TFs in symbiotic nitrogen fixation has been reviewed (Udvardi *et al.*, 2007), so far little attention has been given to the role of TFs in AMs. Given the major reprogramming of gene expression that accompanies mycorrhizal symbiosis, it will be interesting to explore the role of mycorrhizal-induced TFs in the symbiosis and to determine whether the specificity of each root symbiosis (i.e. AM versus RNS) is regulated by specific TFs. An interesting target for further work is the MYB-like transcription factor, *LjMyb*, that is expressed exclusively in arbusculated cells (Fig. 4). *Lotus* MYBs, which are upregulated during nodule development (Colebatch *et al.*, 2004), exhibit low homology with the *LjMyb* sequence detected here, pointing to a symbiosis-specific regulation.

The molecular basis of root formation and growth has been analysed in great detail in model plants such as *Arabidopsis*, rice and maize (Hochholdinger & Zimmermann, 2008). By contrast, the molecular mechanisms that control root development in the presence of AM fungi are poorly known (Olah *et al.*, 2005; Osmont *et al.*, 2007). In this context, the findings that four members of the SCARECROW (SCR) subfamily of GRAS TFs are upregulated at 28 dpi is noteworthy. SCR and SHORTROOT regulate the radial pattern of the root and control the identity of endodermal and cortical cells (Dolan, 2007). Plant GRAS TFs are required for legume nodule development and symbiotic nitrogen fixation by rhizobia (Udvardi *et al.*, 2007), and it will be intriguing to learn whether members of this family of TFs are also required for mycorrhizal symbiosis, for example in avoiding endodermal colonization. It is noteworthy that SCR TFs are also upregulated during the formation of the short ectomycorrhizal tips (Laajanen *et al.*, 2007), supporting the hypothesis that mycorrhizal fungi stimulate root branching via specific TFs.

These and other TFs identified here are likely to regulate different, complementary processes required for the development and functioning of mycorrhizal roots.

Arbuscule development requires the activation of genes involved in the interface construction

Arbuscule development changes the host cell architecture to a great extent, as a novel apoplastic compartment is produced by plasma membrane proliferation and cell wall deposition all around the arbscular branches (Bonfante, 2001). A large number of sequences that are induced during the symbiosis in *Lotus* are related to protein turnover (i.e. proteases), membrane dynamics and cell wall synthesis, providing strong support for the notion that plant cells play an active role in accommodating their fungal symbionts via membrane proliferation and cell wall construction.

Among these sequences, some code for plasma membrane proteins (LjMlo2; LjCesA; LjCel1) and their transcripts were detected exclusively in arbusculated cells and not elsewhere in roots, using microdissected cells.

Mlo proteins are integral plasma membrane proteins with seven hydrophobic, membrane-spanning helices (Devoto *et al.*, 1999). In barley, the *Mlo* gene is essential for a successful penetration of the host cell wall by powdery mildew fungi. A loss of function-mutation confers a broad-spectrum resistance against powdery mildew fungi as well as a hypersusceptibility to other pathogens as *Magnaporthe grisea* (Jarosch *et al.*, 2003). Similar to AM fungi, powdery mildew fungi penetrate the plant cell wall but not the plasma membrane, which, after invagination, hosts the so-called haustorium (Green *et al.*, 2002). Functionally speaking, the haustorium is similar to the arbuscule, even though nutrients only flow in the direction of

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

the pathogen. Barley Mlo-mediated resistance requires the presence of the *Ror2* gene (Freialdenhoven *et al.*, 1996; Collins *et al.*, 2003), which codes for a syntaxin of the superfamily of the SNARE proteins that play key roles in membrane fusion events during vesicle trafficking (Bock *et al.*, 2001). Our array data revealed sevenfold up-regulation of a gene (Ljwgs_016013.1) encoding a protein with a 60% identity to the *A. thaliana* syntaxin SYP 132. The detection of trans-Golgi networks, vesicle trafficking and membrane fusion events in the cortex of both *M. truncatula* and *Daucus carota* (Genre *et al.*, 2008) provide an interesting backdrop to the molecular data revealing up-regulation of putative *Mlo* and syntaxin genes. This begs the question of whether these up-regulated genes/proteins facilitate plant/fungal compatibility in mycorrhizal *Lotus* roots by keeping the fungus 'under (membrane) wraps'.

The up-regulation of genes involved in plant cell wall synthesis during AM symbiosis is well documented (Balestrini & Bonfante, 2005). *MtCel1* expression was located in arbuscule-containing cells of *Medicago* and, considering the membrane domain, it was suggested that MtCel1 could be located in the periarbuscular membrane where it might be involved in assembling the cellulose/hemicellulose matrix at the interface (Liu *et al.*, 2003). Here we found that transcripts of a homologous *Lotus* gene, *LjCel1*, accumulated specifically in the arbusculated cells. Similarly, transcripts of a putative cellulose synthase, *LjCesA*, accumulated in arbuscular cells. CesA proteins are part of the cellulose synthase complex in higher plants (Taylor, 2008), which is a membrane-located enzymatic system that is responsible for cellulose synthesis. We hypothesize that *LjCesA*, whose transcripts appear to be confined to arbusculated cells of roots, plays a key role in cell expansion during arbuscule development, in conjunction with other proteins involved in cell wall remodelling (Balestrini & Bonfante, 2005). According to this hypothesis, the molecular mechanisms activated by the fungal presence and leading to the construction of the interface compartment might also have an additional target, namely the peripheral cell wall (R. Balestrini & P. Bonfante, unpublished).

Up-regulation of genes involved in nutrient transport in mycorrhizal roots

Nutrient exchange between plant and fungi is a hallmark of AM biology, and arbuscules are the major site of such an exchange (Smith & Read, 2008). Recent investigations have convincingly demonstrated that mycorrhiza-specific plant phosphate transporters are localized at the fungal interface (Harrison *et al.*, 2002; Bucher, 2007) and are essential for an active symbiosis (Javot *et al.*, 2007a).

Our results provide new insights into this aspect of the symbiosis: 47 genes related to transport were induced in *Lotus* mycorrhizal roots. Induction of a subset (eight) of these was tested and confirmed by q-RT PCR and phosphate (*LjPT4*), and peptide transporter (*LjPTR*) transcripts were found exclusively in arbusculated cells (Fig. 4). The result for *LjPT4* was consistent with similar data obtained for the putative orthologs in *Medicago* (Harrison *et al.*, 2002) and tomato (Balestrini *et al.*, 2007) and indicates that arbuscules are the fungal structures that release Pi to the host plant. The localization of *LjPTR* transcripts in the arbusculated cells suggests that the corresponding transporter may be involved in nitrogen transfer from the fungus to the plant before or after arbuscules disintegrate. However, we cannot exclude a possible function in signaling, as suggested for the *Arabidopsis* AtPTR3 in pathogenic interactions (Karim *et al.*, 2007). In addition to the phosphate transporters, which are currently considered the functional markers of active AM (Bucher, 2007; Javot *et al.*, 2007b), our investigation revealed activation of many other transporters, including transporters of organic and inorganic nitrogen. Recent data on fungal physiology (Govindarajulu *et al.*, 2005; Cappellazzo *et al.*, 2008; Allen & Shachar-Hill, 2009; Leigh *et al.*, 2009) convincingly demonstrate that AM fungi have the capacities to transfer substantial amounts of nitrogen and sulfur from soil to the host plant. Our work identified sulfate, ammonium, nitrate, and other N-transporters that may be involved in S and N uptake by the plant from the fungus. These transporters represent interesting targets for future studies on nutrient exchange between the two symbionts.

In conclusion, global transcriptional profiling and quantitative RT-PCR performed on highly arbusculated roots of *L. japonicus* have uncovered a panoply of genes involved in AM symbiosis, including regulatory genes controlling transcription, and many others involved in cell wall and membrane biosynthesis and nutrient transport between the plant and fungus. Together, these provide new perspectives on AM development and functioning.

Acknowledgements

We thank Mara Novero for her assistance in sandwich preparation and light microscope pictures, Drs Björn Usadel and Diego Sanchez for the *Lotus japonicus* MapMan mapping file. M. G. was supported by the EU in the frame of the INTEGRAL Project (Marie Curie Research Training Network, Project Reference: 505227). The research was supported by grants to P. B. from INTEGRAL Project, Consiglio Nazionale delle Ricerche (Biodiversity Project) and the San Paolo Company (Torino, Italy).

References

Allan AC, Hellens RP, Laing WA. 2008. MYB transcription factors that colour our fruit. *Trends in Plant Science* **13**: 99–102.

Allen JW, Shachar-Hill Y. 2009. Sulfur transfer through an arbuscular mycorrhiza. *Plant Physiology* **149**: 549–560.

Balestrini R, Bonfante P. 2005. The interface compartment in arbuscular mycorrhizae: a special type of plant cell wall? *Plant Biosystems* **139**: 8–15.

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

Balestrini R, Gomez-Ariza J, Lanfranco L, Bonfante P. 2007. Laser microdissection reveals that transcripts for five plant and one fungal phosphate transporter genes are contemporaneously present in arbusculated cells. *Molecular Plant–Microbe Interactions* **20**: 1055–1062.

Berta G, Trotta A, Fusconi A, Hooker JE, Munro M, Atkinson D, Giovannetti M, Morini S, Fortuna P, Tisserant B *et al.* 1995. Arbuscular mycorrhizal induced changes to plant-growth and root-system morphology in *Prunus cerasifera*. *Tree Physiology* **15**: 281–293.

Bock JB, Matern HT, Peden AA, Scheller RH. 2001. A genomic perspective on membrane compartment organization. *Nature* **409**: 839–841.

Bonfante P. 2001. At the interface between mycorrhizal fungi and plants: the structural organization of cell wall, plasma membrane and cytoskeleton. In: Hock B, ed. *The mycota IX*. Berlin, Germany: Springer-Verlag, 45–61.

Bonfante P, Genre A, Faccio A, Martini I, Schauser L, Stougaard J, Webb J, Parniske M. 2000. The *Lotus japonicus* LjSym4 gene is required for the successful symbiotic infection of root epidermal cells. *Molecular Plant–Microbe Interactions* **13**: 1109–1120.

Brechenmacher L, Weidmann S, van Tuinen D, Chatagnier O, Gianinazzi S, Franken P, Gianinazzi-Pearson V. 2004. Expression profiling of up-regulated plant and fungal genes in early and late stages of *Medicago truncatula-Glomus mosseae* interactions. *Mycorrhiza* **14**: 253–262.

Bucher M. 2007. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. *New Phytologist* **173**: 11–26.

Cappellazzo G, Lanfranco L, Fitz M, Wipf D, Bonfante P. 2008. Characterization of an amino acid permease from the endomycorrhizal fungus *Glomus mosseae*. *Plant Physiology* **147**: 429–437.

Chang S, Puryear J, Cairney J. 1993. Simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biolology Report* **11**: 113–116.

Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi MK. 2004. Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant Journal* **39**: 487–512.

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Huckelhoven R, Stein M, Freialdenhoven A, Somerville SC *et al.* 2003. SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* **425**: 973–977.

Deguchi Y, Banba M, Shimoda Y, Chechetka SA, Suzuri R, Okusako Y, Ooki Y, Toyokura K, Suzuki A, Uchiumi T *et al.* 2007. Transcriptome profiling of *Lotus japonicus* roots during arbuscular mycorrhiza development and comparison with that of nodulation. *DNA Research* **14**: 117–133.

Devoto A, Piffanelli P, Nilsson I, Wallin E, Panstruga R, von Heijne G, Schulze-Lefert P. 1999. Topology, subcellular localization, and sequence diversity of the Mlo family in plants. *Journal of Biological Chemistry* **274**: 34993–35004.

Dolan L. 2007. Plant science. SCARECROWs at the border. *Science* **316**: 377–378.

Duplessis S, Courty PE, Tagu D, Martin F. 2005. Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. *New Phytologist* **165**: 599–611.

Freialdenhoven A, Peterhansel C, Kurth J, Kreuzaler F, Schulze-Lefert P. 1996. Identification of genes required for the function of nonrace-specific mlo resistance to powdery mildew in barley. *Plant Cell* **8**: 5–14.

Frenzel A, Manthey K, Perlick AM, Meyer F, Puhler A, Kuster H, Krajinski F. 2005. Combined transcriptome profiling reveals a novel family of arbuscular mycorrhizal-specific *Medicago truncatula* lectin genes. *Molecular Plant–Microbe Interactions* **18**: 771–782.

Gautier L, Cope L, Bolstad BM, Irizarry RA. 2004. affy – analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **20**: 307–315.

Genre A, Chabaud M, Faccio A, Barker DG, Bonfante P. 2008. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both *Medicago truncatula* and *Daucus carota*. *Plant Cell* **20**: 1407–1420.

Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG. 2005. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *Plant Cell* **17**: 3489–3499.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge YC, Gentry J *et al.* 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology* **5**: R80.1–R80.16.

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C. 1993. Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. *New Phytologist* **125**: 587–593.

Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y. 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* **435**: 819–823.

Green JR, Carver TLW, Gurr SJ. 2002. The formation and function of infection and feeding structures. In: Belanger RR, Bushnell WR, Dik AJ, Carver TLW, eds. *The powdery mildews: a comprehensive treatise*. St Paul, MN, USA: American Phytopathological Society Press, 66–82.

Güimil S, Chang HS, Zhu T, Sesma A, Osbourn A, Roux C, Ionnidis V, Oakeley EJ, Docquier M, Descombes P *et al.* 2005. Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization. *Proceedings of the National Academy of Sciences, USA* **102**: 8066–8070.

Harrison MJ. 2005. Signaling in the arbuscular mycorrhizal symbiosis. *Annual Review of Microbiology* **59**: 19–42.

Harrison MJ, Dewbre GR, Liu J. 2002. A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell* **14**: 2413–2429.

Heller G, Adomas A, Li G, Osborne J, van Zyl L, Sederoff R, Finlay RD, Stenlid J, Asiegbu FO. 2008. Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria bicolor*. *BMC Plant Biology* **8**: 19.

Hewitt EJ. 1966. *Sand and water culture methods used in the study of plant nutrition*. Farnham Royal, UK: Commonwealth Agricultural Bureaux.

Hochholdinger F, Zimmermann R. 2008. Conserved and diverse mechanisms in root development. *Current Opinion in Plant Biology* **11**: 70–74.

Hohnjec N, Vieweg ME, Puhler A, Becker A, Kuster H. 2005. Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different *Glomus* fungi provide insights into the genetic program activated during arbuscular mycorrhiza. *Plant Physiology* **137**: 1283–1301.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Jarosch B, Jansen M, Schaffrath U. 2003. Acquired resistance functions in mlo barley, which is hypersusceptible to *Magnaporthe grisea*. *Molecular Plant–Microbe Interactions* **16**: 107–114.

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ. 2007a. A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences, USA* **104**: 1720–1725.

Javot H, Pumplin N, Harrison MJ. 2007b. Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles. *Plant, Cell & Environment* **30**: 310–322.

Karim S, Holmstrom KO, Mandal A, Dahl P, Hohmann S, Brader G, Palva ET, Pirhonen M. 2007. AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in *Arabidopsis*. *Planta* **225**: 1431–1445.

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

Kistner C, Winzer T, Pitzschke A, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Webb KJ *et al.* 2005. Seven *Lotus japonicus* genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. *Plant Cell* **17**: 2217–2229.

Laajanen K, Vuorinen I, Salo V, Juuti J, Raudaskoski M. 2007. Cloning of *Pinus sylvestris* SCARECROW gene and its expression pattern in the pine root system, mycorrhiza and NPA-treated short roots. *New Phytologist* **175**: 230–243.

Le Quere A, Wright DP, Soderstrom B, Tunlid A, Johansson T. 2005. Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) Fr. *Molecular Plant–Microbe Interactions* **18**: 659–673.

Leigh J, Hodge A, Fitter AH. 2009. Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. *New Phytologist* **181**: 199–207.

Liu JY, Blaylock LA, Endre G, Cho J, Town CD, VandenBosch KA, Harrison MJ. 2003. Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. *Plant Cell* **15**: 2106–2123.

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 Journal* **50**: 529–544.

Maeda D, Ashida K, Iguchi K, Chechetka SA, Hijikata A, Okusako Y, Deguchi Y, Izui K, Hata S. 2006. Knockdown of an arbuscular mycorrhiza-inducible phosphate transporter gene of *Lotus japonicus* suppresses mutualistic symbiosis. *Plant and Cell Physiology* **47**: 807–817.

Manthey K, Krajinski F, Hohnjec N, Firnhaber C, Puhler A, Perlick AM, Kuster H. 2004. Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies a collection of novel genes induced during *Medicago truncatula* root endosymbioses. *Molecular Plant–Microbe Interactions* **17**: 1063–1077.

Martin F, Kohler A, Duplessis S. 2007. Living in harmony in the wood underground: ectomycorrhizal genomics. *Current Opinion in Plant Biology* **10**: 204–210.

Novero M, Faccio A, Genre A, Stougaard J, Webb KJ, Mulder L, Parniske M, Bonfante P. 2002. Dual requirement of the LjSym4 gene for mycorrhizal development in epidermal and cortical cells of *Lotus japonicus* roots. *New Phytologist* **154**: 741–749.

Olah B, Briere C, Becard G, Denarie J, Gough C. 2005. Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. *Plant Journal* **44**: 195–207.

Osmont KS, Sibout R, Hardtke CS. 2007. Hidden branches: developments in root system architecture. *Annual Review of Plant Biology* **58**: 93–113.

Parniske M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews. Microbiology* **6**: 763–775.

Ririe KM, Rasmussen RP, Wittwer CT. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* **245**: 154–160.

Salvioli A, Luimini E, Anca IA, Bianciotto V, Bonfante P. 2008. Simultaneous detection and quantification of the unculturable microbe *Candidatus* Glomeribacter gigasporarum inside its fungal host *Gigaspora margarita*. *New Phytologist* **180**: 248–257.

Sanchez DH, Lippold F, Redestig H, Hannah MA, Erban A, Kramer U, Kopka J, Udvardi MK. 2008. Integrative functional genomics of salt acclimatization in the model legume *Lotus japonicus*. *Plant Journal* **53**: 973–987.

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K *et al.* 2008. Genome structure of the legume *Lotus japonicus*. *DNA Research* **15**: 227–239.

Schußler A, Schwarzott D, Walker C. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research* **105**: 1413–1421.

Siciliano V, Genre A, Balestrini R, Cappellazzo G, Dewit PJGM, Bonfante P. 2007. Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. *Plant Physiology* **144**: 1455–1466.

Smith SE, Read DJ. 2008. *Mycorrhizal symbiosis*. London, UK: Academic.

Smyth GK. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*. **3**: Article3.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

Taylor NG. 2008. Cellulose biosynthesis and deposition in higher plants. *New Phytologist* **178**: 239–252.

Trouvelot A, Kough JL, Gianinazzi-Pearson V. 1986. Mesure du taux de mycorhization VA d'un système radiculaire. In: Gianinazzi-Pearson V, Gianinazzi S, eds. *Recherche de méthodes d'estimation ayant une signification fonctionnelle. Proceedings of the 1st ESM*. Paris, France: INRA Press, 217–221.

Udvardi MK, Kakar K, Wandrey M, Montanari O, Murray J, Andriankaja A, Zhang JY, Benedito V, Hofer JMI, Chueng F *et al.* 2007. Legume transcription factors: global regulators of plant development and response to the environment. *Plant Physiology* **144**: 538–549.

Usadel B, Nagel A, Thimm O, Redestig H, Blaesing OE, Palacios-Rojas N, Selbig J, Hannemann J, Piques MC, Steinhauser D *et al.* 2005. Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of coresponding genes, and comparison with known responses. *Plant Physiology* **138**: 1195–1204.

Wulf A, Manthey K, Doll J, Perlick AM, Linke B, Bekel T, Meyer F, Franken P, Kuster H, Krajinski F. 2003. Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*. *Molecular Plant–Microbe Interactions* **16**: 306–314.

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31**: 3406–3415.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Microscopic images of cotton blue stained mycorrhized roots 4 and 28 d post-inoculation.

Fig. S2 Comparison of the subtracted data sets 28 dpi–4 dpi and 4 dpi–28 dpi to the previous published microarray studies.

Fig. S3 MapMan overview window – cellular response.

Fig. S4 MapMan overview window – metabolism.

Fig. S5 MapMan overview window – regulation.

Fig. S6 MapMan overview window – transport.

Table S1 *Lotus japonicus/Gigaspora margarita* average mycorrhization rates

Table S2 *Lotus japonicus/Gigaspora margarita* average root fresh weight

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)

Table S3 Probe-sets regulated in both the 4 dpi (d post-inoculation) and 28 dpi mycorrhized roots

Table S4 Subtraction of the 28 dpi from the 4 dpi regulated probe-sets

Table S5 Subtraction of the 4 dpi from the 28 dpi regulated probe-sets

Table S6 Primer sequences used in this study

Table S7 28 dpi–4 dpi regulated *Lotus japonicus* genes with their orthologous genes from the Liu *et al.* (2007) mycorrhizal 'core-set'

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

About *New Phytologist*

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at **www.newphytologist.org**.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *Early View* – our average submission to decision time is just 29 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £139 in Europe/$259 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (**newphytol@lancaster.ac.uk**; tel +44 1524 594691) or, for a local contact in North America, the US Office (**newphytol@ornl.gov**; tel +1 865 576 5261).

© The Authors (2009)
Journal compilation © *New Phytologist* (2009)